Biotechnology Foundations, 2nd Edition

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PREFACE

Preface

Jack O'Grady

Welcome to Biotechnology Foundations, 2nd Edition, 2019. This textbook was created to provide free open access teaching and learning resources for the Introduction to Biotechnology courses at Austin Community College, Biotechnology Program. This book provides the foundation of chemistry, biology, and microbiology needed to build biotechnology laboratory science workforce skills. The goal of this book is to encourage both faculty and student adoption and active, engaged use in the classroom and provide the resources students need to succeed. This book is available online at the OpenStax CNX platform: **https://cnx.org/contents/XcbB5HTY (https://cnx.org/contents/XcbB5HTY)**

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1 | THE STUDY OF LIFE

1.1 | Introduction

Textbook introduction and summary: Given the broad definition of biotechnology applications and products, it is easy to see how there is enormous overlap within the fields of cellular biology, microbiology, chemistry, and biomedical engineering. It is the goal of this textbook to provide foundational knowledge to begin building your biotechnology toolkit and enter an exciting career of making a difference through biotechnology.

INTRODUCTION

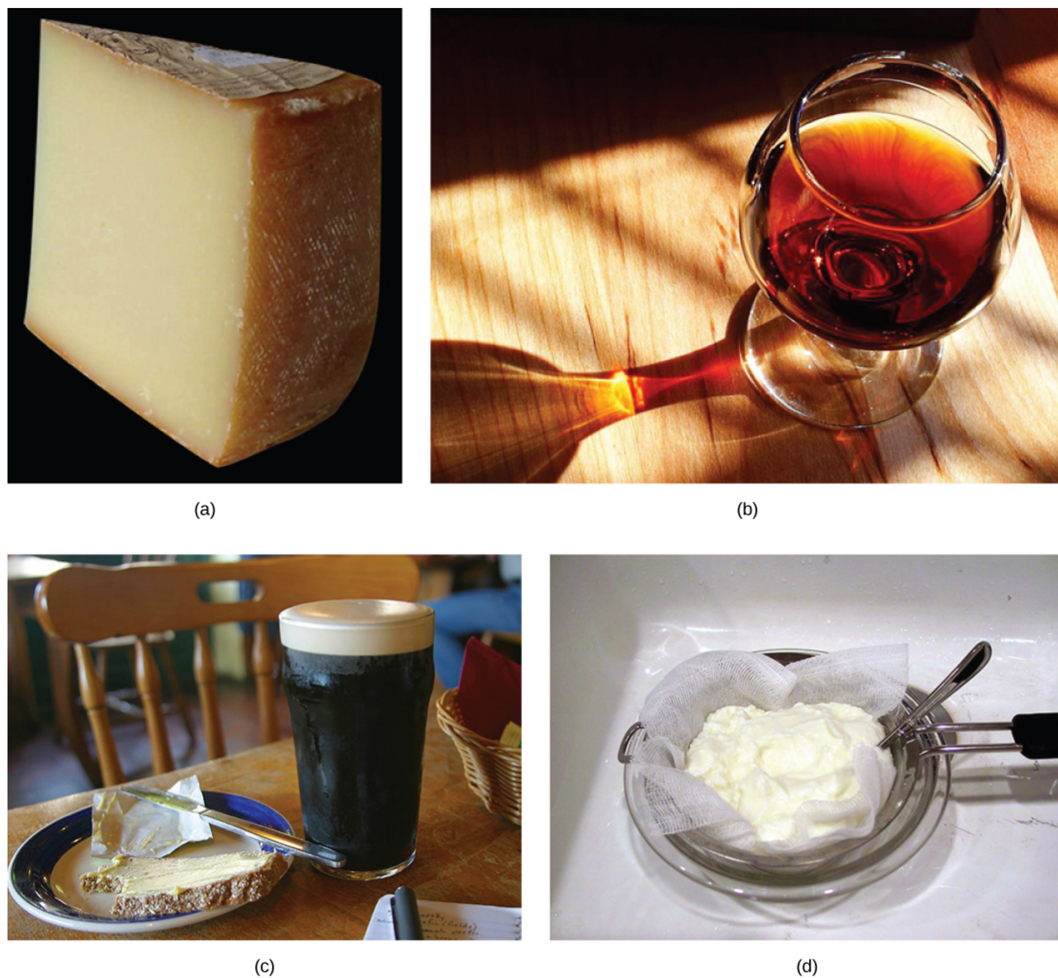

Figure 1.1 Some foods produced by microorganisms. Some of the products derived from the use of prokaryotes in early biotechnology include (a) cheese, (b) wine, (c) beer and bread, and (d) yogurt. (credit bread: modification of work by F. Rodrigo/Wikimedia Commons; credit wine: modification of work by Jon Sullivan; credit beer and bread: modification of work by Kris Miller; credit yogurt: modification of work by Jon Sullivan)

According to the United Nations Convention on Biological Diversity, ***biotechnology is "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use."*** The concept of "specific use" typically involves a commercial application or benefit to humanity. Genetic engineering, artificial selection, antibiotic production, and cell culture are current topics of study in biotechnology and will be described in later chapters. However, humans were

using microbes to create useful products long before Karl Ereky, a Hungarian engineer, coined the term **biotechnology (https://en.wikipedia.org/wiki/Biotechnology)** . Some of the products of this early biotechnology are as familiar as cheese, bread, wine, beer, and yogurt, which employ both bacteria and other microbes, such as yeast, a fungus (Figure).

Early Biotechnology: Cheese, Bread, Wine, Beer, and Yogurt

Cheese production began around 4,000 to 7,000 years ago when humans began to breed animals and process their milk. Fermentation, in this case, preserves nutrients: Milk will spoil relatively quickly, but when processed as cheese, it is more stable. As for ***beer***, the oldest records of brewing are about 6,000 years old and were an integral part of the Sumerian culture. Evidence indicates that the Sumerians discovered fermentation by chance. ***Wine*** has been produced for about 4,500 years, and evidence suggests that cultured milk products, like yogurt, have existed for at least 4,000 years.

In the early twentieth century, scientists gained a greater understanding of microbiology and explored ways of manufacturing specific products. In 1917, Chaim Weizmann first used a pure microbiological culture in an industrial process that of manufacturing corn starch using *Clostridium acetobutylicum*, to produce acetone, which was used to manufacture explosives during World War I. Shortly thereafter, in 1928, Alexander Fleming discovered the mold ***Penicillium***. His work led to the purification of the ***antibiotic*** compound formed by the mold by Howard Florey, Ernst Boris Chain, and Norman Heatley – to form what we today know as ***penicillin***. In 1940, penicillin became available for medicinal use to treat bacterial infections in humans.

The New Biotechnology

The field of modern biotechnology is generally thought of as having been born in 1971 when Paul Berg's (Stanford) experiments in ***gene splicing*** had early success. Herbert W. Boyer (Univ. Calif. at San Francisco) and Stanley N. Cohen (Stanford) significantly advanced the new technology in 1972 by transferring genetic material into a bacterium, such that the imported material would be reproduced, giving birth to the field of recombinant DNA technology. The commercial viability of a biotechnology industry was significantly expanded on June 16, 1980, when ***the United States Supreme Court ruled that a genetically modified microorganism could be patented***. Technology breakthroughs since the 1980s, such as Polymerase Chain Reaction, Sanger Sequencing, Whole Genome Sequencing, and more recently, CRISPR have brought forth a new age of Biotechnology and products.

Textbook Overview

Given the broad definition of biotechnology applications and products, it is easy to see how there is enormous overlap within the fields of cellular biology, microbiology, chemistry, and biomedical engineering. It is the goal of this textbook to provide foundational knowledge to begin building your biotechnology toolkit and enter an exciting career of making a difference through biotechnology.

This textbook first explores the fundamentals of laboratory science and biotechnology. Unit 1 begins with a dive into the foundation of biology and chemistry by asking what is life and what are the molecules of life? The end of the unit offers a primer into laboratory science; preparing solutions and operating basic lab equipment. Unit 2 brings readers through a vital tool of biomanufacturing, understanding the growth and control of microbes. Many biotechnology products are made by harnessing the work of microbes, and this unit explores this invisible and intriguing world of microorganisms. The final unit, molecular biotechnology, offers a more in-depth look at how biological molecules such as DNA and protein are manipulated into creating useful products.

References:

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Wikipedia: **https://en.wikipedia.org/wiki/Biotechnology (https://en.wikipedia.org/wiki/Biotechnology)**

1.2 | Themes and Concepts of Biology

By the end of this section, you will be able to:

- Identify and describe the properties of life
- Describe the levels of organization among living things
- List examples of different sub disciplines in biology

Biology is the science that studies life. What exactly is life? This may sound like a silly question with an obvious answer, but it is not easy to define life. For example, a branch of biology called virology studies viruses, which exhibit some of the characteristics of living entities but lack others. It turns out that although viruses can attack living organisms, cause diseases, and even reproduce, they do not meet the criteria that biologists use to define life.

From its earliest beginnings, biology has wrestled with four questions: What are the shared properties that make something “alive”? How do those various living things function? When faced with the remarkable diversity of life, how do we organize the different kinds of organisms so that we can better understand them? And, finally—what biologists ultimately seek to understand—how did this diversity arise and how is it continuing? As new organisms are discovered every day, biologists continue to seek answers to these and other questions.

Properties of Life

All groups of living organisms share several key characteristics or functions: order, sensitivity or response to stimuli, reproduction, adaptation, growth and development, regulation/homeostasis, and energy processing. When viewed together, these eight characteristics serve to define life.

Order

Organisms are highly organized structures that consist of one or more cells. Even very simple, single-celled organisms are remarkably complex. Inside each cell, atoms make up molecules. These in turn make up cell components or organelles. Multicellular organisms, which may consist of millions of individual cells, have an advantage over single-celled organisms in that their cells can be specialized to perform specific functions, and even sacrificed in certain situations for the good of the organism as a whole. How these specialized cells come together to form organs such as the heart, lung, or skin in organisms like the toad shown in **Figure 1.2** will be discussed later.

Figure 1.2 A toad represents a highly organized structure consisting of cells, tissues, organs, and organ systems. (credit: "Ivengo(RUS)"/Wikimedia Commons)

Sensitivity or Response to Stimuli

Organisms respond to diverse stimuli. For example, plants can bend toward a source of light or respond to touch (**Figure 1.3**). Even tiny bacteria can move toward or away from chemicals (a process called chemotaxis) or light (phototaxis). Movement toward a stimulus is considered a positive response, while movement away from a stimulus is considered a negative response.

Figure 1.3 The leaves of this sensitive plant (*Mimosa pudica*) will instantly droop and fold when touched. After a few minutes, the plant returns to its normal state. (credit: Alex Lomas)

Watch this **video (http://openstax.org/l/thigmonasty)** to see how the sensitive plant responds to a touch stimulus.

Reproduction

Single-celled organisms reproduce by first duplicating their DNA, which is the genetic material, and then dividing it equally as the cell prepares to divide to form two new cells. Many multicellular organisms (those made up of more than one cell) produce specialized reproductive cells that will form new individuals. When reproduction occurs, DNA containing genes is passed along to an organism's offspring. These genes are the reason that the offspring will belong to the same species and will have characteristics similar to the parent, such as fur color and blood type.

Adaptation

All living organisms exhibit a "fit" to their environment. Biologists refer to this fit as adaptation and it is a consequence of evolution by natural selection, which operates in every lineage of reproducing organisms. Examples of adaptations are diverse and unique, from heat-resistant Archaea that live in boiling hot springs to the tongue length of a nectar-feeding moth that matches the size of the flower from which it feeds. Adaptations enhance the reproductive potential of the individual exhibiting them, including their ability to survive to reproduce. Adaptations are not constant. As an environment changes, natural selection causes the characteristics of the individuals in a population to track those changes.

Growth and Development

Organisms grow and develop according to specific instructions coded for by their genes. These genes provide instructions that will direct cellular growth and development, ensuring that a species' young (**Figure 1.4**) will grow up to exhibit many of the same characteristics as its parents.

Figure 1.4 Although no two look alike, these kittens have inherited genes from both parents and share many of the same characteristics. (credit: Pieter & Renée Lanser)

Regulation/Homeostasis

Even the smallest organisms are complex and require multiple regulatory mechanisms to coordinate internal functions, such as the transport of nutrients, response to stimuli, and coping with environmental stresses. For example, organ systems such as the digestive or circulatory systems perform specific functions like carrying oxygen throughout the body, removing wastes, delivering nutrients to every cell, and cooling the body.

To function properly, cells require appropriate conditions such as proper temperature, pH, and concentrations of diverse chemicals. These conditions may, however, change from one moment to the next. Organisms are able to maintain internal conditions within a narrow range almost constantly, despite environmental changes, through a process called **homeostasis** or “steady state”—the ability of an organism to maintain constant internal conditions. For example, many organisms regulate their body temperature in a process known as thermoregulation. Organisms that live in cold climates, such as the polar bear (**Figure 1.5**), have body structures that help them withstand low temperatures and conserve body heat. In hot climates, organisms have methods (such as perspiration in humans or panting in dogs) that help them to shed excess body heat.

Figure 1.5 Polar bears and other mammals living in ice-covered regions maintain their body temperature by generating heat and reducing heat loss through thick fur and a dense layer of fat under their skin. (credit: "longhorndave"/Flickr)

Energy Processing

All organisms (such as the California condor shown in **Figure 1.6**) use a source of energy for their metabolic activities. Some organisms capture energy from the Sun and convert it into chemical energy in food; others use chemical energy from molecules they take in.

Figure 1.6 A lot of energy is required for a California condor to fly. Chemical energy derived from food is used to power flight. California condors are an endangered species; scientists have strived to place a wing tag on each bird to help them identify and locate each individual bird. (credit: Pacific Southwest Region U.S. Fish and Wildlife)

Evolution

The diversity of life on Earth is a result of mutations, or random changes in hereditary material over time. These mutations allow the possibility for organisms to adapt to a changing environment. An organism that evolves characteristics fit for the environment will have greater reproductive success, subject to the forces of natural selection.

Levels of Organization of Living Things

Living things are highly organized and structured, following a hierarchy on a scale from small to large. The **atom** is the smallest and most fundamental unit of matter that retains the properties of an element. It consists of a nucleus surrounded by electrons. Atoms form molecules. A **molecule** is a chemical structure consisting of at least two atoms held together by a chemical bond. Many molecules that are biologically important are **macromolecules**, large molecules that are typically formed by combining smaller units called monomers. An example of a macromolecule is deoxyribonucleic acid (DNA) (**Figure 1.7**), which contains the instructions for the functioning of the organism that contains it.

Figure 1.7 A molecule, like this large DNA molecule, is composed of atoms. (credit: "Brian0918"/Wikimedia Commons)

To see an animation of this DNA molecule, click **here (http://openstax.org/l/rotating_DNA2)** .

Some cells contain aggregates of macromolecules surrounded by membranes; these are called **organelles**. Organelles are small structures that exist within cells and perform specialized functions. All living things are made of cells; the **cell** itself is the smallest fundamental unit of structure and function in living organisms. (This requirement is why viruses are not considered living: they are not made of cells. To make new viruses, they have to invade and hijack a living cell; only then can they obtain the materials they need to reproduce.) Some organisms consist of a single cell and others are multicellular. Cells are classified as prokaryotic or eukaryotic. **Prokaryotes** are single-celled organisms that lack organelles surrounded by a membrane and do not have nuclei surrounded by nuclear membranes; in contrast, the cells of **eukaryotes** do have membrane-bound organelles and nuclei.

In most multicellular organisms, cells combine to make **tissues**, which are groups of similar cells carrying out the same function. **Organs** are collections of tissues grouped together based on a common function. Organs are present not only in animals but also in plants. An **organ system** is a higher level of organization that consists of functionally related organs. For example vertebrate animals have many organ systems, such as the circulatory system that transports blood throughout the body and to and from the lungs; it includes organs such as the heart and blood vessels. **Organisms** are individual living entities. For example, each tree in a forest is an organism. Single-celled prokaryotes and single-celled eukaryotes are also considered organisms and are typically referred to as microorganisms.

Visual Connection

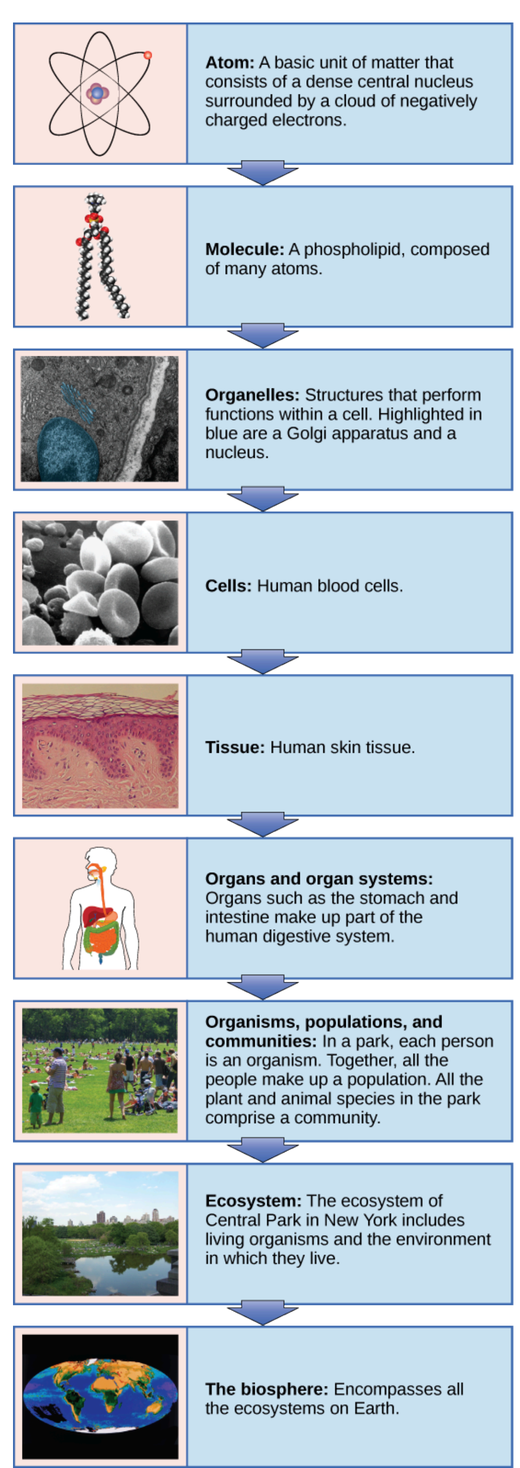


Figure 1.8 From an atom to the entire Earth, biology examines all aspects of life. (credit "molecule": modification of work by Jane Whitney; credit "organelles": modification of work by Louisa Howard; credit "cells": modification of work by Bruce Wetzel, Harry Schaefer, National Cancer Institute; credit "tissue": modification of work by "Kilbad"/Wikimedia Commons; credit "organs": modification of work by Mariana Ruiz Villareal, Joaquim Alves Gaspar; credit "organisms": modification of work by Peter Dutton; credit "ecosystem": modification of work by "gigi4791"/Flickr; credit "biosphere": modification of work by NASA)

Which of the following statements is false?

a. Tissues exist within organs which exist within organ systems.

b. Communities exist within populations which exist within ecosystems.

c. Organelles exist within cells which exist within tissues.

d. Communities exist within ecosystems which exist in the biosphere.

All the individuals of a species living within a specific area are collectively called a **population**. For example, a forest may include many white pine trees. All of these pine trees represent the population of white pine trees in this forest. Different populations may live in the same specific area. For example, the forest with the pine trees includes populations of flowering plants and also insects and microbial populations. A **community** is the set of populations inhabiting a particular area. For instance, all of the trees, flowers, insects, and other populations in a forest form the forest's community. The forest itself is an ecosystem. An **ecosystem** consists of all the living things in a particular area together with the abiotic, or non-living, parts of that environment such as nitrogen in the soil or rainwater. At the highest level of organization (Figure 1.8), the **biosphere** is the collection of all ecosystems, and it represents the zones of life on Earth. It includes land, water, and portions of the atmosphere.

The Diversity of Life

The science of biology is very broad in scope because there is a tremendous diversity of life on Earth. The source of this diversity is **evolution**, the process of gradual change during which new species arise from older species. Evolutionary biologists study the evolution of living things in everything from the microscopic world to ecosystems.

In the 18th century, a scientist named Carl Linnaeus first proposed organizing the known species of organisms into a hierarchical taxonomy. In this system, species that are most similar to each other are put together within a grouping known as a genus. Furthermore, similar genera (the plural of genus) are put together within a family. This grouping continues until all organisms are collected together into groups at the highest level. The current taxonomic system now has eight levels in its hierarchy, from lowest to highest, they are: species, genus, family, order, class, phylum, kingdom, domain. Thus species are grouped within genera, genera are grouped within families, families are grouped within orders, and so on (Figure 1.9).

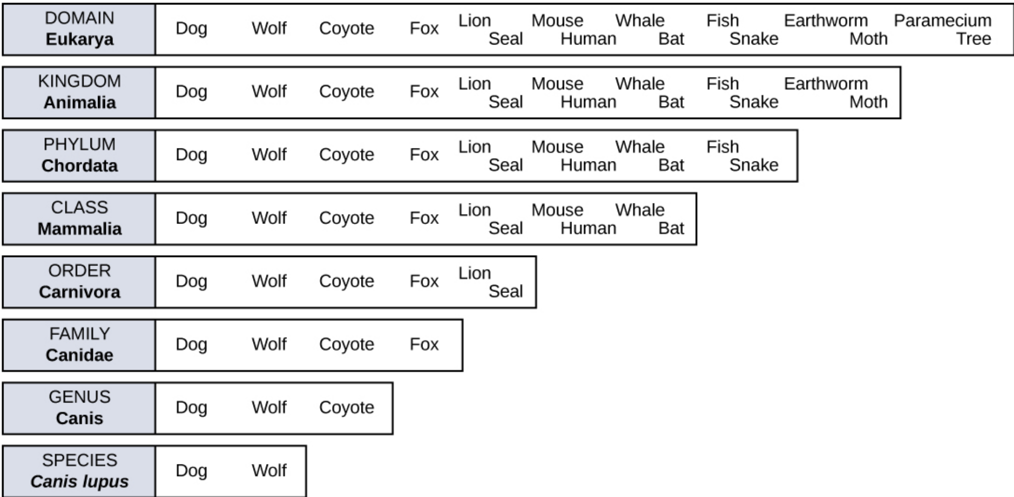


Figure 1.9 This diagram shows the levels of taxonomic hierarchy for a dog, from the broadest category—domain—to the most specific—species.

The highest level, domain, is a relatively new addition to the system since the 1970s. Scientists now recognize three domains of life, the Eukarya, the Archaea, and the Bacteria. The domain Eukarya contains organisms that have cells with nuclei. It includes the kingdoms of fungi, plants, animals, and several kingdoms of protists. The Archaea, are single-celled organisms without nuclei and include many extremophiles that live in harsh environments like hot springs. The Bacteria are another quite different group of single-celled organisms without nuclei (Figure 1.10). Both the Archaea and the Bacteria are prokaryotes, an informal name for cells without nuclei. The recognition in the 1970s that certain "bacteria," now known as the Archaea, were as different genetically and biochemically from other bacterial cells as they were from eukaryotes, motivated the recommendation to divide life into three domains. This dramatic change in our knowledge of the tree of life demonstrates that classifications are not permanent and will change when new information becomes available.

In addition to the hierarchical taxonomic system, Linnaeus was the first to name organisms using two unique

names, now called the binomial naming system. Before Linnaeus, the use of common names to refer to organisms caused confusion because there were regional differences in these common names. Binomial names consist of the genus name (which is capitalized) and the species name (all lower-case). Both names are set in italics when they are printed. Every species is given a unique binomial which is recognized the world over, so that a scientist in any location can know which organism is being referred to. For example, the North American blue jay is known uniquely as *Cyanocitta cristata*. Our own species is *Homo sapiens*.

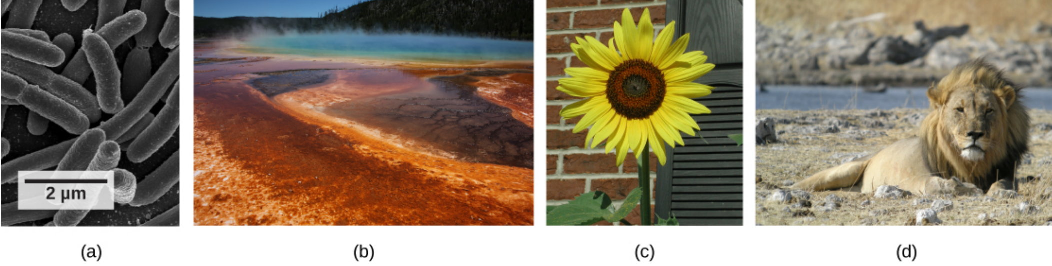


Figure 1.10 These images represent different domains. The scanning electron micrograph shows (a) bacterial cells belong to the domain Bacteria, while the (b) extremophiles, seen all together as colored mats in this hot spring, belong to domain Archaea. Both the (c) sunflower and (d) lion are part of domain Eukarya. (credit a: modification of work by Rocky Mountain Laboratories, NIAID, NIH; credit b: modification of work by Steve Jurvetson; credit c: modification of work by Michael Arrighi; credit d: modification of work by Frank Vassen)

evolution IN ACTION

Carl Woese and the Phylogenetic Tree

The evolutionary relationships of various life forms on Earth can be summarized in a phylogenetic tree. A **phylogenetic tree** is a diagram showing the evolutionary relationships among biological species based on similarities and differences in genetic or physical traits or both. A phylogenetic tree is composed of branch points, or nodes, and branches. The internal nodes represent ancestors and are points in evolution when, based on scientific evidence, an ancestor is thought to have diverged to form two new species. The length of each branch can be considered as estimates of relative time.

In the past, biologists grouped living organisms into five kingdoms: animals, plants, fungi, protists, and bacteria. The pioneering work of American microbiologist Carl Woese in the early 1970s has shown, however, that life on Earth has evolved along three lineages, now called domains—Bacteria, Archaea, and Eukarya. Woese proposed the domain as a new taxonomic level and Archaea as a new domain, to reflect the new phylogenetic tree (Figure 1.11). Many organisms belonging to the Archaea domain live under extreme conditions and are called extremophiles. To construct his tree, Woese used genetic relationships rather than similarities based on morphology (shape). Various genes were used in phylogenetic studies. Woese's tree was constructed from comparative sequencing of the genes that are universally distributed, found in some slightly altered form in every organism, conserved (meaning that these genes have remained only slightly changed throughout evolution), and of an appropriate length.

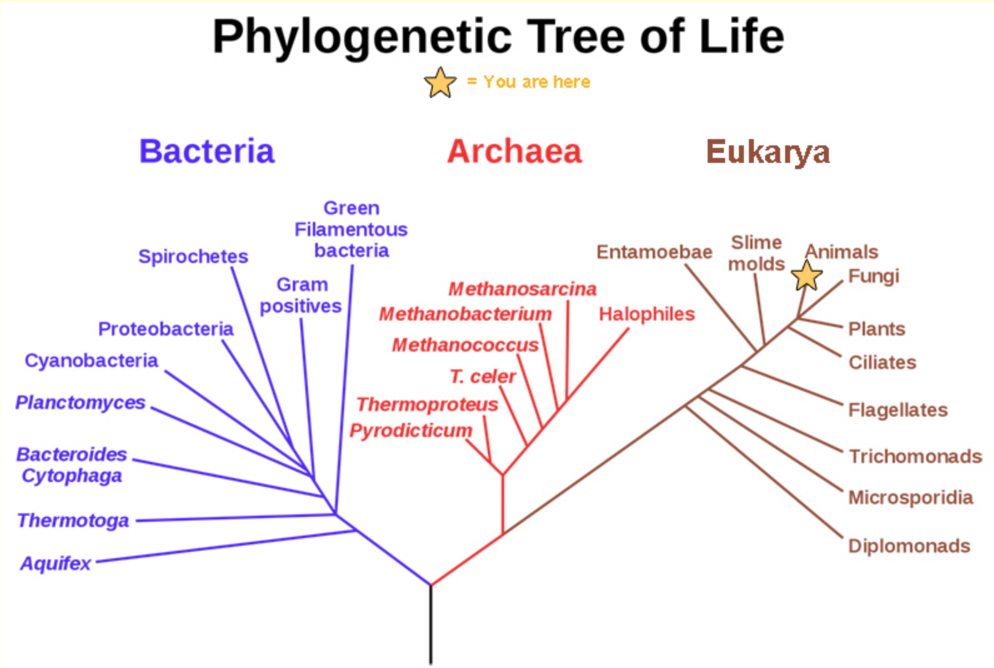


Figure 1.11 This phylogenetic tree was constructed by microbiologist Carl Woese using genetic relationships. The tree shows the separation of living organisms into three domains: Bacteria, Archaea, and Eukarya. Bacteria and Archaea are organisms without a nucleus or other organelles surrounded by a membrane and, therefore, are prokaryotes. (credit: modification of work by Eric Gaba)

Branches of Biological Study

The scope of biology is broad and therefore contains many branches and sub disciplines. Biologists may pursue one of those sub disciplines and work in a more focused field. For instance, molecular biology studies biological processes at the molecular level, including interactions among molecules such as DNA, RNA, and proteins, as well as the way they are regulated. Microbiology is the study of the structure and function of microorganisms. It is quite a broad branch itself, and depending on the subject of study, there are also microbial physiologists, ecologists, and geneticists, among others.

Another field of biological study, neurobiology, studies the biology of the nervous system, and although it is considered a branch of biology, it is also recognized as an interdisciplinary field of study known as neuroscience. Because of its interdisciplinary nature, this sub discipline studies different functions of the nervous system using molecular, cellular, developmental, medical, and computational approaches.

Figure 1.12 Researchers work on excavating dinosaur fossils at a site in Castellón, Spain. (credit: Mario Modesto)

Paleontology, another branch of biology, uses fossils to study life's history (**Figure 1.12**). Zoology and botany are the study of animals and plants, respectively. Biologists can also specialize as biotechnologists, ecologists, or physiologists, to name just a few areas. Biotechnologists apply the knowledge of biology to create useful products. Ecologists study the interactions of organisms in their environments. Physiologists study the workings of cells, tissues and organs. This is just a small sample of the many fields that biologists can pursue. From our own bodies to the world we live in, discoveries in biology can affect us in very direct and important ways. We depend on these discoveries for our health, our food sources, and the benefits provided by our ecosystem. Because of this, knowledge of biology can benefit us in making decisions in our day-to-day lives.

The development of technology in the twentieth century that continues today, particularly the technology to describe and manipulate the genetic material, DNA, has transformed biology. This transformation will allow biologists to continue to understand the history of life in greater detail, how the human body works, our human origins, and how humans can survive as a species on this planet despite the stresses caused by our increasing numbers. Biologists continue to decipher huge mysteries about life suggesting that we have only begun to understand life on the planet, its history, and our relationship to it. For this and other reasons, the knowledge of biology gained through this textbook and other printed and electronic media should be a benefit in whichever field you enter.

careers IN ACTION

Forensic Scientist

Forensic science is the application of science to answer questions related to the law. Biologists as well as chemists and biochemists can be forensic scientists. Forensic scientists provide scientific evidence for use in courts, and their job involves examining trace material associated with crimes. Interest in forensic science has increased in the last few years, possibly because of popular television shows that feature forensic scientists on the job. Also, the development of molecular techniques and the establishment of DNA databases have updated the types of work that forensic scientists can do. Their job activities are primarily related to crimes against people such as murder, rape, and assault. Their work involves analyzing samples such as hair, blood, and other body fluids and also processing DNA (Figure 1.13) found in many different environments and materials. Forensic scientists also analyze other biological evidence left at crime scenes, such as insect parts or pollen grains. Students who want to pursue careers in forensic science will most likely be required to take chemistry and biology courses as well as some intensive math courses.

Figure 1.13 This forensic scientist works in a DNA extraction room at the U.S. Army Criminal Investigation Laboratory. (credit: U.S. Army CID Command Public Affairs)

Section Summary

Biology is the science of life. All living organisms share several key properties such as order, sensitivity or response to stimuli, reproduction, adaptation, growth and development, regulation, homeostasis, and energy processing. Living things are highly organized following a hierarchy that includes atoms, molecules, organelles, cells, tissues, organs, and organ systems. Organisms, in turn, are grouped as populations, communities, ecosystems, and the biosphere. Evolution is the source of the tremendous biological diversity on Earth today. A diagram called a phylogenetic tree can be used to show evolutionary relationships among organisms. Biology is very broad and includes many branches and sub disciplines. Examples include molecular biology, microbiology, neurobiology, zoology, and botany, among others.

Art Connections

Exercise 1.1

Figure 1.8 Which of the following statements is false?

A. Tissues exist within organs which exist within organ systems.

B. Communities exist within populations which exist within ecosystems.
C. Organelles exist within cells which exist within tissues.
D. Communities exist within ecosystems which exist in the biosphere.

Solution

Figure 1.8 B

Multiple Choice

Exercise 1.2

The smallest unit of biological structure that meets the functional requirements of “living” is the ________.

a. organ
b. organelle
c. cell
d. macromolecule

Solution

C

Exercise 1.3

Which of the following sequences represents the hierarchy of biological organization from the most complex to the least complex level?

a. organelle, tissue, biosphere, ecosystem, population
b. organ, organism, tissue, organelle, molecule
c. organism, community, biosphere, molecule, tissue, organ
d. biosphere, ecosystem, community, population, organism

Solution

D

Free Response

Exercise 1.4

Using examples, explain how biology can be studied from a microscopic approach to a global approach.

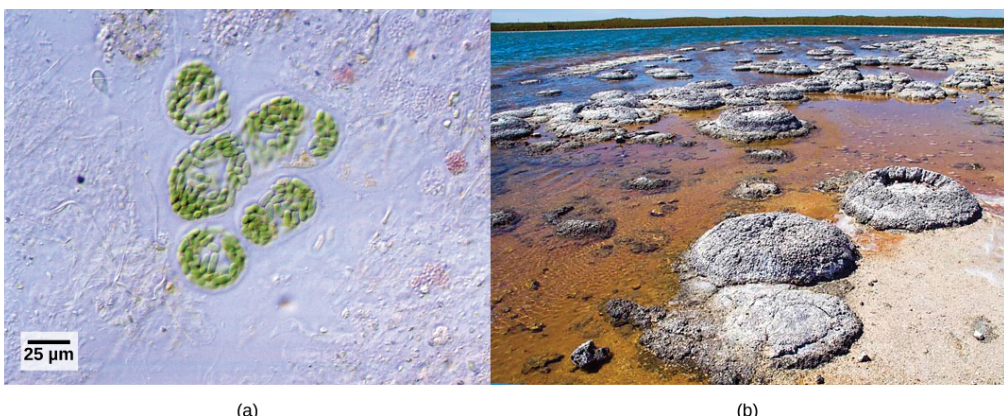
Solution

Researchers can approach biology from the smallest to the largest, and everything in between. For instance, an ecologist may study a population of individuals, the population’s community, the community’s ecosystem, and the ecosystem’s part in the biosphere. When studying an individual organism, a biologist could examine the cell and its organelles, the tissues that the cells make up, the organs and their respective organ systems, and the sum total—the organism itself.

1.3 | The Process of Science

By the end of this section, you will be able to:

- Identify the shared characteristics of the natural sciences
- Understand the process of scientific inquiry
- Compare inductive reasoning with deductive reasoning
- Describe the goals of basic science and applied science



(a) (b)

Figure 1.14 Formerly called blue-green algae, the (a) cyanobacteria seen through a light microscope are some of Earth's oldest life forms. These (b) stromatolites along the shores of Lake Thetis in Western Australia are ancient structures formed by the layering of cyanobacteria in shallow waters. (credit a: modification of work by NASA; scale-bar data from Matt Russell; credit b: modification of work by Ruth Ellison)

Like geology, physics, and chemistry, biology is a science that gathers knowledge about the natural world. Specifically, biology is the study of life. The discoveries of biology are made by a community of researchers who work individually and together using agreed-on methods. In this sense, biology, like all sciences is a social enterprise like politics or the arts. The methods of science include careful observation, record keeping, logical and mathematical reasoning, experimentation, and submitting conclusions to the scrutiny of others. Science also requires considerable imagination and creativity; a well-designed experiment is commonly described as elegant, or beautiful. Like politics, science has considerable practical implications and some science is dedicated to practical applications, such as the prevention of disease (see **Figure 1.15**). Other science proceeds largely motivated by curiosity. Whatever its goal, there is no doubt that science, including biology, has transformed human existence and will continue to do so.

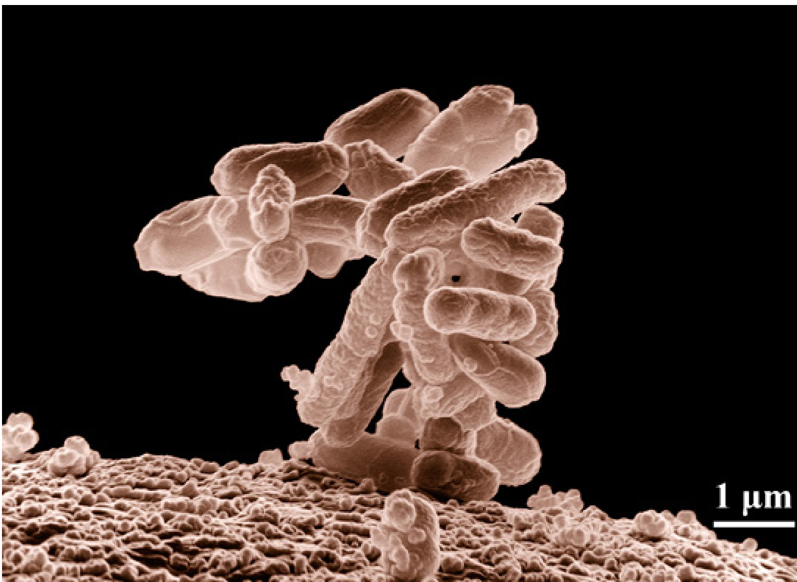


Figure 1.15 Biologists may choose to study *Escherichia coli* (*E. coli*), a bacterium that is a normal resident of our digestive tracts but which is also sometimes responsible for disease outbreaks. In this micrograph, the bacterium is visualized using a scanning electron microscope and digital colorization. (credit: Eric Erbe; digital colorization by Christopher Pooley, USDA-ARS)

The Nature of Science

Biology is a science, but what exactly is science? What does the study of biology share with other scientific disciplines? **Science** (from the Latin *scientia,* meaning "knowledge") can be defined as knowledge about the natural world.

Science is a very specific way of learning, or knowing, about the world. The history of the past 500 years demonstrates that science is a very powerful way of knowing about the world; it is largely responsible for the technological revolutions that have taken place during this time. There are however, areas of knowledge and human experience that the methods of science cannot be applied to. These include such things as answering purely moral questions, aesthetic questions, or what can be generally categorized as spiritual questions. Science cannot investigate these areas because they are outside the realm of material phenomena, the phenomena of matter and energy, and cannot be observed and measured.

The **scientific method** is a method of research with defined steps that include experiments and careful observation. The steps of the scientific method will be examined in detail later, but one of the most important aspects of this method is the testing of hypotheses. A **hypothesis** is a suggested explanation for an event, which can be tested. Hypotheses, or tentative explanations, are generally produced within the context of a **scientific theory**. A scientific theory is a generally accepted, thoroughly tested and confirmed explanation for a set of observations or phenomena. Scientific theory is the foundation of scientific knowledge. In addition, in many scientific disciplines (less so in biology) there are **scientific laws**, often expressed in mathematical formulas, which describe how elements of nature will behave under certain specific conditions. There is not an evolution of hypotheses through theories to laws as if they represented some increase in certainty about the world. Hypotheses are the day-to-day material that scientists work with and they are developed within the context of theories. Laws are concise descriptions of parts of the world that are amenable to formulaic or mathematical description.

Natural Sciences

What would you expect to see in a museum of natural sciences? Frogs? Plants? Dinosaur skeletons? Exhibits about how the brain functions? A planetarium? Gems and minerals? Or maybe all of the above? Science includes such diverse fields as astronomy, biology, computer sciences, geology, logic, physics, chemistry, and mathematics (**Figure 1.16**). However, those fields of science related to the physical world and its phenomena and processes are considered **natural sciences**. Thus, a museum of natural sciences might contain any of the items listed above.

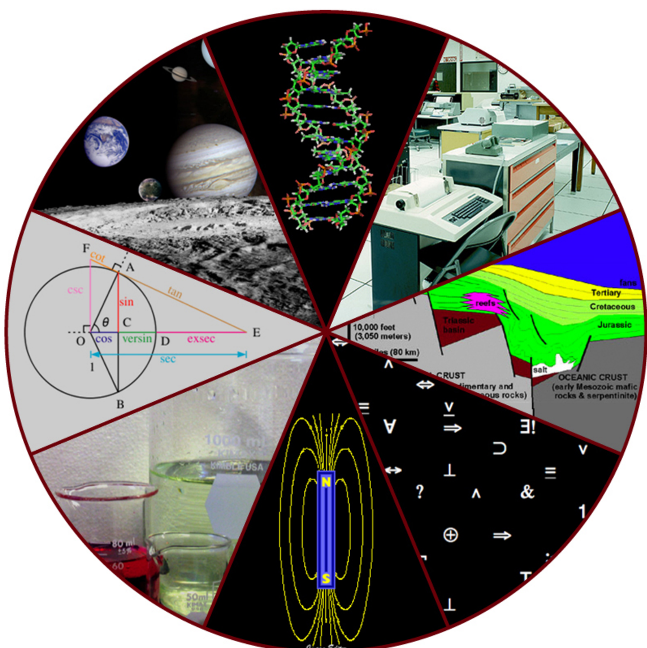


Figure 1.16 Some fields of science include astronomy, biology, computer science, geology, logic, physics, chemistry, and mathematics. (credit: "Image Editor"/Flickr)

There is no complete agreement when it comes to defining what the natural sciences include. For some experts, the natural sciences are astronomy, biology, chemistry, earth science, and physics. Other scholars choose to divide natural sciences into **life sciences**, which study living things and include biology, and **physical sciences**, which study nonliving matter and include astronomy, physics, and chemistry. Some disciplines such as biophysics and biochemistry build on two sciences and are interdisciplinary.

Scientific Inquiry

One thing is common to all forms of science: an ultimate goal "to know." Curiosity and inquiry are the driving forces for the development of science. Scientists seek to understand the world and the way it operates. Two methods of logical thinking are used: inductive reasoning and deductive reasoning.

Inductive reasoning is a form of logical thinking that uses related observations to arrive at a general conclusion. This type of reasoning is common in descriptive science. A life scientist such as a biologist makes observations and records them. These data can be qualitative (descriptive) or quantitative (consisting of numbers), and the raw data can be supplemented with drawings, pictures, photos, or videos. From many observations, the scientist can infer conclusions (inductions) based on evidence. Inductive reasoning involves formulating generalizations inferred from careful observation and the analysis of a large amount of data. Brain studies often work this way. Many brains are observed while people are doing a task. The part of the brain that lights up, indicating activity, is then demonstrated to be the part controlling the response to that task.

Deductive reasoning or deduction is the type of logic used in hypothesis-based science. In deductive reasoning, the pattern of thinking moves in the opposite direction as compared to inductive reasoning. **Deductive reasoning** is a form of logical thinking that uses a general principle or law to predict specific results. From those general principles, a scientist can deduce and predict the specific results that would be valid as long as the general principles are valid. For example, a prediction would be that if the climate is becoming warmer in a region, the distribution of plants and animals should change. Comparisons have been made between distributions in the past and the present, and the many changes that have been found are consistent with a warming climate. Finding the change in distribution is evidence that the climate change conclusion is a valid one.

Both types of logical thinking are related to the two main pathways of scientific study: descriptive science and hypothesis-based science. **Descriptive** (or discovery) **science** aims to observe, explore, and discover, while **hypothesis-based science** begins with a specific question or problem and a potential answer or solution that can be tested. The boundary between these two forms of study is often blurred, because most scientific endeavors combine both approaches. Observations lead to questions, questions lead to forming a hypothesis as a possible answer to those questions, and then the hypothesis is tested. Thus, descriptive science and hypothesis-based science are in continuous dialogue.

Hypothesis Testing

Biologists study the living world by posing questions about it and seeking science-based responses. This approach is common to other sciences as well and is often referred to as the scientific method. The scientific method was used even in ancient times, but it was first documented by England's Sir Francis Bacon (1561–1626) (**Figure 1.17**), who set up inductive methods for scientific inquiry. The scientific method is not exclusively used by biologists but can be applied to almost anything as a logical problem-solving method.

Figure 1.17 Sir Francis Bacon is credited with being the first to document the scientific method.

The scientific process typically starts with an observation (often a problem to be solved) that leads to a question. Let's think about a simple problem that starts with an observation and apply the scientific method to solve the problem. One Monday morning, a student arrives at class and quickly discovers that the classroom is too warm. That is an observation that also describes a problem: the classroom is too warm. The student then asks a question: "Why is the classroom so warm?"

Recall that a hypothesis is a suggested explanation that can be tested. To solve a problem, several hypotheses may be proposed. For example, one hypothesis might be, "The classroom is warm because no one turned on the air conditioning." But there could be other responses to the question, and therefore other hypotheses may be proposed. A second hypothesis might be, "The classroom is warm because there is a power failure, and so the air conditioning doesn't work."

Once a hypothesis has been selected, a prediction may be made. A prediction is similar to a hypothesis but it typically has the format "If . . . then . . . ." For example, the prediction for the first hypothesis might be, "*If* the student turns on the air conditioning, *then* the classroom will no longer be too warm."

A hypothesis must be testable to ensure that it is valid. For example, a hypothesis that depends on what a bear thinks is not testable, because it can never be known what a bear thinks. It should also be **falsifiable**, meaning that it can be disproven by experimental results. An example of an unfalsifiable hypothesis is "Botticelli's *Birth of Venus* is beautiful." There is no experiment that might show this statement to be false. To test a hypothesis, a researcher will conduct one or more experiments designed to eliminate one or more of the hypotheses. This is important. A hypothesis can be disproven, or eliminated, but it can never be proven. Science does not deal in proofs like mathematics. If an experiment fails to disprove a hypothesis, then we find support for that explanation, but this is not to say that down the road a better explanation will not be found, or a more carefully designed experiment will be found to falsify the hypothesis.

Each experiment will have one or more variables and one or more controls. A **variable** is any part of the experiment that can vary or change during the experiment. A **control** is a part of the experiment that does not change. Look for the variables and controls in the example that follows. As a simple example, an experiment might be conducted to test the hypothesis that phosphate limits the growth of algae in freshwater ponds. A series

of artificial ponds are filled with water and half of them are treated by adding phosphate each week, while the other half are treated by adding a salt that is known not to be used by algae. The variable here is the phosphate (or lack of phosphate), the experimental or treatment cases are the ponds with added phosphate and the control ponds are those with something inert added, such as the salt. Just adding something is also a control against the possibility that adding extra matter to the pond has an effect. If the treated ponds show lesser growth of algae, then we have found support for our hypothesis. If they do not, then we reject our hypothesis. Be aware that rejecting one hypothesis does not determine whether or not the other hypotheses can be accepted; it simply eliminates one hypothesis that is not valid (Figure 1.18). Using the scientific method, the hypotheses that are inconsistent with experimental data are rejected.

In recent years a new approach of testing hypotheses has developed as a result of an exponential growth of data deposited in various databases. Using computer algorithms and statistical analyses of data in databases, a new field of so-called "data research" (also referred to as "in silico" research) provides new methods of data analyses and their interpretation. This will increase the demand for specialists in both biology and computer science, a promising career opportunity.

Visual Connection

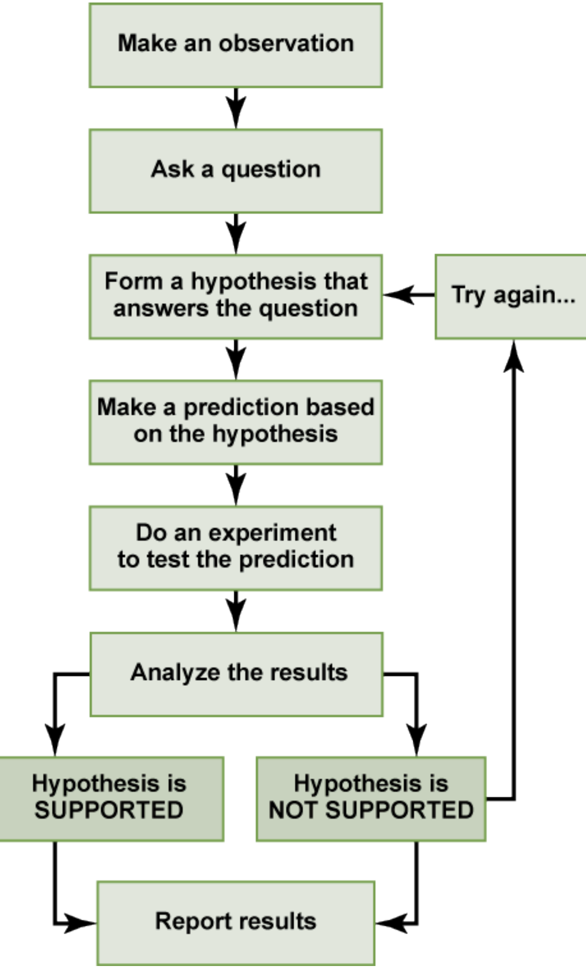


Figure 1.18 The scientific method is a series of defined steps that include experiments and careful observation. If a hypothesis is not supported by data, a new hypothesis can be proposed.

In the example below, the scientific method is used to solve an everyday problem. Which part in the example below is the hypothesis? Which is the prediction? Based on the results of the experiment, is the hypothesis supported? If it is not supported, propose some alternative hypotheses.

1. My toaster doesn’t toast my bread.
2. Why doesn’t my toaster work?
3. There is something wrong with the electrical outlet.

4. If something is wrong with the outlet, my coffeemaker also won’t work when plugged into it.
5. I plug my coffeemaker into the outlet.
6. My coffeemaker works.

In practice, the scientific method is not as rigid and structured as it might at first appear. Sometimes an experiment leads to conclusions that favor a change in approach; often, an experiment brings entirely new scientific questions to the puzzle. Many times, science does not operate in a linear fashion; instead, scientists continually draw inferences and make generalizations, finding patterns as their research proceeds. Scientific reasoning is more complex than the scientific method alone suggests.

Basic and Applied Science

The scientific community has been debating for the last few decades about the value of different types of science. Is it valuable to pursue science for the sake of simply gaining knowledge, or does scientific knowledge only have worth if we can apply it to solving a specific problem or bettering our lives? This question focuses on the differences between two types of science: basic science and applied science.

Basic science or “pure” science seeks to expand knowledge regardless of the short-term application of that knowledge. It is not focused on developing a product or a service of immediate public or commercial value. The immediate goal of basic science is knowledge for knowledge’s sake, though this does not mean that in the end it may not result in an application.

In contrast, **applied science** or “technology,” aims to use science to solve real-world problems, making it possible, for example, to improve a crop yield, find a cure for a particular disease, or save animals threatened by a natural disaster. In applied science, the problem is usually defined for the researcher.

Some individuals may perceive applied science as “useful” and basic science as “useless.” A question these people might pose to a scientist advocating knowledge acquisition would be, “What for?” A careful look at the history of science, however, reveals that basic knowledge has resulted in many remarkable applications of great value. Many scientists think that a basic understanding of science is necessary before an application is developed; therefore, applied science relies on the results generated through basic science. Other scientists think that it is time to move on from basic science and instead to find solutions to actual problems. Both approaches are valid. It is true that there are problems that demand immediate attention; however, few solutions would be found without the help of the knowledge generated through basic science.

One example of how basic and applied science can work together to solve practical problems occurred after the discovery of DNA structure led to an understanding of the molecular mechanisms governing DNA replication. Strands of DNA, unique in every human, are found in our cells, where they provide the instructions necessary for life. During DNA replication, new copies of DNA are made, shortly before a cell divides to form new cells. Understanding the mechanisms of DNA replication enabled scientists to develop laboratory techniques that are now used to identify genetic diseases, pinpoint individuals who were at a crime scene, and determine paternity. Without basic science, it is unlikely that applied science could exist.

Another example of the link between basic and applied research is the Human Genome Project, a study in which each human chromosome was analyzed and mapped to determine the precise sequence of DNA subunits and the exact location of each gene. (The gene is the basic unit of heredity represented by a specific DNA segment that codes for a functional molecule.) Other organisms have also been studied as part of this project to gain a better understanding of human chromosomes. The Human Genome Project (**Figure 1.19**) relied on basic research carried out with non-human organisms and, later, with the human genome. An important end goal eventually became using the data for applied research seeking cures for genetically related diseases.

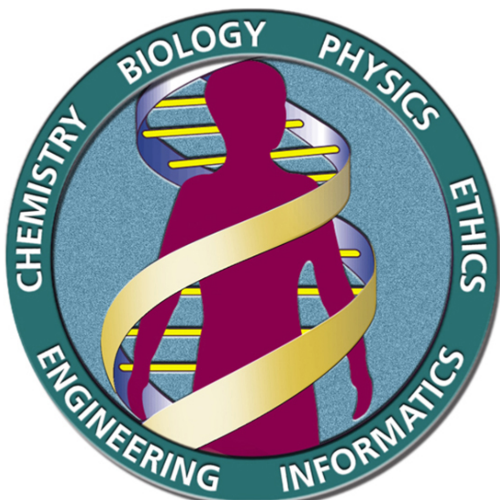


Figure 1.19 The Human Genome Project was a 13-year collaborative effort among researchers working in several different fields of science. The project was completed in 2003. (credit: the U.S. Department of Energy Genome Programs)

While research efforts in both basic science and applied science are usually carefully planned, it is important to note that some discoveries are made by serendipity, that is, by means of a fortunate accident or a lucky surprise. Penicillin was discovered when biologist Alexander Fleming accidentally left a petri dish of *Staphylococcus* bacteria open. An unwanted mold grew, killing the bacteria. The mold turned out to be *Penicillium*, and a new antibiotic was discovered. Even in the highly organized world of science, luck—when combined with an observant, curious mind—can lead to unexpected breakthroughs.

Reporting Scientific Work

Whether scientific research is basic science or applied science, scientists must share their findings for other researchers to expand and build upon their discoveries. Communication and collaboration within and between sub disciplines of science are key to the advancement of knowledge in science. For this reason, an important aspect of a scientist's work is disseminating results and communicating with peers. Scientists can share results by presenting them at a scientific meeting or conference, but this approach can reach only the limited few who are present. Instead, most scientists present their results in peer-reviewed articles that are published in scientific journals. **Peer-reviewed articles** are scientific papers that are reviewed, usually anonymously by a scientist's colleagues, or peers. These colleagues are qualified individuals, often experts in the same research area, who judge whether or not the scientist's work is suitable for publication. The process of peer review helps to ensure that the research described in a scientific paper or grant proposal is original, significant, logical, and thorough. Grant proposals, which are requests for research funding, are also subject to peer review. Scientists publish their work so other scientists can reproduce their experiments under similar or different conditions to expand on the findings.

There are many journals and the popular press that do not use a peer-review system. A large number of online open-access journals, journals with articles available without cost, are now available many of which use rigorous peer-review systems, but some of which do not. Results of any studies published in these forums without peer review are not reliable and should not form the basis for other scientific work. In one exception, journals may allow a researcher to cite a personal communication from another researcher about unpublished results with the cited author's permission.

Section Summary

Biology is the science that studies living organisms and their interactions with one another and their environments. Science attempts to describe and understand the nature of the universe in whole or in part. Science has many fields; those fields related to the physical world and its phenomena are considered natural sciences.

A hypothesis is a tentative explanation for an observation. A scientific theory is a well-tested and consistently verified explanation for a set of observations or phenomena. A scientific law is a description, often in the form of a mathematical formula, of the behavior of an aspect of nature under certain circumstances. Two types of

logical reasoning are used in science. Inductive reasoning uses results to produce general scientific principles. Deductive reasoning is a form of logical thinking that predicts results by applying general principles. The common thread throughout scientific research is the use of the scientific method. Scientists present their results in peer-reviewed scientific papers published in scientific journals.

Science can be basic or applied. The main goal of basic science is to expand knowledge without any expectation of short-term practical application of that knowledge. The primary goal of applied research, however, is to solve practical problems.

Art Connections

Exercise 1.5

Figure 1.18 In the example below, the scientific method is used to solve an everyday problem. Which part in the example below is the hypothesis? Which is the prediction? Based on the results of the experiment, is the hypothesis supported? If it is not supported, propose some alternative hypotheses.

1. My toaster doesn't toast my bread.
2. Why doesn't my toaster work?
3. There is something wrong with the electrical outlet.
4. If something is wrong with the outlet, my coffeemaker also won't work when plugged into it.
5. I plug my coffeemaker into the outlet.
6. My coffeemaker works.

Solution

Figure 1.18 The hypothesis is #3 (there is something wrong with the electrical outlet), and the prediction is #4 (if something is wrong with the outlet, then the coffeemaker also won't work when plugged into the outlet). The original hypothesis is not supported, as the coffee maker works when plugged into the outlet. Alternative hypotheses may include (1) the toaster might be broken or (2) the toaster wasn't turned on.

Multiple Choice

Exercise 1.6

A suggested and testable explanation for an event is called a ________.

a. hypothesis
b. variable
c. theory
d. control

Solution

A

Exercise 1.7

The type of logical thinking that uses related observations to arrive at a general conclusion is called ________.

a. deductive reasoning
b. the scientific method
c. hypothesis-based science
d. inductive reasoning

Solution

D

Free Response

Exercise 1.8

Give an example of how applied science has had a direct effect on your daily life.

Solution

Answers will vary. One example of how applied science has had a direct effect on daily life is the presence of vaccines. Vaccines to prevent diseases such polio, measles, tetanus, and even the influenza affect daily life by contributing to individual and societal health.

KEY TERMS

applied science a form of science that solves real-world problems

atom a basic unit of matter that cannot be broken down by normal chemical reactions

basic science science that seeks to expand knowledge regardless of the short-term application of that knowledge

biology the study of living organisms and their interactions with one another and their environments

biosphere a collection of all ecosystems on Earth

cell the smallest fundamental unit of structure and function in living things

community a set of populations inhabiting a particular area

control a part of an experiment that does not change during the experiment

deductive reasoning a form of logical thinking that uses a general statement to predict specific results

descriptive science a form of science that aims to observe, explore, and find things out

ecosystem all living things in a particular area together with the abiotic, nonliving parts of that environment

eukaryote an organism with cells that have nuclei and membrane-bound organelles

evolution the process of gradual change in a population that can also lead to new species arising from older species

falsifiable able to be disproven by experimental results

homeostasis the ability of an organism to maintain constant internal conditions

hypothesis a suggested explanation for an event, which can be tested

hypothesis-based science a form of science that begins with a specific explanation that is then tested

inductive reasoning a form of logical thinking that uses related observations to arrive at a general conclusion

life science a field of science, such as biology, that studies living things

macromolecule a large molecule typically formed by the joining of smaller molecules

molecule a chemical structure consisting of at least two atoms held together by a chemical bond

natural science a field of science that studies the physical world, its phenomena, and processes

organ a structure formed of tissues operating together to perform a common function

organ system the higher level of organization that consists of functionally related organs

organelle a membrane-bound compartment or sac within a cell

organism an individual living entity

peer-reviewed article a scientific report that is reviewed by a scientist's colleagues before publication

phylogenetic tree a diagram showing the evolutionary relationships among biological species based on similarities and differences in genetic or physical traits or both

physical science a field of science, such as astronomy, physics, and chemistry, that studies nonliving matter

population all individuals within a species living within a specific area

prokaryote a unicellular organism that lacks a nucleus or any other membrane-bound organelle

science knowledge that covers general truths or the operation of general laws, especially when acquired and tested by the scientific method

scientific law a description, often in the form of a mathematical formula, for the behavior of some aspect of nature under certain specific conditions

scientific method a method of research with defined steps that include experiments and careful observation

scientific theory a thoroughly tested and confirmed explanation for observations or phenomena

tissue a group of similar cells carrying out the same function

variable a part of an experiment that can vary or change

2 | THE BUILDING BLOCKS OF LIFE

Figure 2.1 Foods such as bread, fruit, and cheese are rich sources of biological macromolecules. (credit: modification of work by Bengt Nyman)

Chapter Outline

2.1: The Building Blocks of Molecules

2.2: Water

2.3: Human Blood Buffering Systems

2.4: Biological Molecules

2.5: Friedrich Miescher and the Structure of Nucleic Acids

Introduction

The elements carbon, hydrogen, nitrogen, oxygen, sulfur, and phosphorus are the key building blocks of the chemicals found in living things. They form the carbohydrates, nucleic acids, proteins, and lipids (all of which will be defined later in this chapter) that are the fundamental molecular components of all organisms. In this chapter, we will discuss these important building blocks and learn how the unique properties of the atoms of different elements affect their interactions with other atoms to form the molecules of life.

Food provides an organism with nutrients—the matter it needs to survive. Many of these critical nutrients come in the form of biological macromolecules, or large molecules necessary for life. These macromolecules are built from different combinations of smaller organic molecules. What specific types of biological macromolecules do living things require? How are these molecules formed? What functions do they serve? In this chapter, we will explore these questions.

2.1 | The Building Blocks of Molecules

By the end of this section, you will be able to:

- Describe matter and elements
- Describe the interrelationship between protons, neutrons, and electrons, and the ways in which electrons can be donated or shared between atoms

At its most fundamental level, life is made up of matter. **Matter** occupies space and has mass. All matter is composed of **elements**, substances that cannot be broken down or transformed chemically into other substances. Each element is made of atoms, each with a constant number of protons and unique properties. A total of 118 elements have been defined; however, only 92 occur naturally, and fewer than 30 are found in living cells. The remaining 26 elements are unstable and, therefore, do not exist for very long or are theoretical and have yet to be detected.

Each element is designated by its chemical symbol (such as H, N, O, C, and Na), and possesses unique properties. These unique properties allow elements to combine and to bond with each other in specific ways.

Atoms

An atom is the smallest component of an element that retains all of the chemical properties of that element. For example, one hydrogen atom has all of the properties of the element hydrogen, such as it exists as a gas at room temperature, and it bonds with oxygen to create a water molecule. Hydrogen atoms cannot be broken down into anything smaller while still retaining the properties of hydrogen. If a hydrogen atom were broken down into subatomic particles, it would no longer have the properties of hydrogen.

At the most basic level, all organisms are made of a combination of elements. They contain atoms that combine together to form molecules. In multicellular organisms, such as animals, molecules can interact to form cells that combine to form tissues, which make up organs. These combinations continue until entire multicellular organisms are formed.

All atoms contain protons, electrons, and neutrons (Figure 2.2). The most common isotope of hydrogen (H) is the only exception and is made of one proton and one electron with no neutrons. A **proton** is a positively charged particle that resides in the **nucleus** (the core of the atom) of an atom and has a mass of 1 and a charge of +1. An **electron** is a negatively charged particle that travels in the space around the nucleus. In other words, it resides outside of the nucleus. It has a negligible mass and has a charge of –1.

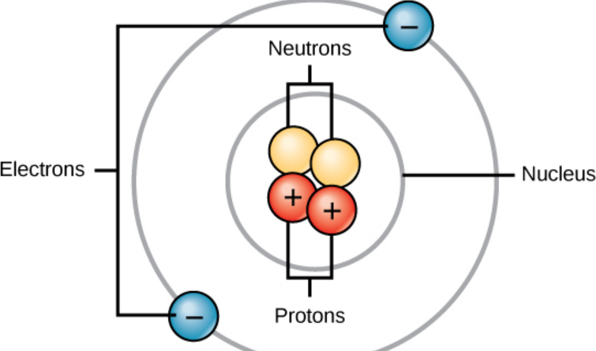


Figure 2.2 Atoms are made up of protons and neutrons located within the nucleus, and electrons surrounding the nucleus.

Neutrons, like protons, reside in the nucleus of an atom. They have a mass of 1 and no charge. The positive (protons) and negative (electrons) charges balance each other in a neutral atom, which has a net zero charge.

Because protons and neutrons each have a mass of 1, the mass of an atom is equal to the number of protons and neutrons of that atom. The number of electrons does not factor into the overall mass, because their mass is so small.

As stated earlier, each element has its own unique properties. Each contains a different number of protons and neutrons, giving it its own atomic number and mass number. The **atomic number** of an element is equal to the number of protons that element contains. The **mass number**, or atomic mass, is the number of protons plus the number of neutrons of that element. Therefore, it is possible to determine the number of neutrons by subtracting the atomic number from the mass number.

These numbers provide information about the elements and how they will react when combined. Different elements have different melting and boiling points, and are in different states (liquid, solid, or gas) at room temperature. They also combine in different ways. Some form specific types of bonds, whereas others do not. How they combine is based on the number of electrons present. Because of these characteristics, the elements are arranged into the **periodic table of elements**, a chart of the elements that includes the atomic number and relative atomic mass of each element. The periodic table also provides key information about the properties of elements (Figure 2.2)—often indicated by color-coding. The arrangement of the table also shows how the electrons in each element are organized and provides important details about how atoms will react with each other to form molecules.

Isotopes are different forms of the same element that have the same number of protons, but a different number of neutrons. Some elements, such as carbon, potassium, and uranium, have naturally occurring isotopes. Carbon-12, the most common isotope of carbon, contains six protons and six neutrons. Therefore, it has a mass number of 12 (six protons and six neutrons) and an atomic number of 6 (which makes it carbon). Carbon-14 contains six protons and eight neutrons. Therefore, it has a mass number of 14 (six protons and eight neutrons) and an atomic number of 6, meaning it is still the element carbon. These two alternate forms of carbon are isotopes. Some isotopes are unstable and will lose protons, other subatomic particles, or energy to form more stable elements. These are called **radioactive isotopes** or radioisotopes.

Visual Connection

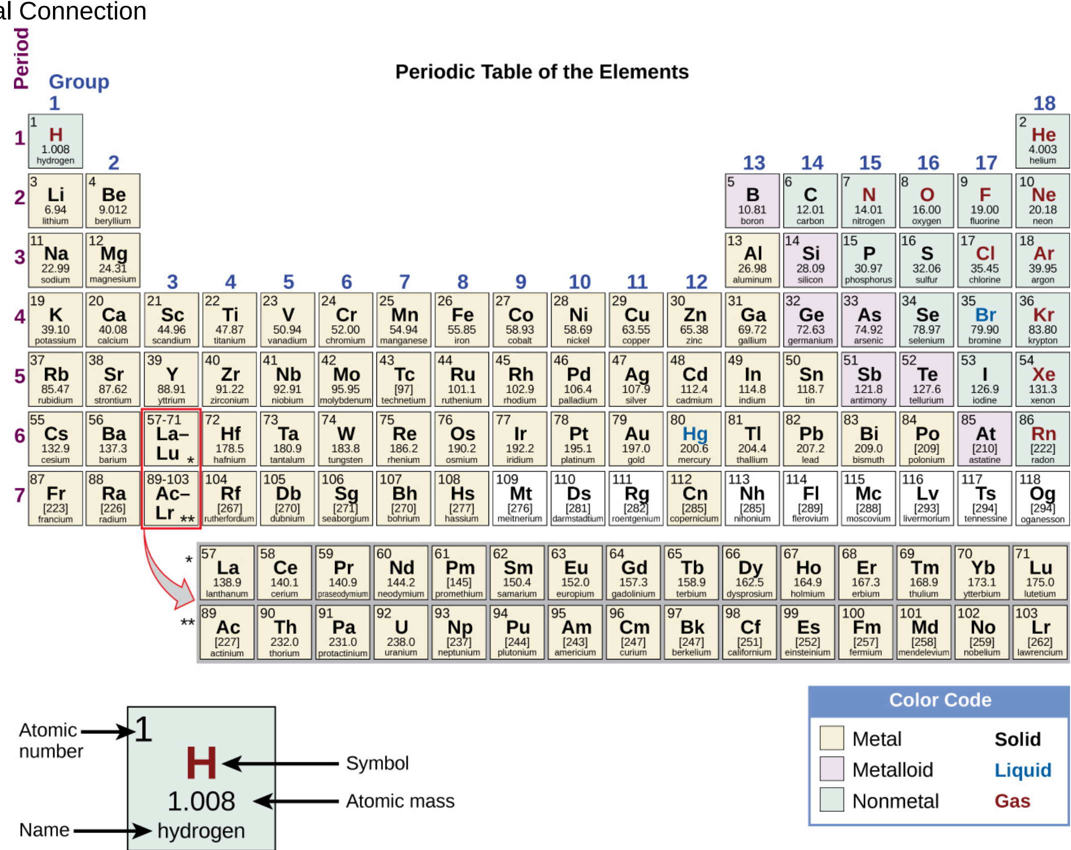


Figure 2.3 Arranged in columns and rows based on the characteristics of the elements, the periodic table provides key information about the elements and how they might interact with each other to form molecules. Most periodic tables provide a key or legend to the information they contain.

How many neutrons do (K) potassium-39 and potassium-40 have, respectively?

evolution IN ACTION

Carbon Dating

Carbon-14 (^{14}C) is a naturally occurring radioisotope that is created in the atmosphere by cosmic rays. This is a continuous process, so more ^{14}C is always being created. As a living organism develops, the relative level of ^{14}C in its body is equal to the concentration of ^{14}C in the atmosphere. When an organism dies, it is no longer ingesting ^{14}C, so the ratio will decline. ^{14}C decays to ^{14}N by a process called beta decay; it gives off energy in this slow process.

After approximately 5,730 years, only one-half of the starting concentration of ^{14}C will have been converted to ^{14}N. The time it takes for half of the original concentration of an isotope to decay to its more stable form is called its half-life. Because the half-life of ^{14}C is long, it is used to age formerly living objects, such as fossils. Using the ratio of the ^{14}C concentration found in an object to the amount of ^{14}C detected in the atmosphere, the amount of the isotope that has not yet decayed can be determined. Based on this amount, the age of the fossil can be calculated to about 50,000 years (**Figure 2.4**). Isotopes with longer half-lives, such as potassium-40, are used to calculate the ages of older fossils. Through the use of carbon dating, scientists can reconstruct the ecology and biogeography of organisms living within the past 50,000 years.

Figure 2.4 The age of remains that contain carbon and are less than about 50,000 years old, such as this pygmy mammoth, can be determined using carbon dating. (credit: Bill Faulkner/NPS)

To learn more about atoms and isotopes, and how you can tell one isotope from another, visit this **site (http://openstax.org/l/isotopes)** and run the simulation.

Chemical Bonds

How elements interact with one another depends on how their electrons are arranged and how many openings for electrons exist at the outermost region where electrons are present in an atom. Electrons exist at energy

levels that form shells around the nucleus. The closest shell can hold up to two electrons. The closest shell to the nucleus is always filled first, before any other shell can be filled. Hydrogen has one electron; therefore, it has only one spot occupied within the lowest shell. Helium has two electrons; therefore, it can completely fill the lowest shell with its two electrons. If you look at the periodic table, you will see that hydrogen and helium are the only two elements in the first row. This is because they only have electrons in their first shell. Hydrogen and helium are the only two elements that have the lowest shell and no other shells.

The second and third energy levels can hold up to eight electrons. The eight electrons are arranged in four pairs and one position in each pair is filled with an electron before any pairs are completed.

Looking at the periodic table again (**Figure 2.3**), you will notice that there are seven rows. These rows correspond to the number of shells that the elements within that row have. The elements within a particular row have increasing numbers of electrons as the columns proceed from left to right. Although each element has the same number of shells, not all of the shells are completely filled with electrons. If you look at the second row of the periodic table, you will find lithium (Li), beryllium (Be), boron (B), carbon (C), nitrogen (N), oxygen (O), fluorine (F), and neon (Ne). These all have electrons that occupy only the first and second shells. Lithium has only one electron in its outermost shell, beryllium has two electrons, boron has three, and so on, until the entire shell is filled with eight electrons, as is the case with neon.

Not all elements have enough electrons to fill their outermost shells, but an atom is at its most stable when all of the electron positions in the outermost shell are filled. Because of these vacancies in the outermost shells, we see the formation of **chemical bonds**, or interactions between two or more of the same or different elements that result in the formation of molecules. To achieve greater stability, atoms will tend to completely fill their outer shells and will bond with other elements to accomplish this goal by sharing electrons, accepting electrons from another atom, or donating electrons to another atom. Because the outermost shells of the elements with low atomic numbers (up to calcium, with atomic number 20) can hold eight electrons, this is referred to as the **octet rule**. An element can donate, accept, or share electrons with other elements to fill its outer shell and satisfy the octet rule.

When an atom does not contain equal numbers of protons and electrons, it is called an **ion**. Because the number of electrons does not equal the number of protons, each ion has a net charge. Positive ions are formed by losing electrons and are called **cations**. Negative ions are formed by gaining electrons and are called **anions**.

For example, sodium only has one electron in its outermost shell. It takes less energy for sodium to donate that one electron than it does to accept seven more electrons to fill the outer shell. If sodium loses an electron, it now has 11 protons and only 10 electrons, leaving it with an overall charge of +1. It is now called a sodium ion.

The chlorine atom has seven electrons in its outer shell. Again, it is more energy-efficient for chlorine to gain one electron than to lose seven. Therefore, it tends to gain an electron to create an ion with 17 protons and 18 electrons, giving it a net negative (–1) charge. It is now called a chloride ion. This movement of electrons from one element to another is referred to as **electron transfer**. As **Figure 2.5** illustrates, a sodium atom (Na) only has one electron in its outermost shell, whereas a chlorine atom (Cl) has seven electrons in its outermost shell. A sodium atom will donate its one electron to empty its shell, and a chlorine atom will accept that electron to fill its shell, becoming chloride. Both ions now satisfy the octet rule and have complete outermost shells. Because the number of electrons is no longer equal to the number of protons, each is now an ion and has a +1 (sodium) or –1 (chloride) charge.

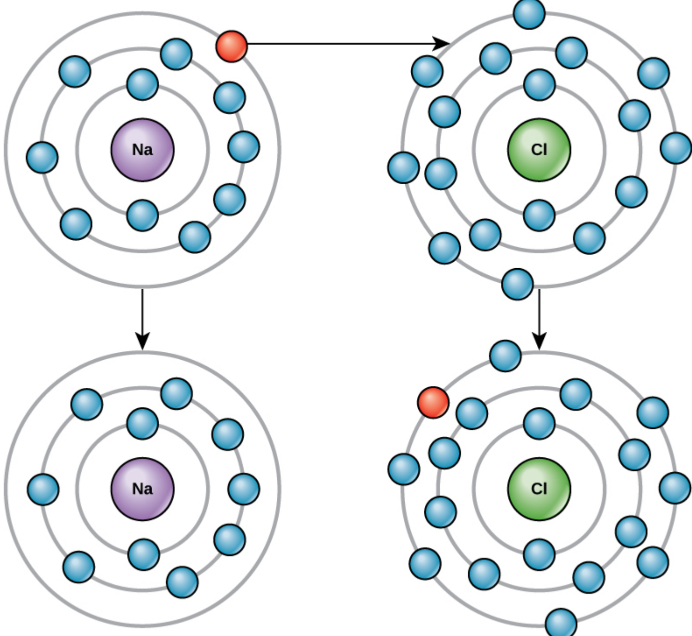


Figure 2.5 Elements tend to fill their outermost shells with electrons. To do this, they can either donate or accept electrons from other elements.

Ionic Bonds

There are four types of bonds or interactions: ionic, covalent, hydrogen bonds, and van der Waals interactions. Ionic and covalent bonds are strong interactions that require a larger energy input to break apart. When an element donates an electron from its outer shell, as in the sodium atom example above, a positive ion is formed. The element accepting the electron is now negatively charged. Because positive and negative charges attract, these ions stay together and form an **ionic bond**, or a bond between ions. The elements bond together with the electron from one element staying predominantly with the other element. When Na^+ and Cl^- ions combine to produce NaCl, an electron from a sodium atom stays with the other seven from the chlorine atom, and the sodium and chloride ions attract each other in a lattice of ions with a net zero charge.

Covalent Bonds

Another type of strong chemical bond between two or more atoms is a **covalent bond**. These bonds form when an electron is shared between two elements and are the strongest and most common form of chemical bond in living organisms. Covalent bonds form between the elements that make up the biological molecules in our cells. Unlike ionic bonds, covalent bonds do not dissociate in water.

The hydrogen and oxygen atoms that combine to form water molecules are bound together by covalent bonds. The electron from the hydrogen atom divides its time between the outer shell of the hydrogen atom and the incomplete outer shell of the oxygen atom. To completely fill the outer shell of an oxygen atom, two electrons from two hydrogen atoms are needed, hence the subscript “2” in H_2O. The electrons are shared between the atoms, dividing their time between them to “fill” the outer shell of each. This sharing is a lower energy state for all of the atoms involved than if they existed without their outer shells filled.

There are two types of covalent bonds: polar and nonpolar. **Nonpolar covalent bonds** form between two atoms of the same element or between different elements that share the electrons equally. For example, an oxygen atom can bond with another oxygen atom to fill their outer shells. This association is nonpolar because the electrons will be equally distributed between each oxygen atom. Two covalent bonds form between the two oxygen atoms because oxygen requires two shared electrons to fill its outermost shell. Nitrogen atoms will form three covalent bonds (also called triple covalent) between two atoms of nitrogen because each nitrogen atom needs three electrons to fill its outermost shell. Another example of a nonpolar covalent bond is found in the methane (CH_4) molecule. The carbon atom has four electrons in its outermost shell and needs four more to fill it. It gets these four from four hydrogen atoms, each atom providing one. These elements all share the electrons equally, creating four nonpolar covalent bonds (**Figure 2.6**).

In a **polar covalent bond**, the electrons shared by the atoms spend more time closer to one nucleus than to the other nucleus. Because of the unequal distribution of electrons between the different nuclei, a slightly positive

(δ+) or slightly negative (δ–) charge develops. The covalent bonds between hydrogen and oxygen atoms in water are polar covalent bonds. The shared electrons spend more time near the oxygen nucleus, giving it a small negative charge, than they spend near the hydrogen nuclei, giving these molecules a small positive charge.

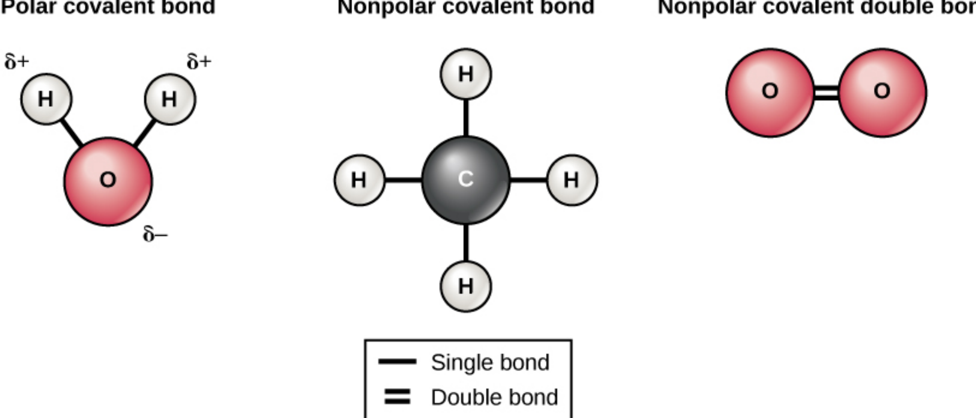


Figure 2.6 The water molecule (left) depicts a polar bond with a slightly positive charge on the hydrogen atoms and a slightly negative charge on the oxygen. Examples of nonpolar bonds include methane (middle) and oxygen (right).

Hydrogen Bonds

Ionic and covalent bonds are strong bonds that require considerable energy to break. However, not all bonds between elements are ionic or covalent bonds. Weaker bonds can also form. These are attractions that occur between positive and negative charges that do not require much energy to break. Two weak bonds that occur frequently are hydrogen bonds and van der Waals interactions. These bonds give rise to the unique properties of water and the unique structures of DNA and proteins.

When polar covalent bonds containing a hydrogen atom form, the hydrogen atom in that bond has a slightly positive charge. This is because the shared electron is pulled more strongly toward the other element and away from the hydrogen nucleus. Because the hydrogen atom is slightly positive (δ+), it will be attracted to neighboring negative partial charges (δ–). When this happens, a weak interaction occurs between the δ+ charge of the hydrogen atom of one molecule and the δ– charge of the other molecule. This interaction is called a **hydrogen bond**. This type of bond is common; for example, the liquid nature of water is caused by the hydrogen bonds between water molecules (Figure 2.7). Hydrogen bonds give water the unique properties that sustain life. If it were not for hydrogen bonding, water would be a gas rather than a liquid at room temperature.

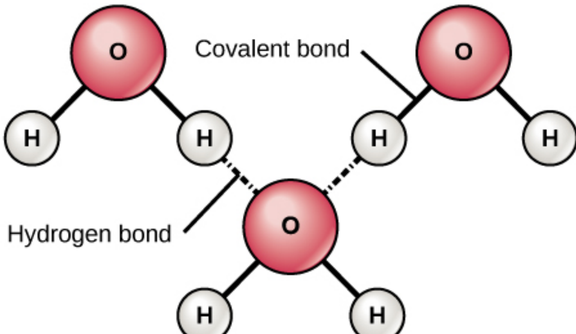


Figure 2.7 Hydrogen bonds form between slightly positive (*δ*+) and slightly negative (*δ*–) charges of polar covalent molecules, such as water.

Hydrogen bonds can form between different molecules and they do not always have to include a water molecule. Hydrogen atoms in polar bonds within any molecule can form bonds with other adjacent molecules. For example, hydrogen bonds hold together two long strands of DNA to give the DNA molecule its characteristic double-stranded structure. Hydrogen bonds are also responsible for some of the three-dimensional structure of proteins.

van der Waals Interactions

Like hydrogen bonds, **van der Waals interactions** are weak attractions or interactions between molecules. They occur between polar, covalently bound, atoms in different molecules. Some of these weak attractions are caused by temporary partial charges formed when electrons move around a nucleus. These weak interactions between

molecules are important in biological systems.

Radiography Technician

Have you or anyone you know ever had a magnetic resonance imaging (MRI) scan, a mammogram, or an X-ray? These tests produce images of your soft tissues and organs (as with an MRI or mammogram) or your bones (as happens in an X-ray) by using either radiowaves or special isotopes (radiolabeled or fluorescently labeled) that are ingested or injected into the body. These tests provide data for disease diagnoses by creating images of your organs or skeletal system.

MRI imaging works by subjecting hydrogen nuclei, which are abundant in the water in soft tissues, to fluctuating magnetic fields, which cause them to emit their own magnetic field. This signal is then read by sensors in the machine and interpreted by a computer to form a detailed image.

Some radiography technologists and technicians specialize in computed tomography, MRI, and mammography. They produce films or images of the body that help medical professionals examine and diagnose. Radiologists work directly with patients, explaining machinery, preparing them for exams, and ensuring that their body or body parts are positioned correctly to produce the needed images. Physicians or radiologists then analyze the test results.

Radiography technicians can work in hospitals, doctors' offices, or specialized imaging centers. Training to become a radiography technician happens at hospitals, colleges, and universities that offer certificates, associate's degrees, or bachelor's degrees in radiography.

2.2 | Water

By the end of this section, you will be able to:

- Describe the properties of water that are critical to maintaining life

Do you ever wonder why scientists spend time looking for water on other planets? It is because water is essential to life; even minute traces of it on another planet can indicate that life could or did exist on that planet. Water is one of the more abundant molecules in living cells and the one most critical to life as we know it. Approximately 60–70 percent of your body is made up of water. Without it, life simply would not exist.

Water Is Polar

The hydrogen and oxygen atoms within water molecules form polar covalent bonds. The shared electrons spend more time associated with the oxygen atom than they do with hydrogen atoms. There is no overall charge to a water molecule, but there is a slight positive charge on each hydrogen atom and a slight negative charge on the oxygen atom. Because of these charges, the slightly positive hydrogen atoms repel each other and form the unique shape seen in **Figure 2.7**. Each water molecule attracts other water molecules because of the positive and negative charges in the different parts of the molecule. Water also attracts other polar molecules (such as sugars), forming hydrogen bonds. When a substance readily forms hydrogen bonds with water, it can dissolve in water and is referred to as **hydrophilic** ("water-loving"). Hydrogen bonds are not readily formed with nonpolar substances like oils and fats (**Figure 2.8**). These nonpolar compounds are **hydrophobic** ("water-fearing") and will not dissolve in water.

Figure 2.8 As this macroscopic image of oil and water show, oil is a nonpolar compound and, hence, will not dissolve in water. Oil and water do not mix. (credit: Gautam Dogra)

Water Stabilizes Temperature

The hydrogen bonds in water allow it to absorb and release heat energy more slowly than many other substances. **Temperature** is a measure of the motion (kinetic energy) of molecules. As the motion increases, energy is higher and thus temperature is higher. Water absorbs a great deal of energy before its temperature rises. Increased energy disrupts the hydrogen bonds between water molecules. Because these bonds can be created and disrupted rapidly, water absorbs an increase in energy and temperature changes only minimally. This means that water moderates temperature changes within organisms and in their environments. As energy input continues, the balance between hydrogen-bond formation and destruction swings toward the destruction side. More bonds are broken than are formed. This process results in the release of individual water molecules at the surface of the liquid (such as a body of water, the leaves of a plant, or the skin of an organism) in a process called **evaporation**. Evaporation of sweat, which is 90 percent water, allows for cooling of an organism, because breaking hydrogen bonds requires an input of energy and takes heat away from the body.

Conversely, as molecular motion decreases and temperatures drop, less energy is present to break the hydrogen bonds between water molecules. These bonds remain intact and begin to form a rigid, lattice-like structure (e.g., ice) (Figure 2.9**a**). When frozen, ice is less dense than liquid water (the molecules are farther apart). This means that ice floats on the surface of a body of water (Figure 2.9**b**). In lakes, ponds, and oceans, ice will form on the surface of the water, creating an insulating barrier to protect the animal and plant life beneath from freezing in the water. If this did not happen, plants and animals living in water would freeze in a block of ice and could not move freely, making life in cold temperatures difficult or impossible.

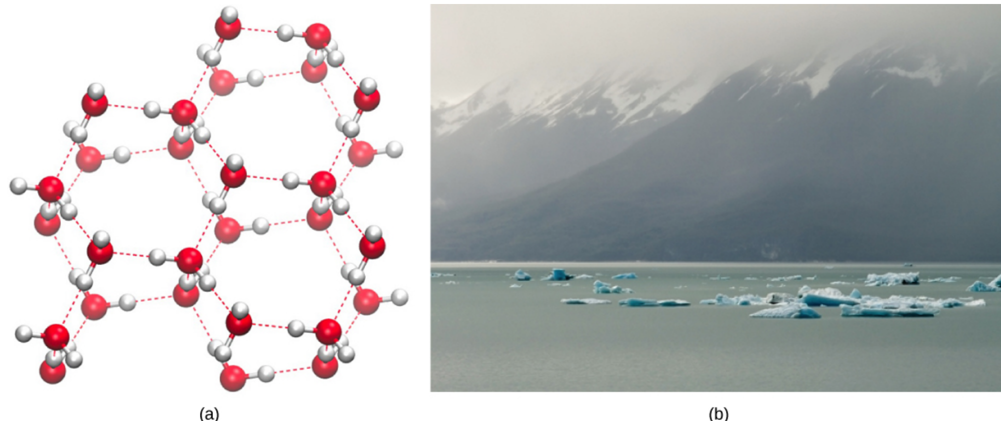


Figure 2.9 (a) The lattice structure of ice makes it less dense than the freely flowing molecules of liquid water. Ice's lower density enables it to (b) float on water. (credit a: modification of work by Jane Whitney; credit b: modification of work by Carlos Ponte)

Click **here (http://openstax.org/l/ice_lattice)** to see a 3-D animation of the structure of an ice lattice.

Water Is an Excellent Solvent

Because water is polar, with slight positive and negative charges, ionic compounds and polar molecules can readily dissolve in it. Water is, therefore, what is referred to as a **solvent**—a substance capable of dissolving another substance. The charged particles will form hydrogen bonds with a surrounding layer of water molecules. This is referred to as a sphere of hydration and serves to keep the particles separated or dispersed in the water. In the case of table salt (NaCl) mixed in water (**Figure 2.10**), the sodium and chloride ions separate, or dissociate, in the water, and spheres of hydration are formed around the ions. A positively charged sodium ion is surrounded by the partially negative charges of oxygen atoms in water molecules. A negatively charged chloride ion is surrounded by the partially positive charges of hydrogen atoms in water molecules. These spheres of hydration are also referred to as hydration shells. The polarity of the water molecule makes it an effective solvent and is important in its many roles in living systems.

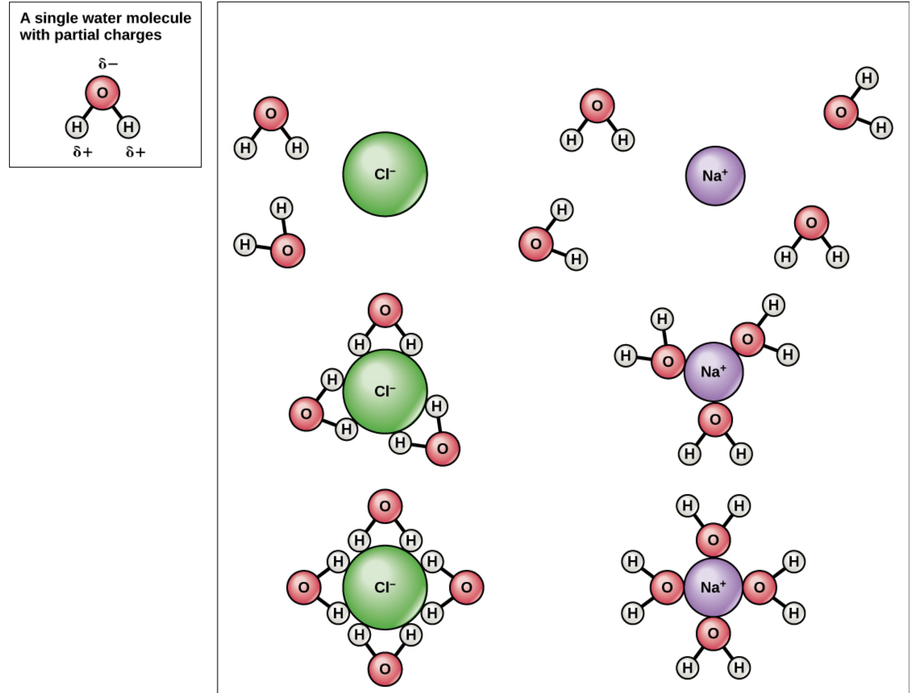


Figure 2.10 When table salt (NaCl) is mixed in water, spheres of hydration form around the ions.

Water Is Cohesive

Have you ever filled up a glass of water to the very top and then slowly added a few more drops? Before it overflows, the water actually forms a dome-like shape above the rim of the glass. This water can stay above the glass because of the property of **cohesion**. In cohesion, water molecules are attracted to each other (because of hydrogen bonding), keeping the molecules together at the liquid-air (gas) interface, although there is no more room in the glass. Cohesion gives rise to **surface tension**, the capacity of a substance to withstand rupture when placed under tension or stress. When you drop a small scrap of paper onto a droplet of water, the paper floats on top of the water droplet, although the object is denser (heavier) than the water. This occurs because of the surface tension that is created by the water molecules. Cohesion and surface tension keep the water molecules intact and the item floating on the top. It is even possible to “float” a steel needle on top of a glass of water if you place it gently, without breaking the surface tension (**Figure 2.11**).

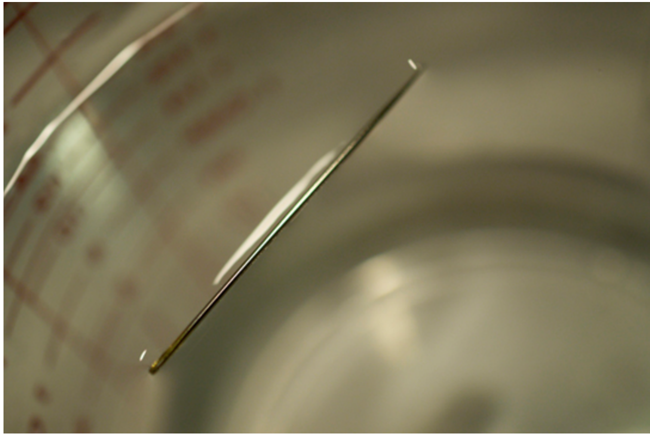


Figure 2.11 The weight of a needle on top of water pulls the surface tension downward; at the same time, the surface tension of the water is pulling it up, suspending the needle on the surface of the water and keeping it from sinking. Notice the indentation in the water around the needle. (credit: Cory Zanker)

These cohesive forces are also related to the water's property of **adhesion**, or the attraction between water molecules and other molecules. This is observed when water "climbs" up a straw placed in a glass of water. You will notice that the water appears to be higher on the sides of the straw than in the middle. This is because the water molecules are attracted to the straw and therefore adhere to it.

Cohesive and adhesive forces are important for sustaining life. For example, because of these forces, water can flow up from the roots to the tops of plants to feed the plant.

To learn more about water, visit the U.S. Geological Survey Water Science for Schools: All About Water! **website. (http://openstax.org/l/about_water)**

Buffers, pH, Acids, and Bases

The pH of a solution is a measure of its acidity or bascicity. You have probably used **litmus paper**, paper that has been treated with a natural water-soluble dye so it can be used as a pH indicator, to test how much acid or base (basicity) exists in a solution. You might have even used some to make sure the water in an outdoor swimming pool is properly treated. In both cases, this pH test measures the amount of hydrogen ions that exists in a given solution. High concentrations of hydrogen ions yield a low pH, whereas low levels of hydrogen ions result in a high pH. The overall concentration of hydrogen ions is inversely related to its pH and can be measured on the **pH scale** (Figure 2.12). Therefore, the more hydrogen ions present, the lower the pH; conversely, the fewer hydrogen ions, the higher the pH.

The pH scale ranges from 0 to 14. A change of one unit on the pH scale represents a change in the concentration of hydrogen ions by a factor of 10, a change in two units represents a change in the concentration of hydrogen ions by a factor of 100. Thus, small changes in pH represent large changes in the concentrations of hydrogen ions. Pure water is neutral. It is neither acidic nor basic, and has a pH of 7.0. Anything below 7.0 (ranging from 0.0 to 6.9) is acidic, and anything above 7.0 (from 7.1 to 14.0) is alkaline. The blood in your veins is slightly alkaline (pH = 7.4). The environment in your stomach is highly acidic (pH = 1 to 2). Orange juice is mildly acidic (pH = approximately 3.5), whereas baking soda is basic (pH = 9.0).

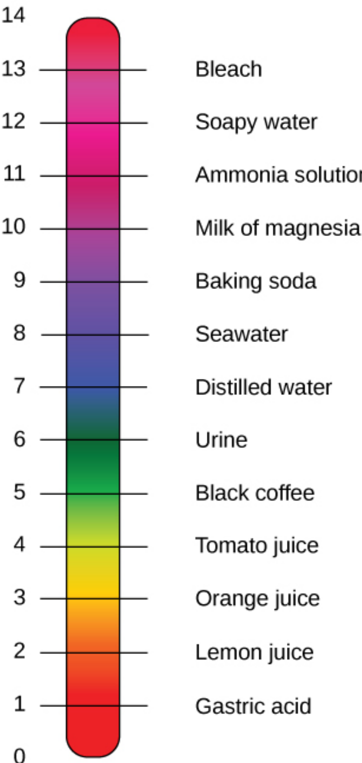


Figure 2.12 The pH scale measures the amount of hydrogen ions (H^+) in a substance. (credit: modification of work by Edward Stevens)

Acids are substances that provide hydrogen ions (H^+) and lower pH, whereas **bases** provide hydroxide ions ($OH^–$) and raise pH. The stronger the acid, the more readily it donates H^+. For example, hydrochloric acid and lemon juice are very acidic and readily give up H^+ when added to water. Conversely, bases are those substances that readily donate $OH^–$. The $OH^–$ ions combine with H^+ to produce water, which raises a substance's pH. Sodium hydroxide and many household cleaners are very alkaline and give up $OH^–$ rapidly when placed in water, thereby raising the pH.

Most cells in our bodies operate within a very narrow window of the pH scale, typically ranging only from 7.2 to 7.6. If the pH of the body is outside of this range, the respiratory system malfunctions, as do other organs in the body. Cells no longer function properly, and proteins will break down. Deviation outside of the pH range can induce coma or even cause death.

So how is it that we can ingest or inhale acidic or basic substances and not die? Buffers are the key. **Buffers** readily absorb excess H^+ or $OH^–$, keeping the pH of the body carefully maintained in the aforementioned narrow range. Carbon dioxide is part of a prominent buffer system in the human body; it keeps the pH within the proper range. This buffer system involves carbonic acid (H_2CO_3) and bicarbonate ($HCO_3^–$) anion. If too much H^+ enters the body, bicarbonate will combine with the H^+ to create carbonic acid and limit the decrease in pH. Likewise, if too much $OH^–$ is introduced into the system, carbonic acid will rapidly dissociate into bicarbonate and H^+ ions. The H^+ ions can combine with the $OH^–$ ions, limiting the increase in pH. While carbonic acid is an important product in this reaction, its presence is fleeting because the carbonic acid is released from the body as carbon dioxide gas each time we breathe. Without this buffer system, the pH in our bodies would fluctuate too much and we would fail to survive.

2.3 | Human Blood Buffering Systems

By the end of this section, you will be able to do the following:

- Describe how oxygen is bound to hemoglobin and transported to body tissues
- Explain how carbon dioxide is transported from body tissues to the lungs

Buffering System in Action: Bicarbonate

There are many critical buffering systems in the human body responsible for maintaining homeostasis. In the following example, you will explore the transport of oxygen in the blood and factors that affect oxygen binding to hemoglobin and how the bicarbonate buffer system carries CO2 to keep your blood pH in balance.

Once the oxygen diffuses across the alveoli, it enters the bloodstream and is transported to the tissues where it is unloaded, and carbon dioxide diffuses out of the blood and into the alveoli to be expelled from the body. Although gas exchange is a continuous process, the oxygen and carbon dioxide are transported by different mechanisms.

Transport of Oxygen in the Blood

Although oxygen dissolves in blood, only a small amount of oxygen is transported this way. Only 1.5 percent of oxygen in the blood is dissolved directly into the blood itself. Most oxygen—98.5 percent—is bound to a protein called hemoglobin and carried to the tissues.

Hemoglobin

Hemoglobin, or Hb, is a protein molecule found in red blood cells (erythrocytes) made of four subunits: two alpha subunits and two beta subunits (Figure 2.13). Each subunit surrounds a central **heme group** that contains iron and binds one oxygen molecule, allowing each hemoglobin molecule to bind four oxygen molecules. Molecules with more oxygen bound to the heme groups are brighter red. As a result, oxygenated arterial blood where the Hb is carrying four oxygen molecules is bright red, while venous blood that is deoxygenated is darker red.

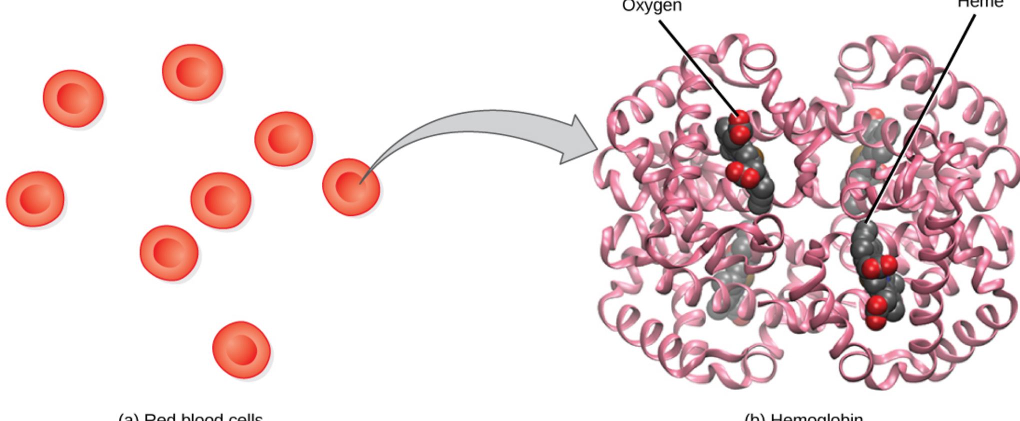


Figure 2.13 The protein inside (a) red blood cells that carries oxygen to cells and carbon dioxide to the lungs is (b) hemoglobin. Hemoglobin is made up of four symmetrical subunits and four heme groups. Iron associated with the heme binds oxygen. It is the iron in hemoglobin that gives blood its red color.

It is easier to bind a second and third oxygen molecule to Hb than the first molecule. This is because the hemoglobin molecule changes its shape, or conformation, as oxygen binds. The fourth oxygen is then more difficult to bind. The binding of oxygen to hemoglobin can be plotted as a function of the partial pressure of oxygen in the blood (x-axis) versus the relative Hb-oxygen saturation (y-axis). The resulting graph—an **oxygen dissociation curve**—is sigmoidal, or S-shaped (Figure 2.14). As the partial pressure of oxygen increases, the hemoglobin becomes increasingly saturated with oxygen.

Visual Connection

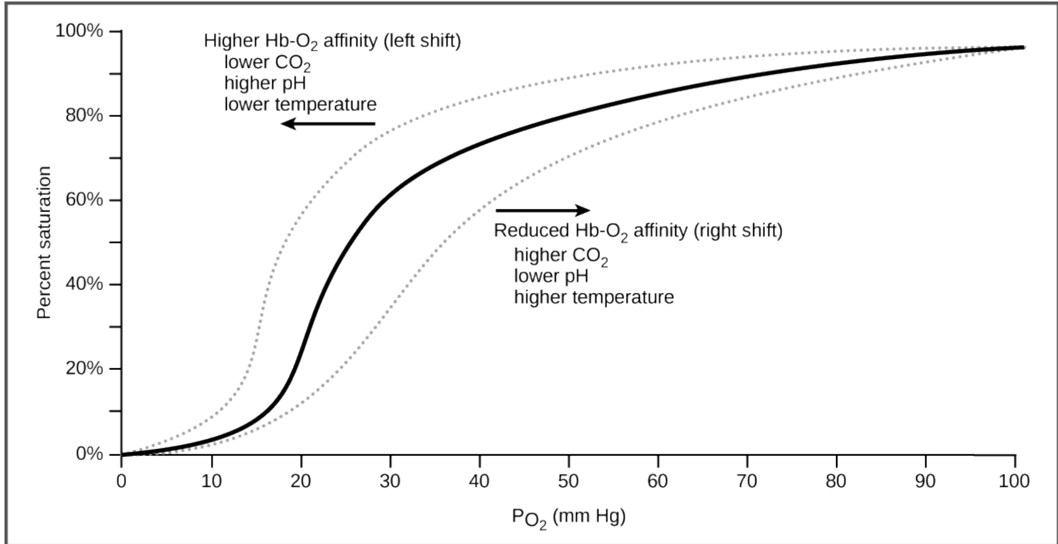


Figure 2.14 The oxygen dissociation curve demonstrates that, as the partial pressure of oxygen increases, more oxygen binds hemoglobin. However, the affinity of hemoglobin for oxygen may shift to the left or the right depending on environmental conditions.

The kidneys are responsible for removing excess H+ ions from the blood. If the kidneys fail, what would happen to blood pH and to hemoglobin affinity for oxygen?

Factors That Affect Oxygen Binding

The **oxygen-carrying capacity** of hemoglobin determines how much oxygen is carried in the blood. In addition to P_{O_2}, other environmental factors and diseases can affect oxygen carrying capacity and delivery.

Carbon dioxide levels, blood pH, and body temperature affect oxygen-carrying capacity (**Figure 2.14**). When carbon dioxide is in the blood, it reacts with water to form bicarbonate (HCO_3^-) and hydrogen ions (H^+). As the level of carbon dioxide in the blood increases, more H^+ is produced and the pH decreases. This increase in carbon dioxide and subsequent decrease in pH reduce the affinity of hemoglobin for oxygen. The oxygen dissociates from the Hb molecule, shifting the oxygen dissociation curve to the right. Therefore, more oxygen is needed to reach the same hemoglobin saturation level as when the pH was higher. A similar shift in the curve also results from an increase in body temperature. Increased temperature, such as from increased activity of skeletal muscle, causes the affinity of hemoglobin for oxygen to be reduced.

Diseases like sickle cell anemia and thalassemia decrease the blood’s ability to deliver oxygen to tissues and its oxygen-carrying capacity. In **sickle cell anemia**, the shape of the red blood cell is crescent-shaped, elongated, and stiffened, reducing its ability to deliver oxygen (**Figure 2.15**). In this form, red blood cells cannot pass through the capillaries. This is painful when it occurs. **Thalassemia** is a rare genetic disease caused by a defect in either the alpha or the beta subunit of Hb. Patients with thalassemia produce a high number of red blood cells, but these cells have lower-than-normal levels of hemoglobin. Therefore, the oxygen-carrying capacity is diminished.

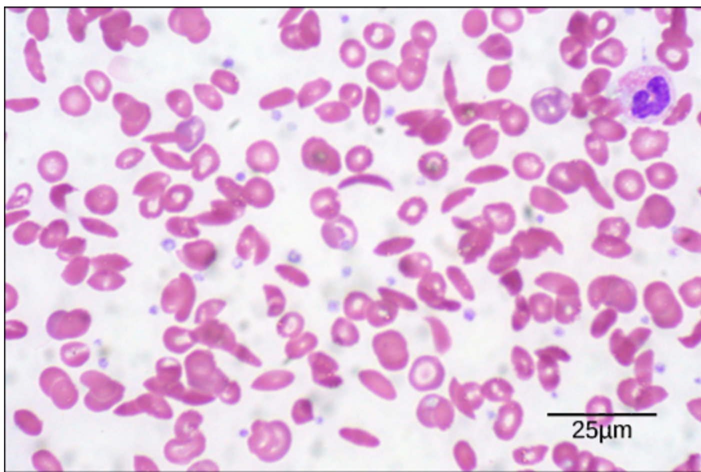


Figure 2.15 Individuals with sickle cell anemia have crescent-shaped red blood cells. (credit: modification of work by Ed Uthman; scale-bar data from Matt Russell)

Transport of Carbon Dioxide in the Blood

Carbon dioxide molecules are transported in the blood from body tissues to the lungs by one of three methods: dissolution directly into the blood, binding to hemoglobin, or carried as a bicarbonate ion. Several properties of carbon dioxide in the blood affect its transport. First, carbon dioxide is more soluble in blood than oxygen. About 5 to 7 percent of all carbon dioxide is dissolved in the plasma. Second, carbon dioxide can bind to plasma proteins or can enter red blood cells and bind to hemoglobin. This form transports about 10 percent of the carbon dioxide. When carbon dioxide binds to hemoglobin, a molecule called **carbaminohemoglobin** is formed. Binding of carbon dioxide to hemoglobin is reversible. Therefore, when it reaches the lungs, the carbon dioxide can freely dissociate from the hemoglobin and be expelled from the body.

Third, the majority of carbon dioxide molecules (85 percent) are carried as part of the **bicarbonate buffer system**. In this system, carbon dioxide diffuses into the red blood cells. **Carbonic anhydrase (CA)** within the red blood cells quickly converts the carbon dioxide into carbonic acid (H_2CO_3). Carbonic acid is an unstable intermediate molecule that immediately dissociates into **bicarbonate ions** $(\mathbf{HCO_3^-})$ and hydrogen (H^+) ions. Since carbon dioxide is quickly converted into bicarbonate ions, this reaction allows for the continued uptake of carbon dioxide into the blood down its concentration gradient. It also results in the production of H^+ ions. If too much H^+ is produced, it can alter blood pH. However, hemoglobin binds to the free H^+ ions and thus limits shifts in pH. The newly synthesized bicarbonate ion is transported out of the red blood cell into the liquid component of the blood in exchange for a chloride ion (Cl^-); this is called the **chloride shift**. When the blood reaches the lungs, the bicarbonate ion is transported back into the red blood cell in exchange for the chloride ion. The H^+ ion dissociates from the hemoglobin and binds to the bicarbonate ion. This produces the carbonic acid intermediate, which is converted back into carbon dioxide through the enzymatic action of CA. The carbon dioxide produced is expelled through the lungs during exhalation.

$$\mathrm{CO_2 + H_2O} \leftrightarrow \underset{\text{(carbonic acid)}}{\mathrm{H_2CO_3}} \leftrightarrow \underset{\text{(bicarbonate)}}{\mathrm{HCO_3 + H^+}}$$

The benefit of the bicarbonate buffer system is that carbon dioxide is “soaked up” into the blood with little change to the pH of the system. This is important because it takes only a small change in the overall pH of the body for severe injury or death to result. The presence of this bicarbonate buffer system also allows for people to travel and live at high altitudes: When the partial pressure of oxygen and carbon dioxide change at high altitudes, the bicarbonate buffer system adjusts to regulate carbon dioxide while maintaining the correct pH in the body.

Carbon Monoxide Poisoning

While carbon dioxide can readily associate and dissociate from hemoglobin, other molecules such as carbon monoxide (CO) cannot. Carbon monoxide has a greater affinity for hemoglobin than oxygen. Therefore, when carbon monoxide is present, it binds to hemoglobin preferentially over oxygen. As a result, oxygen cannot bind to hemoglobin, so very little oxygen is transported through the body (**Figure 2.16**). Carbon monoxide is a colorless, odorless gas and is therefore difficult to detect. It is produced by gas-powered vehicles and tools. Carbon

monoxide can cause headaches, confusion, and nausea; long-term exposure can cause brain damage or death. Administering 100 percent (pure) oxygen is the usual treatment for carbon monoxide poisoning. Administration of pure oxygen speeds up the separation of carbon monoxide from hemoglobin.

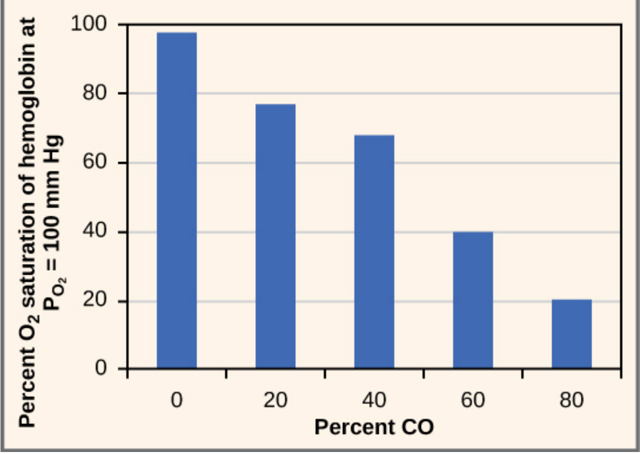


Figure 2.16 As percent CO increases, the oxygen saturation of hemoglobin decreases.

2.4 | Biological Molecules

By the end of this section, you will be able to:

- Describe the ways in which carbon is critical to life
- Explain the impact of slight changes in amino acids on organisms
- Describe the four major types of biological molecules
- Understand the functions of the four major types of molecules

The large molecules necessary for life that are built from smaller organic molecules are called biological **macromolecules**. There are four major classes of biological macromolecules (carbohydrates, lipids, proteins, and nucleic acids), and each is an important component of the cell and performs a wide array of functions. Combined, these molecules make up the majority of a cell's dry mass. Biological macromolecules are organic, meaning that they contain carbon (with some exceptions, like carbon dioxide). In addition, they may contain hydrogen, oxygen, nitrogen, phosphorus, sulfur, and additional minor elements.

Carbon

It is often said that life is "carbon-based." This means that carbon atoms, bonded to other carbon atoms or other elements, form the fundamental components of many, if not most, of the molecules found uniquely in living things. Other elements play important roles in biological molecules, but carbon certainly qualifies as the "foundation" element for molecules in living things. It is the bonding properties of carbon atoms that are responsible for its important role.

Carbon Bonding

Carbon contains four electrons in its outer shell. Therefore, it can form four covalent bonds with other atoms or molecules. The simplest organic carbon molecule is methane (CH_4), in which four hydrogen atoms bind to a carbon atom (**Figure 2.17**).

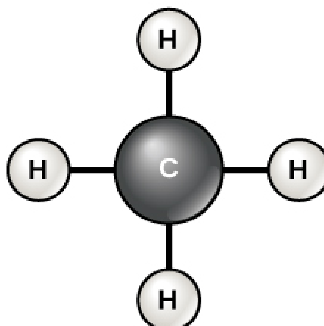


Figure 2.17 Carbon can form four covalent bonds to create an organic molecule. The simplest carbon molecule is methane (CH_4), depicted here.

However, structures that are more complex are made using carbon. Any of the hydrogen atoms can be replaced with another carbon atom covalently bonded to the first carbon atom. In this way, long and branching chains of carbon compounds can be made (**Figure 2.18a**). The carbon atoms may bond with atoms of other elements, such as nitrogen, oxygen, and phosphorus (**Figure 2.18b**). The molecules may also form rings, which themselves can link with other rings (**Figure 2.18c**). This diversity of molecular forms accounts for the diversity of functions of the biological macromolecules and is based to a large degree on the ability of carbon to form multiple bonds with itself and other atoms.

(a)

(b)

(c)

Figure 2.18 These examples show three molecules (found in living organisms) that contain carbon atoms bonded in various ways to other carbon atoms and the atoms of other elements. (a) This molecule of stearic acid has a long chain of carbon atoms. (b) Glycine, a component of proteins, contains carbon, nitrogen, oxygen, and hydrogen atoms. (c) Glucose, a sugar, has a ring of carbon atoms and one oxygen atom.

Carbohydrates

Carbohydrates are macromolecules with which most consumers are somewhat familiar. To lose weight, some individuals adhere to “low-carb” diets. Athletes, in contrast, often “carb-load” before important competitions to ensure that they have sufficient energy to compete at a high level. Carbohydrates are, in fact, an essential part of our diet; grains, fruits, and vegetables are all natural sources of carbohydrates. Carbohydrates provide energy to the body, particularly through glucose, a simple sugar. Carbohydrates also have other important functions in

humans, animals, and plants.

Carbohydrates can be represented by the formula $(CH_2O)_n$, where *n* is the number of carbon atoms in the molecule. In other words, the ratio of carbon to hydrogen to oxygen is 1:2:1 in carbohydrate molecules. Carbohydrates are classified into three subtypes: monosaccharides, disaccharides, and polysaccharides.

Monosaccharides (mono- = “one”; sacchar- = “sweet”) are simple sugars, the most common of which is glucose. In monosaccharides, the number of carbon atoms usually ranges from three to six. Most monosaccharide names end with the suffix -ose. Depending on the number of carbon atoms in the sugar, they may be known as trioses (three carbon atoms), pentoses (five carbon atoms), and hexoses (six carbon atoms).

Monosaccharides may exist as a linear chain or as ring-shaped molecules; in aqueous solutions, they are usually found in the ring form.

The chemical formula for glucose is $C_6H_{12}O_6$. In most living species, glucose is an important source of energy. During cellular respiration, energy is released from glucose, and that energy is used to help make adenosine triphosphate (ATP). Plants synthesize glucose using carbon dioxide and water by the process of photosynthesis, and the glucose, in turn, is used for the energy requirements of the plant. The excess synthesized glucose is often stored as starch that is broken down by other organisms that feed on plants.

Galactose (part of lactose, or milk sugar) and fructose (found in fruit) are other common monosaccharides. Although glucose, galactose, and fructose all have the same chemical formula ($C_6H_{12}O_6$), they differ structurally and chemically (and are known as isomers) because of differing arrangements of atoms in the carbon chain (Figure 2.19).

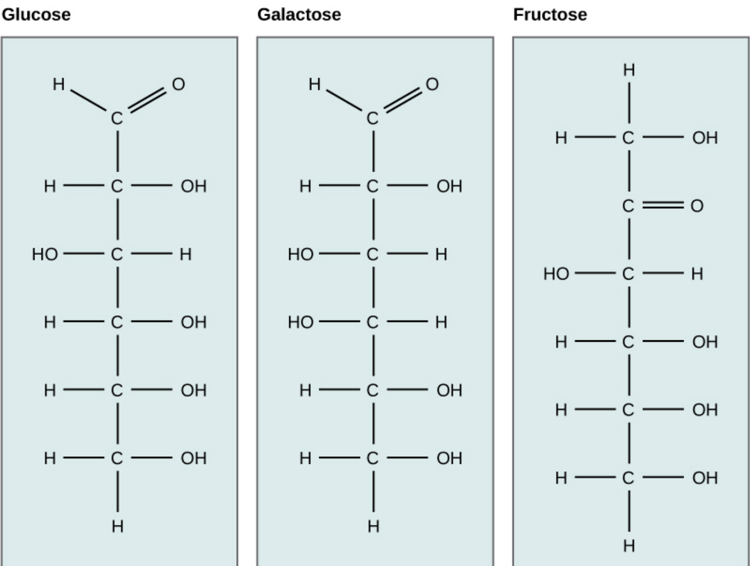


Figure 2.19 Glucose, galactose, and fructose are isomeric monosaccharides, meaning that they have the same chemical formula but slightly different structures.

Disaccharides (di- = “two”) form when two monosaccharides undergo a dehydration reaction (a reaction in which the removal of a water molecule occurs). During this process, the hydroxyl group (–OH) of one monosaccharide combines with a hydrogen atom of another monosaccharide, releasing a molecule of water (H_2O) and forming a covalent bond between atoms in the two sugar molecules.

Common disaccharides include lactose, maltose, and sucrose. Lactose is a disaccharide consisting of the monomers glucose and galactose. It is found naturally in milk. Maltose, or malt sugar, is a disaccharide formed from a dehydration reaction between two glucose molecules. The most common disaccharide is sucrose, or table sugar, which is composed of the monomers glucose and fructose.

A long chain of monosaccharides linked by covalent bonds is known as a **polysaccharide** (poly- = “many”). The chain may be branched or unbranched, and it may contain different types of monosaccharides. Polysaccharides may be very large molecules. Starch, glycogen, cellulose, and chitin are examples of polysaccharides.

Starch is the stored form of sugars in plants and is made up of amylose and amylopectin (both polymers of glucose). Plants are able to synthesize glucose, and the excess glucose is stored as starch in different plant parts, including roots and seeds. The starch that is consumed by animals is broken down into smaller molecules,

such as glucose. The cells can then absorb the glucose.

Glycogen is the storage form of glucose in humans and other vertebrates, and is made up of monomers of glucose. Glycogen is the animal equivalent of starch and is a highly branched molecule usually stored in liver and muscle cells. Whenever glucose levels decrease, glycogen is broken down to release glucose.

Cellulose is one of the most abundant natural biopolymers. The cell walls of plants are mostly made of cellulose, which provides structural support to the cell. Wood and paper are mostly cellulosic in nature. Cellulose is made up of glucose monomers that are linked by bonds between particular carbon atoms in the glucose molecule.

Every other glucose monomer in cellulose is flipped over and packed tightly as extended long chains. This gives cellulose its rigidity and high tensile strength—which is so important to plant cells. Cellulose passing through our digestive system is called dietary fiber. While the glucose-glucose bonds in cellulose cannot be broken down by human digestive enzymes, herbivores such as cows, buffalos, and horses are able to digest grass that is rich in cellulose and use it as a food source. In these animals, certain species of bacteria reside in the digestive system of herbivores and secrete the enzyme cellulase. The appendix also contains bacteria that break down cellulose, giving it an important role in the digestive systems of some ruminants. Cellulases can break down cellulose into glucose monomers that can be used as an energy source by the animal.

Carbohydrates serve other functions in different animals. Arthropods, such as insects, spiders, and crabs, have an outer skeleton, called the exoskeleton, which protects their internal body parts. This exoskeleton is made of the biological macromolecule **chitin**, which is a nitrogenous carbohydrate. It is made of repeating units of a modified sugar containing nitrogen.

Thus, through differences in molecular structure, carbohydrates are able to serve the very different functions of energy storage (starch and glycogen) and structural support and protection (cellulose and chitin) (**Figure 2.20**).

Figure 2.20 Although their structures and functions differ, all polysaccharide carbohydrates are made up of monosaccharides and have the chemical formula $(CH_2O)n$.

Registered Dietitian

Obesity is a worldwide health concern, and many diseases, such as diabetes and heart disease, are becoming more prevalent because of obesity. This is one of the reasons why registered dietitians are increasingly sought after for advice. Registered dietitians help plan food and nutrition programs for individuals in various settings. They often work with patients in health-care facilities, designing nutrition plans to prevent and treat diseases. For example, dietitians may teach a patient with diabetes how to manage blood-sugar levels by eating the correct types and amounts of carbohydrates. Dietitians may also work in nursing homes, schools, and private practices.

To become a registered dietitian, one needs to earn at least a bachelor's degree in dietetics, nutrition, food technology, or a related field. In addition, registered dietitians must complete a supervised internship program and pass a national exam. Those who pursue careers in dietetics take courses in nutrition, chemistry, biochemistry, biology, microbiology, and human physiology. Dietitians must become experts in the chemistry and functions of food (proteins, carbohydrates, and fats).

Lipids

Lipids include a diverse group of compounds that are united by a common feature. **Lipids** are hydrophobic ("water-fearing"), or insoluble in water, because they are nonpolar molecules. This is because they are hydrocarbons that include only nonpolar carbon-carbon or carbon-hydrogen bonds. Lipids perform many different functions in a cell. Cells store energy for long-term use in the form of lipids called fats. Lipids also provide insulation from the environment for plants and animals (**Figure 2.21**). For example, they help keep aquatic birds and mammals dry because of their water-repelling nature. Lipids are also the building blocks of many hormones and are an important constituent of the plasma membrane. Lipids include fats, oils, waxes, phospholipids, and steroids.

Figure 2.21 Hydrophobic lipids in the fur of aquatic mammals, such as this river otter, protect them from the elements. (credit: Ken Bosma)

A **fat** molecule, such as a triglyceride, consists of two main components—glycerol and fatty acids. Glycerol is an organic compound with three carbon atoms, five hydrogen atoms, and three hydroxyl (–OH) groups. Fatty acids have a long chain of hydrocarbons to which an acidic carboxyl group is attached, hence the name "fatty acid." The number of carbons in the fatty acid may range from 4 to 36; most common are those containing 12–18 carbons. In a fat molecule, a fatty acid is attached to each of the three oxygen atoms in the –OH groups of the glycerol molecule with a covalent bond (**Figure 2.22**).

Figure 2.22 Lipids include fats, such as triglycerides, which are made up of fatty acids and glycerol, phospholipids, and steroids.

During this covalent bond formation, three water molecules are released. The three fatty acids in the fat may be similar or dissimilar. These fats are also called **triglycerides** because they have three fatty acids. Some fatty acids have common names that specify their origin. For example, palmitic acid, a saturated fatty acid, is derived from the palm tree. Arachidic acid is derived from *Arachis hypogaea*, the scientific name for peanuts.

Fatty acids may be saturated or unsaturated. In a fatty acid chain, if there are only single bonds between neighboring carbons in the hydrocarbon chain, the fatty acid is saturated. **Saturated fatty acids** are saturated with hydrogen; in other words, the number of hydrogen atoms attached to the carbon skeleton is maximized.

When the hydrocarbon chain contains a double bond, the fatty acid is an **unsaturated fatty acid**.

Most unsaturated fats are liquid at room temperature and are called **oils**. If there is one double bond in the molecule, then it is known as a monounsaturated fat (e.g., olive oil), and if there is more than one double bond, then it is known as a polyunsaturated fat (e.g., canola oil).

Saturated fats tend to get packed tightly and are solid at room temperature. Animal fats with stearic acid and palmitic acid contained in meat, and the fat with butyric acid contained in butter, are examples of saturated fats. Mammals store fats in specialized cells called adipocytes, where globules of fat occupy most of the cell. In plants, fat or oil is stored in seeds and is used as a source of energy during embryonic development.

Unsaturated fats or oils are usually of plant origin and contain unsaturated fatty acids. The double bond causes a bend or a “kink” that prevents the fatty acids from packing tightly, keeping them liquid at room temperature. Olive oil, corn oil, canola oil, and cod liver oil are examples of unsaturated fats. Unsaturated fats help to improve blood cholesterol levels, whereas saturated fats contribute to plaque formation in the arteries, which increases the risk of a heart attack.

In the food industry, oils are artificially hydrogenated to make them semi-solid, leading to less spoilage and increased shelf life. Simply speaking, hydrogen gas is bubbled through oils to solidify them. During this hydrogenation process, double bonds of the *cis*-conformation in the hydrocarbon chain may be converted to double bonds in the *trans*-conformation. This forms a ***trans*-fat** from a *cis*-fat. The orientation of the double bonds affects the chemical properties of the fat (Figure 2.23).

***cis*-fat molecule**

***trans*-fat molecule**

Figure 2.23 During the hydrogenation process, the orientation around the double bonds is changed, making a *trans*-fat from a *cis*-fat. This changes the chemical properties of the molecule.

Margarine, some types of peanut butter, and shortening are examples of artificially hydrogenated *trans*-fats. Recent studies have shown that an increase in *trans*-fats in the human diet may lead to an increase in levels of low-density lipoprotein (LDL), or "bad" cholesterol, which, in turn, may lead to plaque deposition in the arteries, resulting in heart disease. Many fast food restaurants have recently eliminated the use of *trans*-fats, and U.S. food labels are now required to list their *trans*-fat content.

Essential fatty acids are fatty acids that are required but not synthesized by the human body. Consequently, they must be supplemented through the diet. Omega-3 fatty acids fall into this category and are one of only two known essential fatty acids for humans (the other being omega-6 fatty acids). They are a type of polyunsaturated fat and are called omega-3 fatty acids because the third carbon from the end of the fatty acid participates in a double bond.

Salmon, trout, and tuna are good sources of omega-3 fatty acids. Omega-3 fatty acids are important in brain function and normal growth and development. They may also prevent heart disease and reduce the risk of cancer.

Like carbohydrates, fats have received a lot of bad publicity. It is true that eating an excess of fried foods and other "fatty" foods leads to weight gain. However, fats do have important functions. Fats serve as long-term energy storage. They also provide insulation for the body. Therefore, "healthy" unsaturated fats in moderate amounts should be consumed on a regular basis.

Phospholipids are the major constituent of the plasma membrane. Like fats, they are composed of fatty acid chains attached to a glycerol or similar backbone. Instead of three fatty acids attached, however, there are two fatty acids and the third carbon of the glycerol backbone is bound to a phosphate group. The phosphate group is modified by the addition of an alcohol.

A phospholipid has both hydrophobic and hydrophilic regions. The fatty acid chains are hydrophobic and exclude themselves from water, whereas the phosphate is hydrophilic and interacts with water.

Cells are surrounded by a membrane, which has a bilayer of phospholipids. The fatty acids of phospholipids face inside, away from water, whereas the phosphate group can face either the outside environment or the inside of the cell, which are both aqueous.

Steroids and Waxes

Unlike the phospholipids and fats discussed earlier, **steroids** have a ring structure. Although they do not resemble other lipids, they are grouped with them because they are also hydrophobic. All steroids have four, linked carbon rings and several of them, like cholesterol, have a short tail.

Cholesterol is a steroid. Cholesterol is mainly synthesized in the liver and is the precursor of many steroid hormones, such as testosterone and estradiol. It is also the precursor of vitamins E and K. Cholesterol is the precursor of bile salts, which help in the breakdown of fats and their subsequent absorption by cells. Although cholesterol is often spoken of in negative terms, it is necessary for the proper functioning of the body. It is a key

component of the plasma membranes of animal cells.

Waxes are made up of a hydrocarbon chain with an alcohol (–OH) group and a fatty acid. Examples of animal waxes include beeswax and lanolin. Plants also have waxes, such as the coating on their leaves, that helps prevent them from drying out.

For an additional perspective on lipids, explore “Biomolecules: The Lipids” through this interactive **animation (http://openstax.org/l/lipids)** .

Proteins

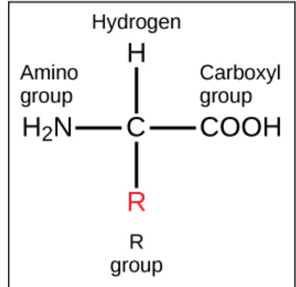
Proteins are one of the most abundant organic molecules in living systems and have the most diverse range of functions of all macromolecules. Proteins may be structural, regulatory, contractile, or protective; they may serve in transport, storage, or membranes; or they may be toxins or enzymes. Each cell in a living system may contain thousands of different proteins, each with a unique function. Their structures, like their functions, vary greatly. They are all, however, polymers of amino acids, arranged in a linear sequence.

The functions of proteins are very diverse because there are 20 different chemically distinct amino acids that form long chains, and the amino acids can be in any order. For example, proteins can function as enzymes or hormones. **Enzymes**, which are produced by living cells, are catalysts in biochemical reactions (like digestion) and are usually proteins. Each enzyme is specific for the substrate (a reactant that binds to an enzyme) upon which it acts. Enzymes can function to break molecular bonds, to rearrange bonds, or to form new bonds. An example of an enzyme is salivary amylase, which breaks down amylose, a component of starch.

Hormones are chemical signaling molecules, usually proteins or steroids, secreted by an endocrine gland or group of endocrine cells that act to control or regulate specific physiological processes, including growth, development, metabolism, and reproduction. For example, insulin is a protein hormone that maintains blood glucose levels.

Proteins have different shapes and molecular weights; some proteins are globular in shape whereas others are fibrous in nature. For example, hemoglobin is a globular protein, but collagen, found in our skin, is a fibrous protein. Protein shape is critical to its function. Changes in temperature, pH, and exposure to chemicals may lead to permanent changes in the shape of the protein, leading to a loss of function or **denaturation** (to be discussed in more detail later). All proteins are made up of different arrangements of the same 20 kinds of amino acids.

Amino acids are the monomers that make up proteins. Each amino acid has the same fundamental structure, which consists of a central carbon atom bonded to an amino group ($–NH_2$), a carboxyl group (–COOH), and a hydrogen atom. Every amino acid also has another variable atom or group of atoms bonded to the central carbon atom known as the R group. The R group is the only difference in structure between the 20 amino acids; otherwise, the amino acids are identical (**Figure 2.24**).



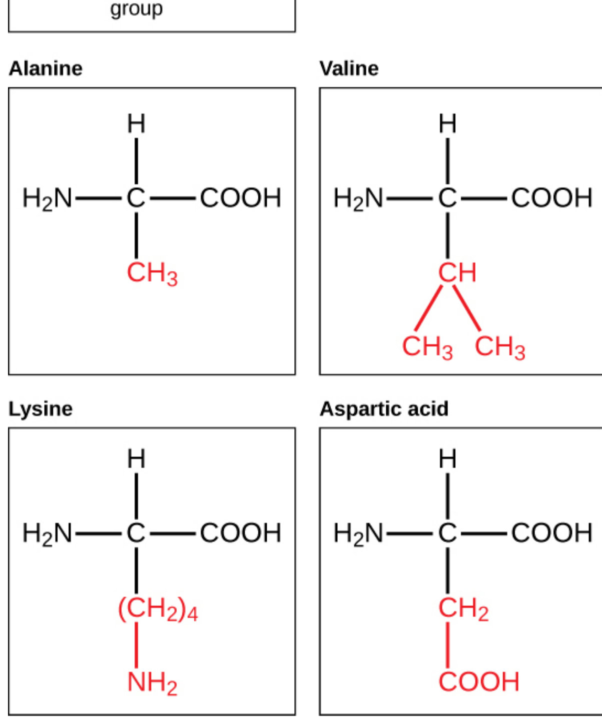


Figure 2.24 Amino acids are made up of a central carbon bonded to an amino group (–NH_2), a carboxyl group (–COOH), and a hydrogen atom. The central carbon's fourth bond varies among the different amino acids, as seen in these examples of alanine, valine, lysine, and aspartic acid.

The chemical nature of the R group determines the chemical nature of the amino acid within its protein (that is, whether it is acidic, basic, polar, or nonpolar).

The sequence and number of amino acids ultimately determine a protein's shape, size, and function. Each amino acid is attached to another amino acid by a covalent bond, known as a peptide bond, which is formed by a dehydration reaction. The carboxyl group of one amino acid and the amino group of a second amino acid combine, releasing a water molecule. The resulting bond is the peptide bond.

The products formed by such a linkage are called polypeptides. While the terms polypeptide and protein are sometimes used interchangeably, a **polypeptide** is technically a polymer of amino acids, whereas the term protein is used for a polypeptide or polypeptides that have combined together, have a distinct shape, and have a unique function.

evolution IN ACTION

The Evolutionary Significance of Cytochrome c

Cytochrome c is an important component of the molecular machinery that harvests energy from glucose. Because this protein's role in producing cellular energy is crucial, it has changed very little over millions of years. Protein sequencing has shown that there is a considerable amount of sequence similarity among cytochrome c molecules of different species; evolutionary relationships can be assessed by measuring the similarities or differences among various species' protein sequences.

For example, scientists have determined that human cytochrome c contains 104 amino acids. For each cytochrome c molecule that has been sequenced to date from different organisms, 37 of these amino acids appear in the same position in each cytochrome c. This indicates that all of these organisms are descended from a common ancestor. On comparing the human and chimpanzee protein sequences, no sequence difference was found. When human and rhesus monkey sequences were compared, a single difference was found in one amino acid. In contrast, human-to-yeast comparisons show a difference in 44 amino acids, suggesting that humans and chimpanzees have a more recent common ancestor than humans and the rhesus monkey, or humans and yeast.

Protein Structure

As discussed earlier, the shape of a protein is critical to its function. To understand how the protein gets its final shape or conformation, we need to understand the four levels of protein structure: primary, secondary, tertiary, and quaternary (**Figure 2.25**).

The unique sequence and number of amino acids in a polypeptide chain is its primary structure. The unique sequence for every protein is ultimately determined by the gene that encodes the protein. Any change in the gene sequence may lead to a different amino acid being added to the polypeptide chain, causing a change in protein structure and function. In sickle cell anemia, the hemoglobin β chain has a single amino acid substitution, causing a change in both the structure and function of the protein. What is most remarkable to consider is that a hemoglobin molecule is made up of two alpha chains and two beta chains that each consist of about 150 amino acids. The molecule, therefore, has about 600 amino acids. The structural difference between a normal hemoglobin molecule and a sickle cell molecule—that dramatically decreases life expectancy in the affected individuals—is a single amino acid of the 600.

Because of this change of one amino acid in the chain, the normally biconcave, or disc-shaped, red blood cells assume a crescent or "sickle" shape, which clogs arteries. This can lead to a myriad of serious health problems, such as breathlessness, dizziness, headaches, and abdominal pain for those who have this disease.

Folding patterns resulting from interactions between the non-R group portions of amino acids give rise to the secondary structure of the protein. The most common are the alpha (α)-helix and beta (β)-pleated sheet structures. Both structures are held in shape by hydrogen bonds. In the alpha helix, the bonds form between every fourth amino acid and cause a twist in the amino acid chain.

In the β-pleated sheet, the "pleats" are formed by hydrogen bonding between atoms on the backbone of the polypeptide chain. The R groups are attached to the carbons, and extend above and below the folds of the pleat. The pleated segments align parallel to each other, and hydrogen bonds form between the same pairs of atoms on each of the aligned amino acids. The α-helix and β-pleated sheet structures are found in many globular and fibrous proteins.

The unique three-dimensional structure of a polypeptide is known as its tertiary structure. This structure is caused by chemical interactions between various amino acids and regions of the polypeptide. Primarily, the interactions among R groups create the complex three-dimensional tertiary structure of a protein. There may be ionic bonds formed between R groups on different amino acids, or hydrogen bonding beyond that involved in the secondary structure. When protein folding takes place, the hydrophobic R groups of nonpolar amino acids lay in the interior of the protein, whereas the hydrophilic R groups lay on the outside. The former types of interactions are also known as hydrophobic interactions.

In nature, some proteins are formed from several polypeptides, also known as subunits, and the interaction of these subunits forms the quaternary structure. Weak interactions between the subunits help to stabilize the overall structure. For example, hemoglobin is a combination of four polypeptide subunits.

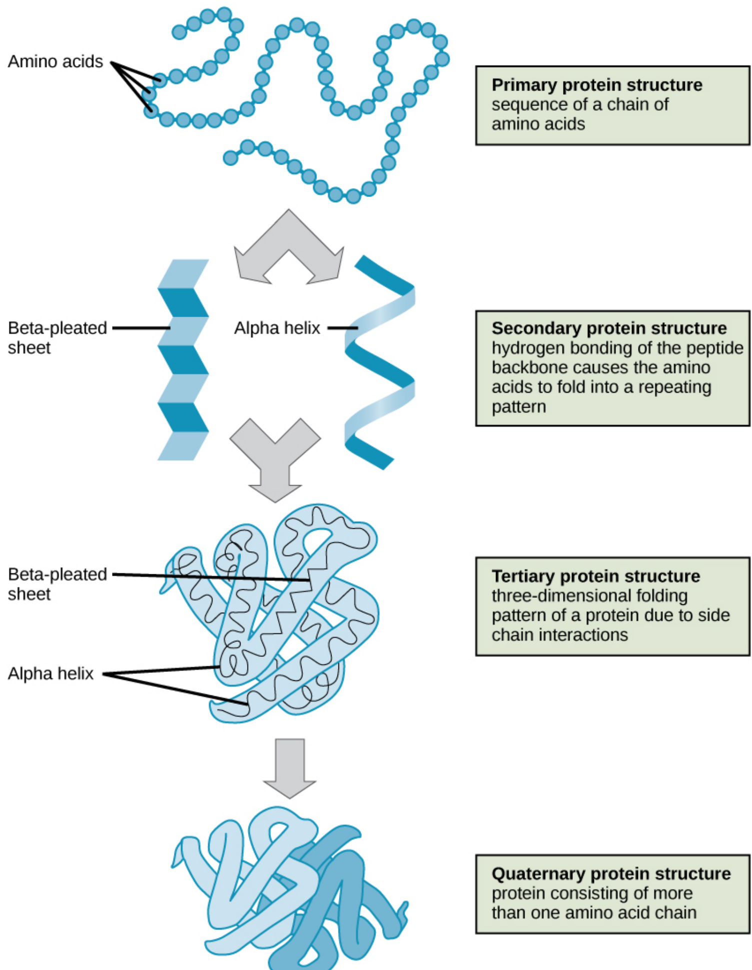


Figure 2.25 The four levels of protein structure can be observed in these illustrations. (credit: modification of work by National Human Genome Research Institute)

Each protein has its own unique sequence and shape held together by chemical interactions. If the protein is subject to changes in temperature, pH, or exposure to chemicals, the protein structure may change, losing its shape in what is known as denaturation as discussed earlier. Denaturation is often reversible because the primary structure is preserved if the denaturing agent is removed, allowing the protein to resume its function. Sometimes denaturation is irreversible, leading to a loss of function. One example of protein denaturation can be seen when an egg is fried or boiled. The albumin protein in the liquid egg white is denatured when placed in a hot pan, changing from a clear substance to an opaque white substance. Not all proteins are denatured at high temperatures; for instance, bacteria that survive in hot springs have proteins that are adapted to function at those temperatures.

For an additional perspective on proteins, explore “Biomolecules: The Proteins” through this interactive **animation (http://openstax.org/l/proteins)** .

Nucleic Acids

Nucleic acids are key macromolecules in the continuity of life. They carry the genetic blueprint of a cell and carry

instructions for the functioning of the cell.

The two main types of **nucleic acids** are **deoxyribonucleic acid (DNA)** and **ribonucleic acid (RNA)**. DNA is the genetic material found in all living organisms, ranging from single-celled bacteria to multicellular mammals.

The other type of nucleic acid, RNA, is mostly involved in protein synthesis. The DNA molecules never leave the nucleus, but instead use an RNA intermediary to communicate with the rest of the cell. Other types of RNA are also involved in protein synthesis and its regulation.

DNA and RNA are made up of monomers known as **nucleotides**. The nucleotides combine with each other to form a polynucleotide, DNA or RNA. Each nucleotide is made up of three components: a nitrogenous base, a pentose (five-carbon) sugar, and a phosphate group (Figure 2.26). Each nitrogenous base in a nucleotide is attached to a sugar molecule, which is attached to a phosphate group.

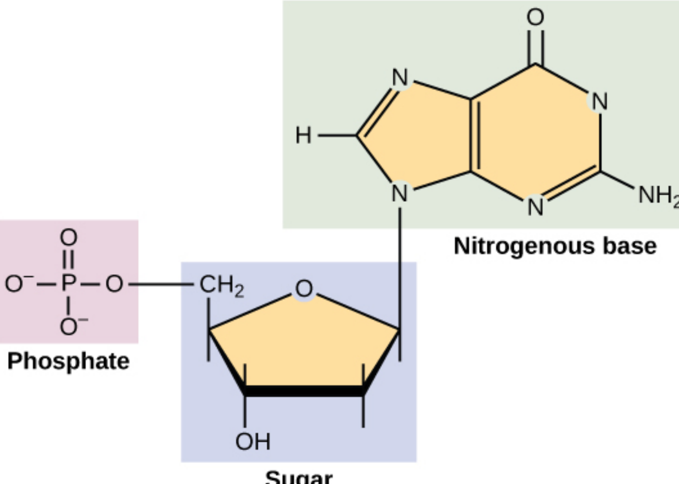


Figure 2.26 A nucleotide is made up of three components: a nitrogenous base, a pentose sugar, and a phosphate group.

DNA Double-Helical Structure

DNA has a double-helical structure (Figure 2.27). It is composed of two strands, or polymers, of nucleotides. The strands are formed with bonds between phosphate and sugar groups of adjacent nucleotides. The strands are bonded to each other at their bases with hydrogen bonds, and the strands coil about each other along their length, hence the “double helix” description, which means a double spiral.

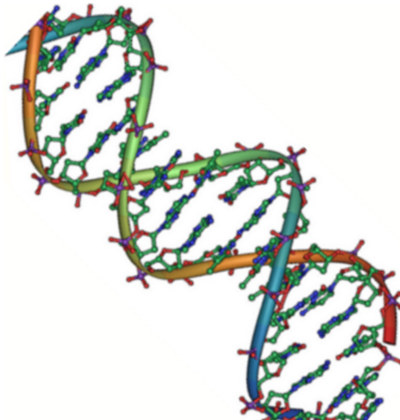


Figure 2.27 The double-helix model shows DNA as two parallel strands of intertwining molecules. (credit: Jerome Walker, Dennis Myts)

The alternating sugar and phosphate groups lie on the outside of each strand, forming the backbone of the DNA. The nitrogenous bases are stacked in the interior, like the steps of a staircase, and these bases pair; the pairs are bound to each other by hydrogen bonds. The bases pair in such a way that the distance between the backbones of the two strands is the same all along the molecule.

2.5 | Friedrich Miescher and the Structure of Nucleic Acids

Johannes Friedrich Miescher (13 August 1844 – 26 August 1895) was a Swiss physician and biologist, and the first researcher to isolate nucleic acid. Friedrich Miescher raised the idea that the nucleic acids could be involved in heredity.

FRIEDRICH MIESCHER & THE STRUCTURE OF NUCLEIC ACIDS

Johannes Friedrich Miescher (13 August 1844 – 26 August 1895) was a Swiss physician and biologist, and the ***first researcher to isolate*** **nucleic acid (https://en.wikipedia.org/wiki/Nucleic_acid)** . Miescher isolated various phosphate-rich chemicals, which he called nuclein (now known as nucleic acids!), from the nuclei of white blood cells in 1869 in Felix Hoppe-Seyler's laboratory at the University of Tübingen, Germany, paving the way for the identification of DNA as the carrier of inheritance. The significance of the discovery, first published in 1871, was not at first apparent, and it was Albrecht Kossel who made the initial inquiries into its chemical structure. Later, ***Friedrich Miescher raised the idea that the nucleic acids could be involved in heredity***.

Figure 2.28

Image: ***Johannes Friedrich Miescher*** (13 August 1844 – 26 August 1895). Image credit **http://www.pbs.org/wgbh/nova/photo51/images/befo-miescher.jpg (http://www.pbs.org/wgbh/nova/photo51/images/befo-miescher.jpg)**

After a typhus infection, Miescher lost hearing in one ear. Miescher felt that his partial deafness would be challenging as a doctor, so he turned to physiological chemistry. He originally wanted to study lymphocytes but was encouraged by Felix Hoppe-Seyler to study neutrophils. He was interested in studying the chemistry of the nucleus. Lymphocytes were difficult to obtain in enough numbers to study, while neutrophils were known to be one of the main and first components in pus and could be obtained from bandages at the nearby hospital. The problem was, however, washing the cells off the bandages without damaging them.

Miescher devised different salt solutions, eventually producing one with sodium sulfate. The cells were filtered. Since centrifuges were not available at the time, the cells were left to settle to the bottom of a beaker. He then tried to isolate the nuclei free of cytoplasm. He subjected the purified nuclei to an alkaline extraction followed by acidification, resulting in the formation of a precipitate that Miescher called nuclein. He found that this contained phosphorus and nitrogen, but not sulfur. The discovery was so unlike anything else at the time that Hoppe-Seyler repeated all of Miescher's research himself before publishing it in his journal. Miescher then went on to study physiology at Leipzig in the laboratory of Carl Ludwig for a year before being appointed a professor of physiology.

Although Miescher and his students aggressively researched nucleic acid chemistry, its function remained

unknown. However, his discovery played an important part in the identification of nucleic acids as the carriers of inheritance. The importance of Miescher's discovery was not apparent until Albrecht Kossel, a German physiologist specializing in the physiological chemistry of the cell and its nucleus and of proteins researched its' chemical structure.

Content & Image Credit:

Content: Edited by Sowjanya Guttula, from Wikipedia: **https://en.wikipedia.org/wiki/Friedrich_Miescher (https://en.wikipedia.org/wiki/Friedrich_Miescher)**

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KEY TERMS

acid a substance that donates hydrogen ions and therefore lowers pH

adhesion the attraction between water molecules and molecules of a different substance

amino acid a monomer of a protein

anion a negative ion formed by gaining electrons

atomic number the number of protons in an atom

base a substance that absorbs hydrogen ions and therefore raises pH

bicarbonate (HCO_3^-) ion ion created when carbonic acid dissociates into H^+ and (HCO_3^-)

bicarbonate buffer system system in the blood that absorbs carbon dioxide and regulates pH levels

buffer a solution that resists a change in pH by absorbing or releasing hydrogen or hydroxide ions

carbaminohemoglobin molecule that forms when carbon dioxide binds to hemoglobin

carbohydrate a biological macromolecule in which the ratio of carbon to hydrogen to oxygen is 1:2:1; carbohydrates serve as energy sources and structural support in cells

carbonic anhydrase (CA) enzyme that catalyzes carbon dioxide and water into carbonic acid

cation a positive ion formed by losing electrons

cellulose a polysaccharide that makes up the cell walls of plants and provides structural support to the cell

chemical bond an interaction between two or more of the same or different elements that results in the formation of molecules

chitin a type of carbohydrate that forms the outer skeleton of arthropods, such as insects and crustaceans, and the cell walls of fungi

chloride shift exchange of chloride for bicarbonate into or out of the red blood cell

cohesion the intermolecular forces between water molecules caused by the polar nature of water; creates surface tension

covalent bond a type of strong bond between two or more of the same or different elements; forms when electrons are shared between elements

denaturation the loss of shape in a protein as a result of changes in temperature, pH, or exposure to chemicals

deoxyribonucleic acid (DNA) a double-stranded polymer of nucleotides that carries the hereditary information of the cell

disaccharide two sugar monomers that are linked together by a glycodsidic bond

electron a negatively charged particle that resides outside of the nucleus in the electron orbital; lacks functional mass and has a charge of –1

electron transfer the movement of electrons from one element to another

element one of 118 unique substances that cannot be broken down into smaller substances and retain the characteristic of that substance; each element has a specified number of protons and unique properties

enzyme a catalyst in a biochemical reaction that is usually a complex or conjugated protein

evaporation the release of water molecules from liquid water to form water vapor

fat a lipid molecule composed of three fatty acids and a glycerol (triglyceride) that typically exists in a solid form at room temperature

glycogen a storage carbohydrate in animals

heme group centralized iron-containing group that is surrounded by the alpha and beta subunits of hemoglobin

hemoglobin molecule in red blood cells that can bind oxygen, carbon dioxide, and carbon monoxide

hormone a chemical signaling molecule, usually a protein or steroid, secreted by an endocrine gland or group of endocrine cells; acts to control or regulate specific physiological processes

hydrogen bond a weak bond between partially positively charged hydrogen atoms and partially negatively charged elements or molecules

hydrophilic describes a substance that dissolves in water; water-loving

hydrophobic describes a substance that does not dissolve in water; water-fearing

ion an atom or compound that does not contain equal numbers of protons and electrons, and therefore has a net charge

ionic bond a chemical bond that forms between ions of opposite charges

isotope one or more forms of an element that have different numbers of neutrons

lipids a class of macromolecules that are nonpolar and insoluble in water

litmus paper filter paper that has been treated with a natural water-soluble dye so it can be used as a pH indicator

macromolecule a large molecule, often formed by polymerization of smaller monomers

mass number the number of protons plus neutrons in an atom

matter anything that has mass and occupies space

monosaccharide a single unit or monomer of carbohydrates

neutron a particle with no charge that resides in the nucleus of an atom; has a mass of 1

nonpolar covalent bond a type of covalent bond that forms between atoms when electrons are shared equally between atoms, resulting in no regions with partial charges as in polar covalent bonds

nucleic acid a biological macromolecule that carries the genetic information of a cell and carries instructions for the functioning of the cell

nucleotide a monomer of nucleic acids; contains a pentose sugar, a phosphate group, and a nitrogenous base

nucleus (chemistry) the dense center of an atom made up of protons and (except in the case of a hydrogen atom) neutrons

octet rule states that the outermost shell of an element with a low atomic number can hold eight electrons

oil an unsaturated fat that is a liquid at room temperature

oxygen dissociation curve curve depicting the affinity of oxygen for hemoglobin

oxygen-carrying capacity amount of oxygen that can be transported in the blood

periodic table of elements an organizational chart of elements, indicating the atomic number and mass

number of each element; also provides key information about the properties of elements

pH scale a scale ranging from 0 to 14 that measures the approximate concentration of hydrogen ions of a substance

phospholipid a major constituent of the membranes of cells; composed of two fatty acids and a phosphate group attached to the glycerol backbone

polar covalent bond a type of covalent bond in which electrons are pulled toward one atom and away from another, resulting in slightly positive and slightly negative charged regions of the molecule

polypeptide a long chain of amino acids linked by peptide bonds

polysaccharide a long chain of monosaccharides; may be branched or unbranched

protein a biological macromolecule composed of one or more chains of amino acids

proton a positively charged particle that resides in the nucleus of an atom; has a mass of 1 and a charge of +1

radioactive isotope an isotope that spontaneously emits particles or energy to form a more stable element

ribonucleic acid (RNA) a single-stranded polymer of nucleotides that is involved in protein synthesis

saturated fatty acid a long-chain hydrocarbon with single covalent bonds in the carbon chain; the number of hydrogen atoms attached to the carbon skeleton is maximized

sickle cell anemia genetic disorder that affects the shape of red blood cells, and their ability to transport oxygen and move through capillaries

solvent a substance capable of dissolving another substance

starch a storage carbohydrate in plants

steroid a type of lipid composed of four fused hydrocarbon rings

surface tension the cohesive force at the surface of a body of liquid that prevents the molecules from separating

temperature a measure of molecular motion

thalassemia rare genetic disorder that results in mutation of the alpha or beta subunits of hemoglobin, creating smaller red blood cells with less hemoglobin

***trans*-fat** a form of unsaturated fat with the hydrogen atoms neighboring the double bond across from each other rather than on the same side of the double bond

triglyceride a fat molecule; consists of three fatty acids linked to a glycerol molecule

unsaturated fatty acid a long-chain hydrocarbon that has one or more than one double bonds in the hydrocarbon chain

van der Waals interaction a weak attraction or interaction between molecules caused by slightly positively charged or slightly negatively charged atoms

CHAPTER SUMMARY

2.1 The Building Blocks of Molecules

Matter is anything that occupies space and has mass. It is made up of atoms of different elements. All of the 92 elements that occur naturally have unique qualities that allow them to combine in various ways to create compounds or molecules. Atoms, which consist of protons, neutrons, and electrons, are the smallest units of an element that retain all of the properties of that element. Electrons can be donated or shared between atoms to create bonds, including ionic, covalent, and hydrogen bonds, as well as van der Waals interactions.

2.2 Water

Water has many properties that are critical to maintaining life. It is polar, allowing for the formation of hydrogen bonds, which allow ions and other polar molecules to dissolve in water. Therefore, water is an excellent solvent. The hydrogen bonds between water molecules give water the ability to hold heat better than many other substances. As the temperature rises, the hydrogen bonds between water continually break and reform, allowing for the overall temperature to remain stable, although increased energy is added to the system. Water's cohesive forces allow for the property of surface tension. All of these unique properties of water are important in the chemistry of living organisms.

The pH of a solution is a measure of the concentration of hydrogen ions in the solution. A solution with a high number of hydrogen ions is acidic and has a low pH value. A solution with a high number of hydroxide ions is basic and has a high pH value. The pH scale ranges from 0 to 14, with a pH of 7 being neutral. Buffers are solutions that moderate pH changes when an acid or base is added to the buffer system. Buffers are important in biological systems because of their ability to maintain constant pH conditions.

2.3 Human Blood Buffering Systems

Hemoglobin is a protein found in red blood cells that is comprised of two alpha and two beta subunits that surround an iron-containing heme group. Oxygen readily binds this heme group. The ability of oxygen to bind increases as more oxygen molecules are bound to heme. Disease states and altered conditions in the body can affect the binding ability of oxygen, and increase or decrease its ability to dissociate from hemoglobin.

Carbon dioxide can be transported through the blood via three methods. It is dissolved directly in the blood, bound to plasma proteins or hemoglobin, or converted into bicarbonate. The majority of carbon dioxide is transported as part of the bicarbonate system. Carbon dioxide diffuses into red blood cells. Inside, carbonic anhydrase converts carbon dioxide into carbonic acid (H_2CO_3), which is subsequently hydrolyzed into bicarbonate (HCO_3^-) and H^+. The H^+ ion binds to hemoglobin in red blood cells, and bicarbonate is transported out of the red blood cells in exchange for a chloride ion. This is called the chloride shift. Bicarbonate leaves the red blood cells and enters the blood plasma. In the lungs, bicarbonate is transported back into the red blood cells in exchange for chloride. The H^+ dissociates from hemoglobin and combines with bicarbonate to form carbonic acid with the help of carbonic anhydrase, which further catalyzes the reaction to convert carbonic acid back into carbon dioxide and water. The carbon dioxide is then expelled from the lungs.

2.4 Biological Molecules

Living things are carbon-based because carbon plays such a prominent role in the chemistry of living things. The four covalent bonding positions of the carbon atom can give rise to a wide diversity of compounds with many functions, accounting for the importance of carbon in living things. Carbohydrates are a group of macromolecules that are a vital energy source for the cell, provide structural support to many organisms, and can be found on the surface of the cell as receptors or for cell recognition. Carbohydrates are classified as monosaccharides, disaccharides, and polysaccharides, depending on the number of monomers in the molecule.

Lipids are a class of macromolecules that are nonpolar and hydrophobic in nature. Major types include fats and oils, waxes, phospholipids, and steroids. Fats and oils are a stored form of energy and can include triglycerides. Fats and oils are usually made up of fatty acids and glycerol.

Proteins are a class of macromolecules that can perform a diverse range of functions for the cell. They help in metabolism by providing structural support and by acting as enzymes, carriers or as hormones. The building blocks of proteins are amino acids. Proteins are organized at four levels: primary, secondary, tertiary, and quaternary. Protein shape and function are intricately linked; any change in shape caused by changes in temperature, pH, or chemical exposure may lead to protein denaturation and a loss of function.

Nucleic acids are molecules made up of repeating units of nucleotides that direct cellular activities such as cell division and protein synthesis. Each nucleotide is made up of a pentose sugar, a nitrogenous base, and a phosphate group. There are two types of nucleic acids: DNA and RNA.

VISUAL CONNECTION QUESTIONS

2.1 The Building Blocks of Molecules

1. Figure 2.3 How many neutrons do (K) potassium-39 and potassium-40 have, respectively?

2.3 Human Blood Buffering Systems

2. Figure 2.14 The kidneys are responsible for removing excess H+ ions from the blood. If the kidneys fail, what would happen to blood pH and to hemoglobin affinity for oxygen?

REVIEW QUESTIONS

3. Magnesium has an atomic number of 12. Which of the following statements is true of a neutral magnesium atom?

a. It has 12 protons, 12 electrons, and 12 neutrons.
b. It has 12 protons, 12 electrons, and six neutrons.
c. It has six protons, six electrons, and no neutrons.
d. It has six protons, six electrons, and six neutrons.

4. Which type of bond represents a weak chemical bond?

a. hydrogen bond
b. ionic bond
c. covalent bond
d. polar covalent bond

5. An isotope of sodium (Na) has a mass number of 22. How many neutrons does it have?

a. 11
b. 12
c. 22
d. 44

6. Which of the following statements is not true?

a. Water is polar.
b. Water stabilizes temperature.
c. Water is essential for life.
d. Water is the most abundant atom in Earth’s atmosphere.

7. Using a pH meter, you find the pH of an unknown solution to be 8.0. How would you describe this solution?

a. weakly acidic
b. strongly acidic
c. weakly basic
d. strongly basic

8. The pH of lemon juice is about 2.0, whereas tomato juice's pH is about 4.0. Approximately how much of an increase in hydrogen ion concentration is there between tomato juice and lemon juice?

a. 2 times
b. 10 times
c. 100 times
d. 1000 times

9. Which of the following will NOT facilitate the transfer of oxygen to tissues?

a. decreased body temperature
b. decreased pH of the blood
c. increased carbon dioxide
d. increased exercise

10. The majority of carbon dioxide in the blood is transported by ________.

a. binding to hemoglobin
b. dissolution in the blood
c. conversion to bicarbonate
d. binding to plasma proteins

11. The majority of oxygen in the blood is transported by ________.

a. dissolution in the blood
b. being carried as bicarbonate ions
c. binding to blood plasma
d. binding to hemoglobin

12. An example of a monosaccharide is ________.

a. fructose
b. glucose
c. galactose
d. all of the above

13. Cellulose and starch are examples of ________.

a. monosaccharides
b. disaccharides
c. lipids
d. polysaccharides

14. Phospholipids are important components of __________.

a. the plasma membrane of cells
b. the ring structure of steroids
c. the waxy covering on leaves
d. the double bond in hydrocarbon chains

15. The monomers that make up proteins are called _________.

a. nucleotides
b. disaccharides
c. amino acids
d. chaperones

CRITICAL THINKING QUESTIONS

16. Why are hydrogen bonds and van der Waals interactions necessary for cells?

17. Why can some insects walk on water?

18. Explain why water is an excellent solvent.

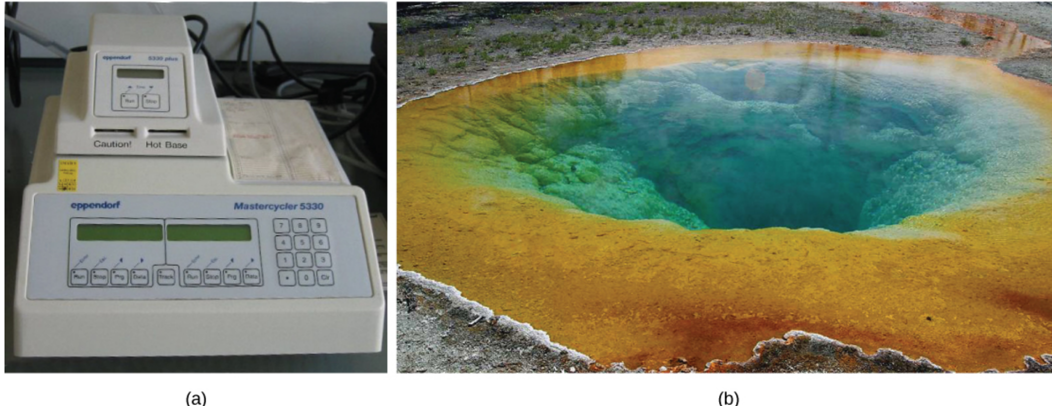
19. What would happen if no carbonic anhydrase were present in red blood cells?

20. How does the administration of 100 percent oxygen save a patient from carbon monoxide poisoning? Why wouldn’t giving carbon dioxide work?

21. Explain at least three functions that lipids serve in plants and/or animals.

22. Explain what happens if even one amino acid is substituted for another in a polypeptide chain. Provide a specific example.

3 | WHAT IS BIOTECHNOLOGY?



(a) (b)

Figure 3.1 (a) A thermal cycler, such as the one shown here, is a basic tool used to study DNA in a process called the polymerase chain reaction (PCR). The polymerase enzyme most often used with PCR comes from a strain of bacteria that lives in (b) the hot springs of Yellowstone National Park. (credit a: modification of work by Magnus Manske; credit b: modification of work by Jon Sullivan)

Chapter Outline

3.1: Cloning and Genetic Engineering
3.2: Biotechnology in Medicine and Agriculture
3.3: The FDA

Introduction

The latter half of the twentieth century began with the discovery of the structure of DNA, then progressed to the development of the basic tools used to study and manipulate DNA. These advances, as well as advances in our understanding of and ability to manipulate cells, have led some to refer to the twenty-first century as the biotechnology century. The rate of discovery and of the development of new applications in medicine, agriculture, and energy is expected to accelerate, bringing huge benefits to humankind and perhaps also significant risks. Many of these developments are expected to raise significant ethical and social questions that human societies have not yet had to consider.

3.1 | Cloning and Genetic Engineering

By the end of this section, you will be able to:

- Explain the basic techniques used to manipulate genetic material
- Explain molecular and reproductive cloning

Biotechnology is the use of artificial methods to modify the genetic material of living organisms or cells to produce novel compounds or to perform new functions. Biotechnology has been used for improving livestock and crops since the beginning of agriculture through selective breeding. Since the discovery of the structure of DNA in 1953, and particularly since the development of tools and methods to manipulate DNA in the 1970s, biotechnology has become synonymous with the manipulation of organisms' DNA at the molecular level. The

primary applications of this technology are in medicine (for the production of vaccines and antibiotics) and in agriculture (for the genetic modification of crops). Biotechnology also has many industrial applications, such as fermentation, the treatment of oil spills, and the production of biofuels, as well as many household applications such as the use of enzymes in laundry detergent.

Manipulating Genetic Material

To accomplish the applications described above, biotechnologists must be able to extract, manipulate, and analyze nucleic acids.

Review of Nucleic Acid Structure

To understand the basic techniques used to work with nucleic acids, remember that nucleic acids are macromolecules made of nucleotides (a sugar, a phosphate, and a nitrogenous base). The phosphate groups on these molecules each have a net negative charge. An entire set of DNA molecules in the nucleus of eukaryotic organisms is called the genome. DNA has two complementary strands linked by hydrogen bonds between the paired bases.

Unlike DNA in eukaryotic cells, RNA molecules leave the nucleus. Messenger RNA (mRNA) is analyzed most frequently because it represents the protein-coding genes that are being expressed in the cell.

Isolation of Nucleic Acids

To study or manipulate nucleic acids, the DNA must first be extracted from cells. Various techniques are used to extract different types of DNA (Figure 3.2). Most nucleic acid extraction techniques involve steps to break open the cell, and then the use of enzymatic reactions to destroy all undesired macromolecules. Cells are broken open using a detergent solution containing buffering compounds. To prevent degradation and contamination, macromolecules such as proteins and RNA are inactivated using enzymes. The DNA is then brought out of solution using alcohol. The resulting DNA, because it is made up of long polymers, forms a gelatinous mass.

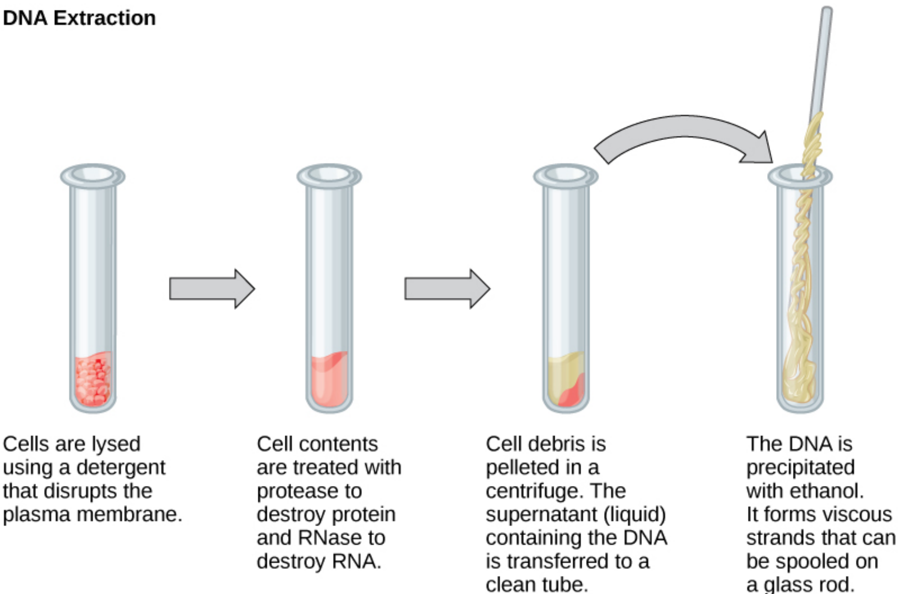


Figure 3.2 This diagram shows the basic method used for the extraction of DNA.

RNA is studied to understand gene expression patterns in cells. RNA is naturally very unstable because enzymes that break down RNA are commonly present in nature. Some are even secreted by our own skin and are very difficult to inactivate. Similar to DNA extraction, RNA extraction involves the use of various buffers and enzymes to inactivate other macromolecules and preserve only the RNA.

Gel Electrophoresis

Because nucleic acids are negatively charged ions at neutral or alkaline pH in an aqueous environment, they can be moved by an electric field. **Gel electrophoresis** is a technique used to separate charged molecules on the basis of size and charge. The nucleic acids can be separated as whole chromosomes or as fragments. The nucleic acids are loaded into a slot at one end of a gel matrix, an electric current is applied, and negatively charged molecules are pulled toward the opposite end of the gel (the end with the positive electrode). Smaller

molecules move through the pores in the gel faster than larger molecules; this difference in the rate of migration separates the fragments on the basis of size. The nucleic acids in a gel matrix are invisible until they are stained with a compound that allows them to be seen, such as a dye. Distinct fragments of nucleic acids appear as bands at specific distances from the top of the gel (the negative electrode end) that are based on their size (**Figure 3.3**). A mixture of many fragments of varying sizes appear as a long smear, whereas uncut genomic DNA is usually too large to run through the gel and forms a single large band at the top of the gel.

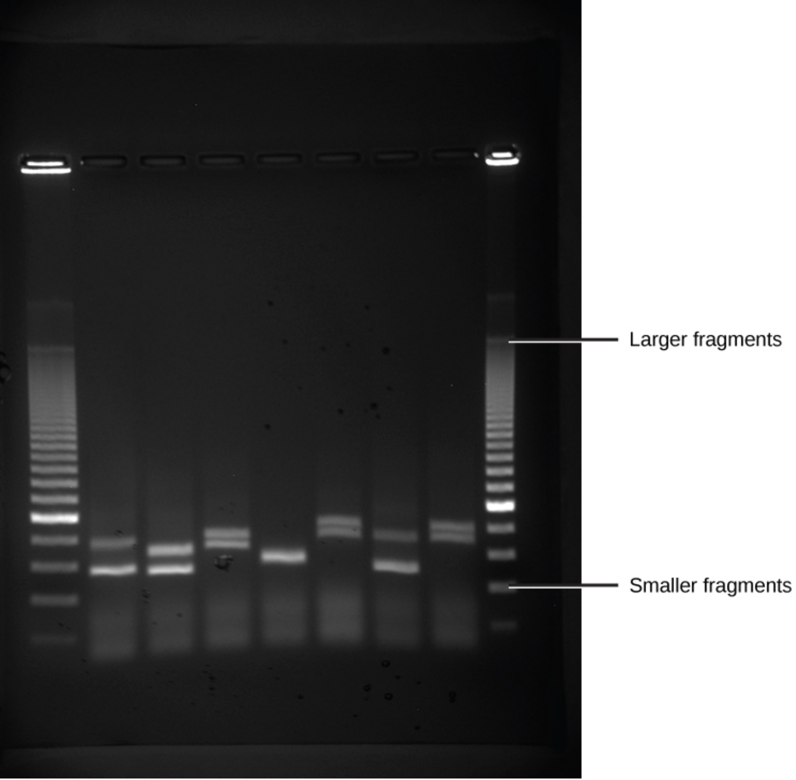


Figure 3.3 Shown are DNA fragments from six samples run on a gel, stained with a fluorescent dye and viewed under UV light. (credit: modification of work by James Jacob, Tompkins Cortland Community College)

Polymerase Chain Reaction

DNA analysis often requires focusing on one or more specific regions of the genome. It also frequently involves situations in which only one or a few copies of a DNA molecule are available for further analysis. These amounts are insufficient for most procedures, such as gel electrophoresis. **Polymerase chain reaction (PCR)** is a technique used to rapidly increase the number of copies of specific regions of DNA for further analyses (**Figure 3.4**). PCR uses a special form of DNA polymerase, the enzyme that replicates DNA, and other short nucleotide sequences called primers that base pair to a specific portion of the DNA being replicated. PCR is used for many purposes in laboratories. These include: 1) the identification of the owner of a DNA sample left at a crime scene; 2) paternity analysis; 3) the comparison of small amounts of ancient DNA with modern organisms; and 4) determining the sequence of nucleotides in a specific region.

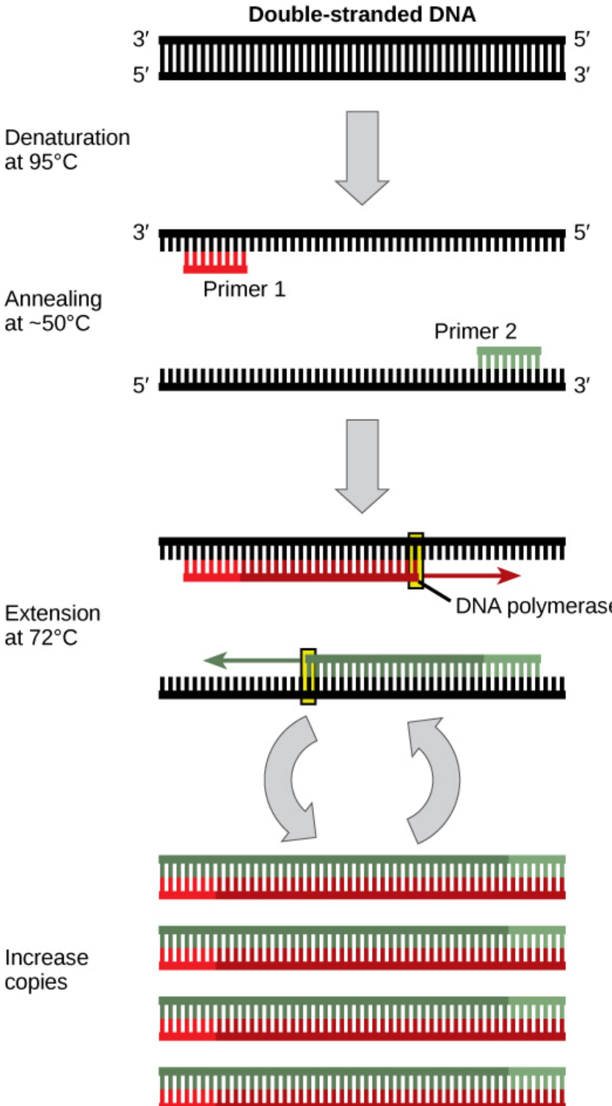


Figure 3.4 Polymerase chain reaction, or PCR, is used to produce many copies of a specific sequence of DNA using a special form of DNA polymerase.

Cloning

In general, **cloning** means the creation of a perfect replica. Typically, the word is used to describe the creation of a genetically identical copy. In biology, the re-creation of a whole organism is referred to as “reproductive cloning.” Long before attempts were made to clone an entire organism, researchers learned how to copy short stretches of DNA—a process that is referred to as molecular cloning.

Molecular Cloning

Cloning allows for the creation of multiple copies of genes, expression of genes, and study of specific genes. To get the DNA fragment into a bacterial cell in a form that will be copied or expressed, the fragment is first inserted into a plasmid. A **plasmid** (also called a vector in this context) is a small circular DNA molecule that replicates independently of the chromosomal DNA in bacteria. In cloning, the plasmid molecules can be used to provide a "vehicle" in which to insert a desired DNA fragment. Modified plasmids are usually reintroduced into a bacterial host for replication. As the bacteria divide, they copy their own DNA (including the plasmids). The inserted DNA fragment is copied along with the rest of the bacterial DNA. In a bacterial cell, the fragment of DNA from the human genome (or another organism that is being studied) is referred to as foreign DNA to differentiate it from the DNA of the bacterium (the host DNA).

Plasmids occur naturally in bacterial populations (such as *Escherichia coli*) and have genes that can contribute favorable traits to the organism, such as antibiotic resistance (the ability to be unaffected by antibiotics).

Plasmids have been highly engineered as vectors for molecular cloning and for the subsequent large-scale production of important molecules, such as insulin. A valuable characteristic of plasmid vectors is the ease with which a foreign DNA fragment can be introduced. These plasmid vectors contain many short DNA sequences that can be cut with different commonly available **restriction enzymes**. Restriction enzymes (also called restriction endonucleases) recognize specific DNA sequences and cut them in a predictable manner; they are naturally produced by bacteria as a defense mechanism against foreign DNA. Many restriction enzymes make staggered cuts in the two strands of DNA, such that the cut ends have a 2- to 4-nucleotide single-stranded overhang. The sequence that is recognized by the restriction enzyme is a four- to eight-nucleotide sequence that is a palindrome. Like with a word palindrome, this means the sequence reads the same forward and backward. In most cases, the sequence reads the same forward on one strand and backward on the complementary strand. When a staggered cut is made in a sequence like this, the overhangs are complementary (**Figure 3.5**).

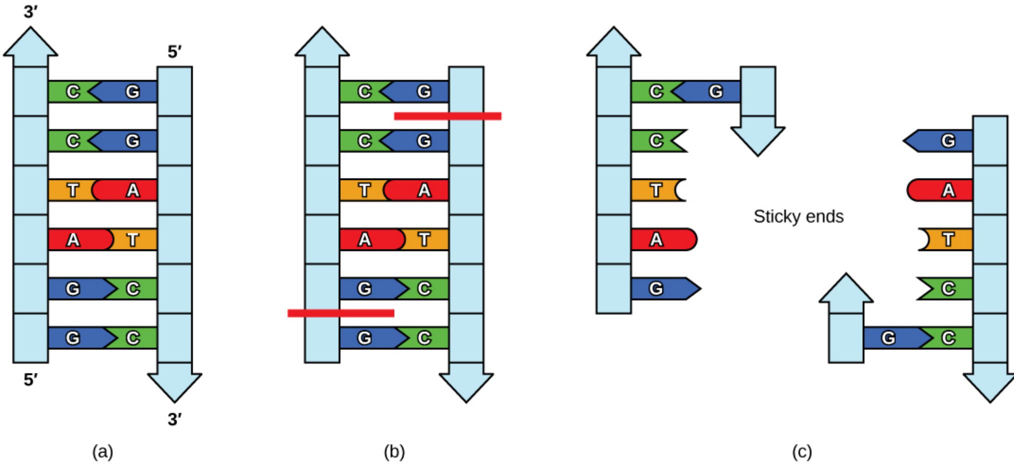


Figure 3.5 In this (a) six-nucleotide restriction enzyme recognition site, notice that the sequence of six nucleotides reads the same in the 5' to 3' direction on one strand as it does in the 5' to 3' direction on the complementary strand. This is known as a palindrome. (b) The restriction enzyme makes breaks in the DNA strands, and (c) the cut in the DNA results in “sticky ends”. Another piece of DNA cut on either end by the same restriction enzyme could attach to these sticky ends and be inserted into the gap made by this cut.

Because these overhangs are capable of coming back together by hydrogen bonding with complementary overhangs on a piece of DNA cut with the same restriction enzyme, these are called “sticky ends.” The process of forming hydrogen bonds between complementary sequences on single strands to form double-stranded DNA is called **annealing**. Addition of an enzyme called DNA ligase, which takes part in DNA replication in cells, permanently joins the DNA fragments when the sticky ends come together. In this way, any DNA fragment can be spliced between the two ends of a plasmid DNA that has been cut with the same restriction enzyme (**Figure 3.6**).

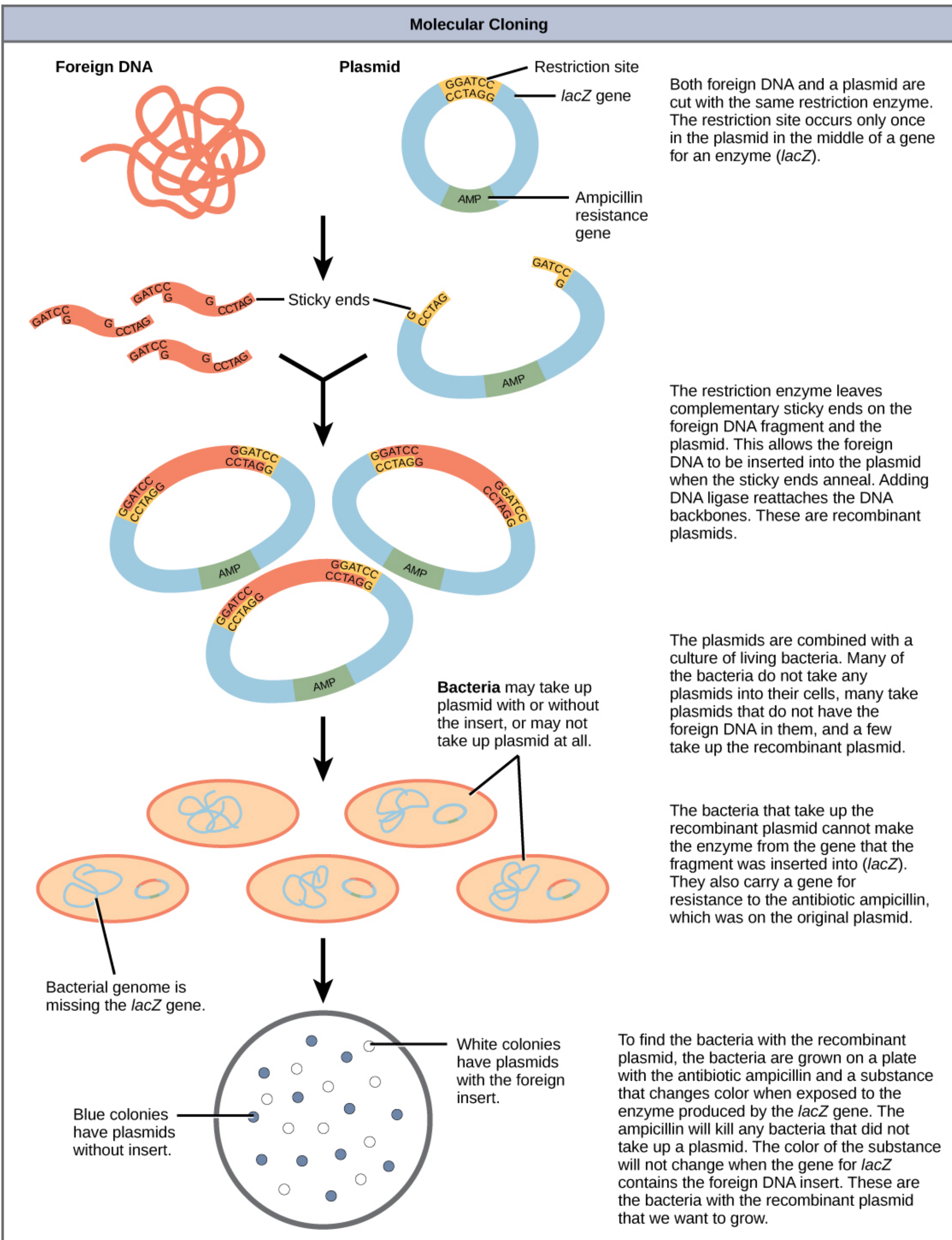


Figure 3.6 This diagram shows the steps involved in molecular cloning.

Plasmids with foreign DNA inserted into them are called **recombinant DNA** molecules because they contain new combinations of genetic material. Proteins that are produced from recombinant DNA molecules are called **recombinant proteins**. Not all recombinant plasmids are capable of expressing genes. Plasmids may also be engineered to express proteins only when stimulated by certain environmental factors, so that scientists can control the expression of the recombinant proteins.

Reproductive Cloning

Reproductive cloning is a method used to make a clone or an identical copy of an entire multicellular organism. Most multicellular organisms undergo reproduction by sexual means, which involves the contribution of DNA from two individuals (parents), making it impossible to generate an identical copy or a clone of either parent. Recent advances in biotechnology have made it possible to reproductively clone mammals in the laboratory.

Natural sexual reproduction involves the union, during fertilization, of a sperm and an egg. Each of these gametes is haploid, meaning they contain one set of chromosomes in their nuclei. The resulting cell, or zygote, is then diploid and contains two sets of chromosomes. This cell divides mitotically to produce a multicellular organism. However, the union of just any two cells cannot produce a viable zygote; there are components in the cytoplasm of the egg cell that are essential for the early development of the embryo during its first few cell divisions. Without these provisions, there would be no subsequent development. Therefore, to produce a new individual, both a diploid genetic complement and an egg cytoplasm are required. The approach to producing an artificially cloned individual is to take the egg cell of one individual and to remove the haploid nucleus. Then a diploid nucleus from a body cell of a second individual, the donor, is put into the egg cell. The egg is then stimulated to divide so that development proceeds. This sounds simple, but in fact it takes many attempts before each of the steps is completed successfully.

The first cloned agricultural animal was Dolly, a sheep who was born in 1996. The success rate of reproductive cloning at the time was very low. Dolly lived for six years and died of a lung tumor (**Figure 3.7**). There was speculation that because the cell DNA that gave rise to Dolly came from an older individual, the age of the DNA may have affected her life expectancy. Since Dolly, several species of animals (such as horses, bulls, and goats) have been successfully cloned.

There have been attempts at producing cloned human embryos as sources of embryonic stem cells. In the procedure, the DNA from an adult human is introduced into a human egg cell, which is then stimulated to divide. The technology is similar to the technology that was used to produce Dolly, but the embryo is never implanted into a surrogate mother. The cells produced are called embryonic stem cells because they have the capacity to develop into many different kinds of cells, such as muscle or nerve cells. The stem cells could be used to research and ultimately provide therapeutic applications, such as replacing damaged tissues. The benefit of cloning in this instance is that the cells used to regenerate new tissues would be a perfect match to the donor of the original DNA. For example, a leukemia patient would not require a sibling with a tissue match for a bone-marrow transplant.

Visual Connection

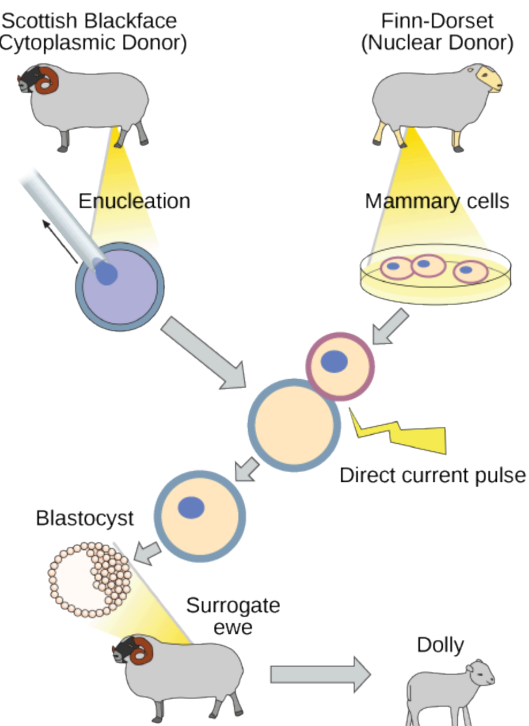


Figure 3.7 Dolly the sheep was the first agricultural animal to be cloned. To create Dolly, the nucleus was removed from a donor egg cell. The enucleated egg was placed next to the other cell, then they were shocked to fuse. They were shocked again to start division. The cells were allowed to divide for several days until an early embryonic stage was reached, before being implanted in a surrogate mother.

Why was Dolly a Finn-Dorset and not a Scottish Blackface sheep?

Genetic Engineering

Using recombinant DNA technology to modify an organism’s DNA to achieve desirable traits is called **genetic engineering**. Addition of foreign DNA in the form of recombinant DNA vectors that are generated by molecular cloning is the most common method of genetic engineering. An organism that receives the recombinant DNA is called a **genetically modified organism** (GMO). If the foreign DNA that is introduced comes from a different species, the host organism is called **transgenic**. Bacteria, plants, and animals have been genetically modified since the early 1970s for academic, medical, agricultural, and industrial purposes. These applications will be examined in more detail in the next module.

Watch this **short video (http://openstax.org/l/transgenic)** explaining how scientists create a transgenic animal.

Although the classic methods of studying the function of genes began with a given phenotype and determined the genetic basis of that phenotype, modern techniques allow researchers to start at the DNA sequence level and ask: "What does this gene or DNA element do?" This technique, called **reverse genetics**, has resulted in reversing the classical genetic methodology. One example of this method is analogous to damaging a body part to determine its function. An insect that loses a wing cannot fly, which means that the wing’s function is flight. The classic genetic method compares insects that cannot fly with insects that can fly, and observes that the non-flying insects have lost wings. Similarly in a reverse genetics approach, mutating or deleting genes provides researchers with clues about gene function. Alternately, reverse genetics can be used to cause a gene to overexpress itself to determine what phenotypic effects may occur.

3.2 | Biotechnology in Medicine and Agriculture

By the end of this section, you will be able to:

- Describe uses of biotechnology in medicine
- Describe uses of biotechnology in agriculture

It is easy to see how biotechnology can be used for medicinal purposes. Knowledge of the genetic makeup of our species, the genetic basis of heritable diseases, and the invention of technology to manipulate and fix mutant genes provides methods to treat diseases. Biotechnology in agriculture can enhance resistance to disease, pests, and environmental stress to improve both crop yield and quality.

Genetic Diagnosis and Gene Therapy

The process of testing for suspected genetic defects before administering treatment is called genetic diagnosis by genetic testing. In some cases in which a genetic disease is present in an individual’s family, family members may be advised to undergo genetic testing. For example, mutations in the *BRCA* genes may increase the likelihood of developing breast and ovarian cancers in women and some other cancers in women and men. A woman with breast cancer can be screened for these mutations. If one of the high-risk mutations is found, her female relatives may also wish to be screened for that particular mutation, or simply be more vigilant for the occurrence of cancers. Genetic testing is also offered for fetuses (or embryos with in vitro fertilization) to determine the presence or absence of disease-causing genes in families with specific debilitating diseases.

See how **human DNA is extracted (http://openstax.org/l/DNA_extraction)** for uses such as genetic testing.

Gene therapy is a genetic engineering technique that may one day be used to cure certain genetic diseases. In its simplest form, it involves the introduction of a non-mutated gene at a random location in the genome to cure a disease by replacing a protein that may be absent in these individuals because of a genetic mutation. The non-mutated gene is usually introduced into diseased cells as part of a vector transmitted by a virus, such as an adenovirus, that can infect the host cell and deliver the foreign DNA into the genome of the targeted cell (**Figure 3.8**). To date, gene therapies have been primarily experimental procedures in humans. A few of these experimental treatments have been successful, but the methods may be important in the future as the factors limiting its success are resolved.

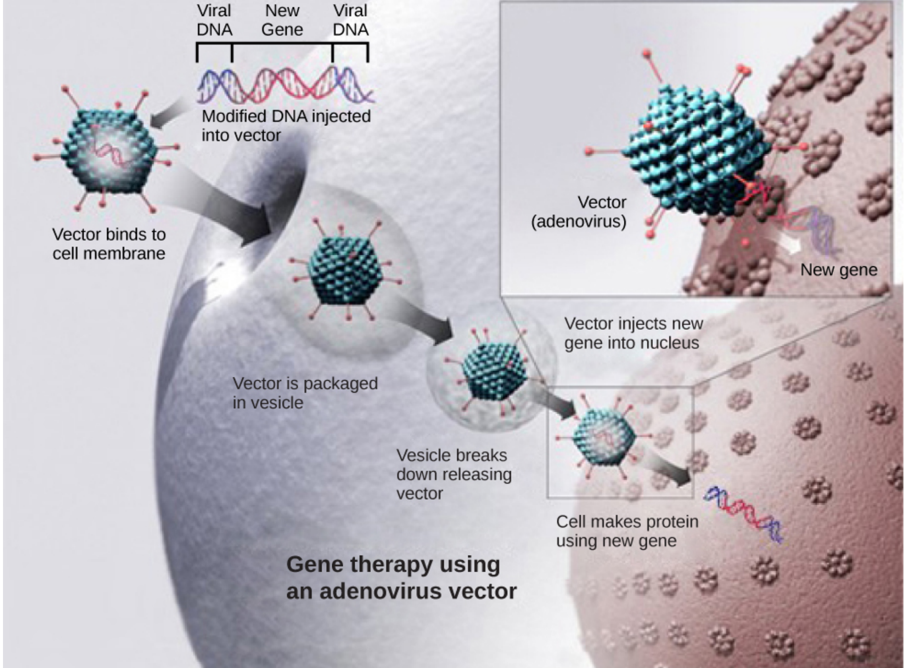


Figure 3.8 This diagram shows the steps involved in curing disease with gene therapy using an adenovirus vector. (credit: modification of work by NIH)

Production of Vaccines, Antibiotics, and Hormones

Traditional vaccination strategies use weakened or inactive forms of microorganisms or viruses to stimulate the immune system. Modern techniques use specific genes of microorganisms cloned into vectors and mass-produced in bacteria to make large quantities of specific substances to stimulate the immune system. The substance is then used as a vaccine. In some cases, such as the H1N1 flu vaccine, genes cloned from the virus have been used to combat the constantly changing strains of this virus.

Antibiotics kill bacteria and are naturally produced by microorganisms such as fungi; penicillin is perhaps the most well-known example. Antibiotics are produced on a large scale by cultivating and manipulating fungal cells. The fungal cells have typically been genetically modified to improve the yields of the antibiotic compound.

Recombinant DNA technology was used to produce large-scale quantities of the human hormone insulin in *E. coli* as early as 1978. Previously, it was only possible to treat diabetes with pig insulin, which caused allergic reactions in many humans because of differences in the insulin molecule. In addition, human growth hormone (HGH) is used to treat growth disorders in children. The HGH gene was cloned from a cDNA (complementary DNA) library and inserted into *E. coli* cells by cloning it into a bacterial vector.

Transgenic Animals

Although several recombinant proteins used in medicine are successfully produced in bacteria, some proteins need a eukaryotic animal host for proper processing. For this reason, genes have been cloned and expressed in animals such as sheep, goats, chickens, and mice. Animals that have been modified to express recombinant DNA are called transgenic animals (**Figure 3.9**).

Figure 3.9 It can be seen that two of these mice are transgenic because they have a gene that causes them to fluoresce under a UV light. The non-transgenic mouse does not have the gene that causes fluorescence. (credit: Ingrid Moen et al.)

Several human proteins are expressed in the milk of transgenic sheep and goats. In one commercial example, the FDA has approved a blood anticoagulant protein that is produced in the milk of transgenic goats for use in humans. Mice have been used extensively for expressing and studying the effects of recombinant genes and mutations.

Transgenic Plants

Manipulating the DNA of plants (creating genetically modified organisms, or GMOs) has helped to create desirable traits such as disease resistance, herbicide, and pest resistance, better nutritional value, and better shelf life (**Figure 3.10**). Plants are the most important source of food for the human population. Farmers developed ways to select for plant varieties with desirable traits long before modern-day biotechnology practices were established.

Figure 3.10 Corn, a major agricultural crop used to create products for a variety of industries, is often modified through plant biotechnology. (credit: Keith Weller, USDA)

Transgenic plants have received DNA from other species. Because they contain unique combinations of genes and are not restricted to the laboratory, transgenic plants and other GMOs are closely monitored by government agencies to ensure that they are fit for human consumption and do not endanger other plant and animal life. Because foreign genes can spread to other species in the environment, particularly in the pollen and seeds of plants, extensive testing is required to ensure ecological stability. Staples like corn, potatoes, and tomatoes were the first crop plants to be genetically engineered.

Transformation of Plants Using Agrobacterium tumefaciens

In plants, tumors caused by the bacterium *Agrobacterium tumefaciens* occur by transfer of DNA from the bacterium to the plant. The artificial introduction of DNA into plant cells is more challenging than in animal cells because of the thick plant cell wall. Researchers used the natural transfer of DNA from *Agrobacterium* to a plant host to introduce DNA fragments of their choice into plant hosts. In nature, the disease-causing *A. tumefaciens* have a set of plasmids that contain genes that integrate into the infected plant cell's genome. Researchers manipulate the plasmids to carry the desired DNA fragment and insert it into the plant genome.

The Organic Insecticide Bacillus thuringiensis

Bacillus thuringiensis (Bt) is a bacterium that produces protein crystals that are toxic to many insect species that feed on plants. Insects that have eaten Bt toxin stop feeding on the plants within a few hours. After the toxin is activated in the intestines of the insects, death occurs within a couple of days. The crystal toxin genes have been cloned from the bacterium and introduced into plants, therefore allowing plants to produce their own crystal Bt toxin that acts against insects. Bt toxin is safe for the environment and non-toxic to mammals (including humans). As a result, it has been approved for use by organic farmers as a natural insecticide. There is some concern, however, that insects may evolve resistance to the Bt toxin in the same way that bacteria evolve resistance to antibiotics.

FlavrSavr Tomato

The first GM crop to be introduced into the market was the FlavrSavr Tomato produced in 1994. Molecular genetic technology was used to slow down the process of softening and rotting caused by fungal infections, which led to increased shelf life of the GM tomatoes. Additional genetic modification improved the flavor of this tomato. The FlavrSavr tomato did not successfully stay in the market because of problems maintaining and shipping the crop.

3.3 | The FDA

This module is a brief history of regulatory milestones for health and human products, including the history and organization of the FDA

THE HISTORY OF PHARMACEUTICAL REGULATIONS

As you just learned in this module, the exposure to a virus can sometimes provide immunity to future infections and is the basis for vaccination. Edward Jenner created the first vaccine in the 1770s, in an unusually unethical way. Small pox was a deadly disease in this time, and he had a theory about protecting people from infection. To test his theory, he gave a small box cowpox, then infected him with small pox. He was right – exposure to the cowpox had protected him from the small pox virus.

The artwork in the figure is titled *The Cow-Pock-or-the Wonderful Effects of the New Inoculation!* By James Gillray in 1802. This cartoon is a caricature of a Smallpox and Inoculation Hospital at St. Pancras, showing cowpox vaccine administered to frightened young women, and cows emerging from different parts of people's bodies. The cartoon inspiration was the controversy over inoculating against the dreaded disease, smallpox. Opponents of vaccination had depicted cases of vaccines developing bovine features, and this is picked up and exaggerated by Gillray. Thanks to an aggressive vaccine campaign, the deadly smallpox virus was eradicated in the 80s. Unfortunately, the **resurgence of anti-vaccination sentiment (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6122668/)** in the U.S. (and worldwide) has returned resulting in unusual and preventable viral outbreaks causing grave concern of scientists globally.

Figure 3.11 small pox vaccine and needle. Image credit: **https://commons.wikimedia.org/wiki/File:Smallpox_vaccine.jpg (https://commons.wikimedia.org/wiki/File:Smallpox_vaccine.jpg)**

Figure 3.12 1802 art work, The Cow-Pock, James Gillray. Images and content credit: **https://simple.wikipedia.org/wiki/Vaccine (https://simple.wikipedia.org/wiki/Vaccine)** , Artwork, By James Gillray - Library of Congress, Prints & Photographs Division, LC-USZC4-3147, Public Domain, **https://simple.wikipedia.org/wiki/Vaccine#/media/File:The_cow_pock.jpg (https://simple.wikipedia.org/wiki/Vaccine#/media/File:The_cow_pock.jpg)**

Consumer Safety and Health

The idea of protecting the safety and health of consumers is not new. There were many societies, such as ancient Rome, where the penalties for producing an unsafe product were quite harsh. In contrast, our modern-day society's view of regulations and protections is that of prevention. Of course, there are still "after-the-fact" remedies available to the consumer. However, ***regulations in modern-day are designed to build quality and safety into the production process***, thereby limiting if not eliminating health and safety concerns before the product reaches the consumer. In this module, we will examine some of the most significant regulations, explore how regulations come to pass, and discuss the role of the Food and Drug Administration (FDA) in regulations of consumer products, especially those in health and wellness.

Regulatory Milestone of the FDA

Pharmaceutical regulation aims to ensure objective characteristics such as safety, effectiveness, honesty in labeling, accurate reporting of side effects (if any). Regulations do not apply to subjective characteristics such as taste, color or texture. There are no regulations, for example, that dictate all aspirin tablets be white. Most of us take product quality for granted. It was not so long ago that substances such as cocaine could be used as ingredients in soda (Coca-Cola) or over-the-counter tonics (**Wikipedia (https://en.wikipedia.org/wiki/Coca-Cola#Coca_%E2%80%93_cocaine)**).

The FDA outlines major **milestones of product regulation (https://www.fda.gov/aboutfda/history/forgshistory/evolvingpowers/ucm2007256.htm)** at their website. Some of the better-known incidences and the regulations that arose, as a result:

The original ***Food Drug and Cosmetic Act (FDCA) of 1906***. The FDCA is intended to prevent the sale of unacceptable foods and drugs rather than regulate safety or effectiveness.

The ***Durham-Humphrey Amendment***, passed in 1951, was the first federal law requiring a physician’s prescription for drugs “unsafe for self-medication.”

The requirement that drugs are proven both safe and effective before release and that such effectiveness is supported by "substantial evidence" is the mandate of the ***Kefauver-Harris Amendments.***

The Orphan Drug Act amended the FDCA as of January 4, 1983, is an act calling for incentives to companies producing orphan drugs (which benefit only a small percentage of the population and unprofitable).

The Drug Price Competition and Patent Term Restoration Act, passed in 1984, made ***generic drugs*** more readily available at the same time provided a way for businesses to recoup some prepatent research costs by factoring research time into the patent life of the drug.

ClinicalTrials.gov (https://clinicaltrials.gov/) was founded in 1999 to provide the public with updated information on enrollment in federally and privately supported clinical research.

What Influences Pharmaceutical Regulations?

Two major influences trigger the enactment of regulations: 1. Consumer tragedy (serious injury, death) resulting from the use of a product, and 2. Advancements in science and technology. Once alerted to either of the above conditions, our lawmakers respond by passing the needed legislation, and enforcement of the law is assigned to the appropriate government agency. It’s important to note the FDA does not create laws, those are enacted by Congress, but they do enforce some of them. The issue of enforcement is not always clear-cut. Several agencies may enforce regulations in certain sectors – for example, Genetically Modified Organisms (GMOs), are overseen by the EPA, FDA, and the USDA. Authority for oversight and regulation of the pharmaceutical industry, however, is quite clear - the primary agency is the Food and Drug Administration (FDA).

Let’s Explore! (https://www.fda.gov/Safety/ReportaProblem/default.htm) As you just learned, consumers play an important role in product quality and public health. Go to the following website to learn how consumers can report problems with FDA-regulated products. **https://www.fda.gov/Safety/ReportaProblem/default.htm (https://www.fda.gov/Safety/ReportaProblem/default.htm)** Look to the Q&A on the left-hand panel - what kinds of products are not overseen by the FDA?

THE FOOD & DRUG ADMINISTRATION

The **FDA (http://www.fda.gov)** is an administrative agency created to regulate food and drug supplies in the United States for the safety and health of its citizens. FDA is an agency within **the Department of Health and Human (http://legacy.cnx.org/content/m71330/1.1/) Services (http://legacy.cnx.org/content/m71330/1.1/)** . Although the FDA has traditionally focused on the US markets, however, with the global market growth (of imports and exports of products and raw materials) the FDA now manages over 2 trillion dollars of goods

manufactured in over 150 nations worldwide (FDA Global engagement report, 2016).

FDA ORGANIZATION

The FDA, like most organizations, change with the economy, political appointments, world harmonization, and emerging technologies and products. The current **FDA organizational hierarchy (https://www.fda.gov/aboutfda/centersoffices/organizationcharts/ucm393155.htm)** consists of the Office of the Commissioner overseeing five offices and directorates. Those offices oversee eight centers. Like the federal government, the FDA possesses the following powers:

- **Legislative:** The FDA has the authority to create and issue rules *(not laws!).*
- **Executive**: The FDA has the power to conduct investigations.
- **Judicial:** The FDA has the jurisdiction to review evidence and make judgments on a product.

FIELD OFFICES & CENTERS

The field offices are located throughout the United States and are *the eyes and ears* of the FDA, and it is from these offices that operational personnel enforce the law. Under the Offices, the FDA has Product Centers headquartered largely in the Washington, D.C. area.

Office of the Commissioner:

The leadership of the agency's scientific activities, communication, legislative liaison, policy and planning, women's and minority health. The current FDA Commissioner is Dr. Scott Gottlieb, was appointed by President Trump, and sworn in May 2017.

National Center for Toxicological Research (NCTR): FDA's research center conducts peer-reviewed research and develops new scientific tools for FDA to improve public health. This research produces innovative tools to assist in solving complex health issues and anticipated toxicological problems and enhances the science of regulatory decision-making at the FDA.

Office of Medical Products and Tobacco:

Provides advice and counsel to the Commissioner on all medical product and tobacco-related programs and issues and oversees the following centers: CDER, CBER, CDRH, and CTP.

Center for Drug Evaluation and Research (CDER): The CDER oversees the regulation of **drugs (https://www.fda.gov/Drugs/default.htm)** . The official definition of a drug is *"an article intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; articles designed to affect the structure or any function of the body of man or other animals."* (FDA, 2018) . CDER regulates over-the-counter and prescription drugs, including biological therapeutics, which act like drugs, and generic drugs.

Ritalin (Figure) is one of many drugs that CDER oversees. In 2018, CDER approved a staggering 59 novel drugs, far surpassing the previous five years. In totality, the FDA has approved over 1600 drugs to date. However, CDER’s reach covers more than just traditional medicines. For example, the FDA classifies all fluoride toothpaste, antiperspirants, and dandruff shampoos as over-the-counter drugs.

Figure 3.13 The FDA product centers oversee many different types of regulated products. Ritalin, a drug approved by the FDA in 1955. Image Credit: **https://fr.m.wikipedia.org/wiki/Fichier:Ritalin-SR-20mg-1000x1000.jpg (https://fr.m.wikipedia.org/wiki/Fichier:Ritalin-SR-20mg-1000x1000.jpg)**

Figure 3.14 dandruff shampoo, the FDA classifies as an over-the-counter drug. Image Credit: Mike Mozart, **https://www.flickr.com/photos/jeepersmedia/16162915122 (https://www.flickr.com/photos/jeepersmedia/16162915122)**

Center for Biologics Evaluation and Research (CBER): The CBER oversees the regulation of **vaccines, blood, and biologics (https://www.fda.gov/BiologicsBloodVaccines/default.htm)** . The official definition of a biological product is "***any virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, or analogous product***" (FDA, 2018). Note, therapeutic biologics, biologics that act like drugs, are overseen by CDER instead. CDER oversees all drugs, even biologic (think, recombinant proteins!)!

Center for Devices and Radiological Health (CDRH): Oversees the regulation of **medical devices (https://www.fda.gov/MedicalDevices/default.htm)** and radiation-emitting products, and includes biotechnology products used in diagnostics, such as HIV or pregnancy tests. You will be surprised to learn about some unusual products that are considered medical devices.

Center for Tobacco Products (CTP): The Center for **Tobacco Products (https://www.fda.gov/TobaccoProducts/default.htm)** (CTP) oversees the implementation of the Family Smoking Prevention and Tobacco Control Act. Some of the Agency's responsibilities under the law include setting performance standards, reviewing premarket applications for new and modified-risk tobacco products, requiring new warning labels, and establishing and enforcing advertising and promotion restrictions.

Office of Foods and Veterinary Medicine:

Leads a functionally unified FDA Foods Program that addresses food and feed safety, nutrition, and other critical areas to achieve public health goals. The Office of Food & Veterinary Medicine oversees two centers, CFSAN and CVM.

Center for Food Safety and Applied Nutrition (CFSAN): CFSAN oversees **food (https://www.fda.gov/Food/default.htm)** safety and purity. It has the power to regulate all domestic and imported food *except for meat, poultry, and eggs* (USDA regulates those). They oversee the safety of food ingredients developed through biotechnology, dietary supplements, food additives, and proper labeling of food. CFSAN is also concerned with food contamination, such as biological pathogens and naturally occurring toxins.

Center for Veterinary Medicine, and (CVM): The **CVM (https://www.fda.gov/AnimalVeterinary/default.htm)** is a product center which oversees regulation of pet food and pet food additives, as well as drugs and biologics for animals, such as oral heart-worm medication and annual vaccines (Figure). The CVM does conduct research that helps FDA ensure the safety of animal drugs, food for animals, and food products made from animals, however, they do not oversee pre-clinical animal studies. Learn more about what the CVM does, watch this video: https://www.fda.gov/AboutFDA/CentersOffices/OfficeofFoods/CVM/ucm245225.htm

Figure 3.15 Dr. Nina Griffin is giving a dog his annual exam and vaccinations. Image credit: **https://www.jble.af.mil/News/Article-Display/Article/843261/langley-veterinary-clinic-keeping-our-furry-friends-healthy/ (https://www.jble.af.mil/News/Article-Display/Article/843261/langley-veterinary-clinic-keeping-our-furry-friends-healthy/)**

Office of Global Regulatory Operations and Policy:

Provides leadership for FDA's domestic and international product quality and safety efforts.

The FDA’s **Office of Regulatory Affairs (ORA)** leads all FDA field activities and provides FDA leadership on imports, inspections, and enforcement policy. ORA supports the agency in the following ways: By inspecting regulated products and manufacturers. ? Performing sample analysis on regulated products, reviewing imported goods, developing policies on compliance and enforcement, and executing the FDA’s Import Strategy and Food Protection Plans.

The **Office of International Programs** leads the FDA’s international effort is divided into Office of the Director, International Agreement Staff, International Relations Staff, International Scientific Activities and Standards Staff, International Planning, and Resources Management Staff. Conflicting regulatory standards made international trade of FDA-regulated products a challenge until an effort began in the 1990s to harmonize international standards so that important medical products could be bought and sold with less regulatory ‘red tape.’ The FDA assists both foreign and domestic manufacturers in compliance with health and safety regulations.

Office of Operations:

Provides agency-wide services including information technology, financial management, procurement, library services, and freedom of information, FDA history, and facilities.

Content Credit:

The FDA: **https://www.fda.gov (https://www.fda.gov)**

Wikipedia: **https://simple.wikipedia.org/wiki/Vaccine (https://simple.wikipedia.org/wiki/Vaccine)**

KEY TERMS

anneal in molecular biology, the process by which two single strands of DNA hydrogen bond at complementary nucleotides to form a double-stranded molecule

biotechnology the use of artificial methods to modify the genetic material of living organisms or cells to produce novel compounds or to perform new functions

cloning the production of an exact copy—specifically, an exact genetic copy—of a gene, cell, or organism

gel electrophoresis a technique used to separate molecules on the basis of their ability to migrate through a semisolid gel in response to an electric current

gene therapy the technique used to cure heritable diseases by replacing mutant genes with good genes

genetic engineering alteration of the genetic makeup of an organism using the molecular methods of biotechnology

genetic testing identifying gene variants in an individual that may lead to a genetic disease in that individual

genetically modified organism (GMO) an organism whose genome has been artificially changed

plasmid a small circular molecule of DNA found in bacteria that replicates independently of the main bacterial chromosome; plasmids code for some important traits for bacteria and can be used as vectors to transport DNA into bacteria in genetic engineering applications

polymerase chain reaction (PCR) a technique used to make multiple copies of DNA

recombinant DNA a combination of DNA fragments generated by molecular cloning that does not exist in nature

recombinant protein a protein that is expressed from recombinant DNA molecules

reproductive cloning cloning of entire organisms

restriction enzyme an enzyme that recognizes a specific nucleotide sequence in DNA and cuts the DNA double strand at that recognition site, often with a staggered cut leaving short single strands or “sticky” ends

reverse genetics a form of genetic analysis that manipulates DNA to disrupt or affect the product of a gene to analyze the gene’s function

transgenic describing an organism that receives DNA from a different species

CHAPTER SUMMARY

3.1 Cloning and Genetic Engineering

Nucleic acids can be isolated from cells for the purposes of further analysis by breaking open the cells and enzymatically destroying all other major macromolecules. Fragmented or whole chromosomes can be separated on the basis of size by gel electrophoresis. Short stretches of DNA can be amplified by PCR. DNA can be cut (and subsequently re-spliced together) using restriction enzymes. The molecular and cellular techniques of biotechnology allow researchers to genetically engineer organisms, modifying them to achieve desirable traits.

Cloning may involve cloning small DNA fragments (molecular cloning), or cloning entire organisms (reproductive cloning). In molecular cloning with bacteria, a desired DNA fragment is inserted into a bacterial plasmid using restriction enzymes and the plasmid is taken up by a bacterium, which will then express the foreign DNA. Using other techniques, foreign genes can be inserted into eukaryotic organisms. In each case, the organisms are called transgenic organisms. In reproductive cloning, a donor nucleus is put into an enucleated egg cell, which is then stimulated to divide and develop into an organism.

In reverse genetics methods, a gene is mutated or removed in some way to identify its effect on the phenotype

of the whole organism as a way to determine its function.

3.2 Biotechnology in Medicine and Agriculture

Genetic testing is performed to identify disease-causing genes, and can be used to benefit affected individuals and their relatives who have not developed disease symptoms yet. Gene therapy—by which functioning genes are incorporated into the genomes of individuals with a non-functioning mutant gene—has the potential to cure heritable diseases. Transgenic organisms possess DNA from a different species, usually generated by molecular cloning techniques. Vaccines, antibiotics, and hormones are examples of products obtained by recombinant DNA technology. Transgenic animals have been created for experimental purposes and some are used to produce some human proteins.

Genes are inserted into plants, using plasmids in the bacterium *Agrobacterium tumefaciens*, which infects plants. Transgenic plants have been created to improve the characteristics of crop plants—for example, by giving them insect resistance by inserting a gene for a bacterial toxin.

VISUAL CONNECTION QUESTIONS

3.1 Cloning and Genetic Engineering

1. Figure 3.7 Why was Dolly a Finn-Dorset and not a Scottish Blackface sheep?

REVIEW QUESTIONS

2. In gel electrophoresis of DNA, the different bands in the final gel form because the DNA molecules ________.

a. are from different organisms
b. have different lengths
c. have different nucleotide compositions
d. have different genes

3. In the reproductive cloning of an animal, the genome of the cloned individual comes from ________.

a. a sperm cell
b. an egg cell
c. any gamete cell
d. a body cell

4. What carries a gene from one organism into a bacteria cell?

a. a plasmid
b. an electrophoresis gel
c. a restriction enzyme
d. polymerase chain reaction

5. What is a genetically modified organism (GMO)?

a. a plant with certain genes removed
b. an organism with an artificially altered genome
c. a hybrid organism
d. any agricultural organism produced by breeding or biotechnology

6. What is the role of *Agrobacterium tumefaciens* in the production of transgenic plants?

a. Genes from *A. tumefaciens* are inserted into plant DNA to give the plant different traits.
b. Transgenic plants have been given resistance to the pest *A. tumefaciens*.
c. *A. tumefaciens* is used as a vector to move genes into plant cells.
d. Plant genes are incorporated into the genome of *Agrobacterium tumefaciens*.

CRITICAL THINKING QUESTIONS

7. What is the purpose and benefit of the polymerase chain reaction?

8. Today, it is possible for a diabetic patient to purchase human insulin from a pharmacist. What technology makes this possible and why is it a benefit over how things used to be?

4 | BIOTECHNICIAN TOOLS: MEASUREMENTS & UNCERTAINTY

4.1 | Introduction

Introduction to measurements section

INTRODUCTION

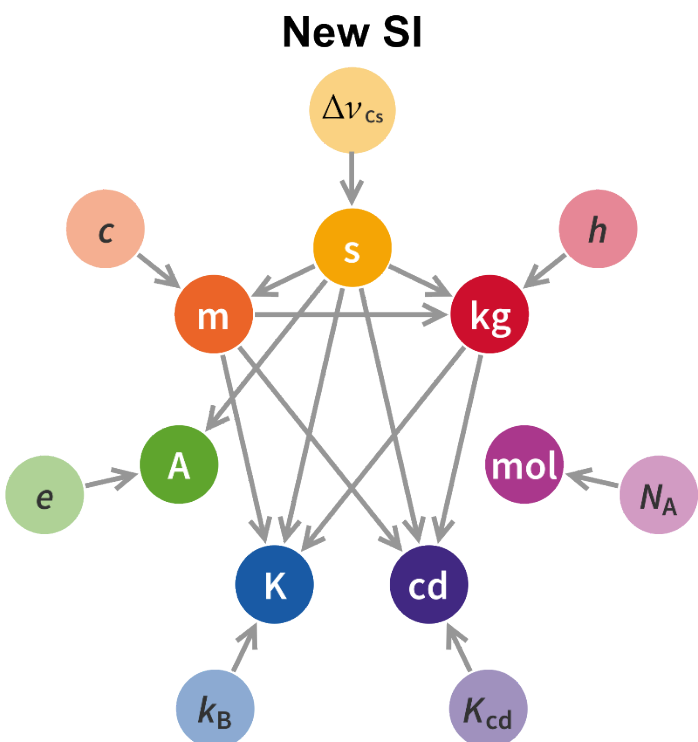


Figure 4.1 The SI system after the 2019 redefinition: Dependence of base unit definitions on physical constants with fixed numerical values and other base units. Image credit: By Emilio Pisanty - Own work, CC BY-SA 4.0, **https://commons.wikimedia.org/w/index.php?curid=50713156 (https://commons.wikimedia.org/w/index.php?curid=50713156)**

In November 2018, the 26th General Conference on Weights and Measures (CGPM) voted unanimously in favor of revised definitions of the SI base units, which the International Committee for Weights and Measures (CIPM) had proposed earlier that year. The new definitions will come into force on May 20th, 2019. ***The kilogram, ampere, Kelvin, and mole will then be defined by setting exact numerical values for the Planck constant (h), the elementary electric charge (e), the Boltzmann constant (k), and the Avogadro constant (NA), respectively***. The meter and candela are already defined by physical constants, subject to correction to their present definitions. The new definitions aim to improve the SI without changing the size of any units, thus ensuring continuity with existing measurements.

To learn more: **https://en.wikipedia.org/wiki/2019_redefinition_of_SI_base_units (https://en.wikipedia.org/wiki/2019_redefinition_of_SI_base_units)**

4.2 | Measurements

By the end of this section, you will be able to:

- Explain the process of measurement
- Identify the three basic parts of a quantity
- Describe the properties and units of length, mass, volume, density, temperature, and time
- Perform basic unit calculations and conversions in the metric and other unit systems

Measurements provide much of the information that informs the hypotheses, theories, and laws describing the behavior of matter and energy in both the macroscopic and microscopic domains of chemistry. Every measurement provides three kinds of information: the size or magnitude of the measurement (a number); a standard of comparison for the measurement (a unit); and an indication of the uncertainty of the measurement. While the number and unit are explicitly represented when a quantity is written, the uncertainty is an aspect of the measurement result that is more implicitly represented and will be discussed later.

The number in the measurement can be represented in different ways, including decimal form and scientific notation. (Scientific notation is also known as exponential notation; a review of this topic can be found in **Appendix B (https://legacy.cnx.org/content/m68860/latest/)** .) For example, the maximum takeoff weight of a Boeing 777-200ER airliner is 298,000 kilograms, which can also be written as 2.98×10^5 kg. The mass of the average mosquito is about 0.0000025 kilograms, which can be written as 2.5×10^{-6} kg.

Units, such as liters, pounds, and centimeters, are standards of comparison for measurements. A 2-liter bottle of a soft drink contains a volume of beverage that is twice that of the accepted volume of 1 liter. The meat used to prepare a 0.25-pound hamburger weighs one-fourth as much as the accepted weight of 1 pound. Without units, a number can be meaningless, confusing, or possibly life threatening. Suppose a doctor prescribes phenobarbital to control a patient's seizures and states a dosage of "100" without specifying units. Not only will this be confusing to the medical professional giving the dose, but the consequences can be dire: 100 mg given three times per day can be effective as an anticonvulsant, but a single dose of 100 g is more than 10 times the lethal amount.

The measurement units for seven fundamental properties ("base units") are listed in **Table 4.1**. The standards for these units are fixed by international agreement, and they are called the **International System of Units** or **SI Units** (from the French, *Le Système International d'Unités*). SI units have been used by the United States National Institute of Standards and Technology (NIST) since 1964. Units for other properties may be derived from these seven base units.

Base Units of the SI System

Property Measured	Name of Unit	Symbol of Unit
length	meter	m
mass	kilogram	kg
time	second	s
temperature	kelvin	K
electric current	ampere	A
amount of substance	mole	mol
luminous intensity	candela	cd

Table 4.1

Everyday measurement units are often defined as fractions or multiples of other units. Milk is commonly packaged in containers of 1 gallon (4 quarts), 1 quart (0.25 gallon), and one pint (0.5 quart). This same approach is used with SI units, but these fractions or multiples are always powers of 10. Fractional or multiple SI units are named using a prefix and the name of the base unit. For example, a length of 1000 meters is also called a kilometer because the prefix *kilo* means “one thousand,” which in scientific notation is 10^3 (1 kilometer = 1000 m = 10^3 m). The prefixes used and the powers to which 10 are raised are listed in **Table 4.2**.

Common Unit Prefixes

Prefix	Symbol	Factor	Example
femto	f	10^{-15}	1 femtosecond (fs) = 1×10^{-15} s (0.000000000000001 s)
pico	p	10^{-12}	1 picometer (pm) = 1×10^{-12} m (0.000000000001 m)
nano	n	10^{-9}	4 nanograms (ng) = 4×10^{-9} g (0.000000004 g)
micro	µ	10^{-6}	1 microliter (µL) = 1×10^{-6} L (0.000001 L)
milli	m	10^{-3}	2 millimoles (mmol) = 2×10^{-3} mol (0.002 mol)
centi	c	10^{-2}	7 centimeters (cm) = 7×10^{-2} m (0.07 m)
deci	d	10^{-1}	1 deciliter (dL) = 1×10^{-1} L (0.1 L)
kilo	k	10^{3}	1 kilometer (km) = 1×10^{3} m (1000 m)
mega	M	10^{6}	3 megahertz (MHz) = 3×10^{6} Hz (3,000,000 Hz)
giga	G	10^{9}	8 gigayears (Gyr) = 8×10^{9} yr (8,000,000,000 yr)
tera	T	10^{12}	5 terawatts (TW) = 5×10^{12} W (5,000,000,000,000 W)

Table 4.2

Need a refresher or more practice with scientific notation? Visit this **site (http://openstax.org/l/16notation)** to go over the basics of scientific notation.

SI Base Units

The initial units of the metric system, which eventually evolved into the SI system, were established in France during the French Revolution. The original standards for the meter and the kilogram were adopted there in 1799 and eventually by other countries. This section introduces four of the SI base units commonly used in chemistry. Other SI units will be introduced in subsequent chapters.

Length

The standard unit of **length** in both the SI and original metric systems is the **meter (m)**. A meter was originally specified as 1/10,000,000 of the distance from the North Pole to the equator. It is now defined as the distance light in a vacuum travels in 1/299,792,458 of a second. A meter is about 3 inches longer than a yard (**Figure 4.2**); one meter is about 39.37 inches or 1.094 yards. Longer distances are often reported in kilometers (1 km = 1000 m = 10^3 m), whereas shorter distances can be reported in centimeters (1 cm = 0.01 m = 10^{-2} m) or millimeters (1 mm = 0.001 m = 10^{-3} m).

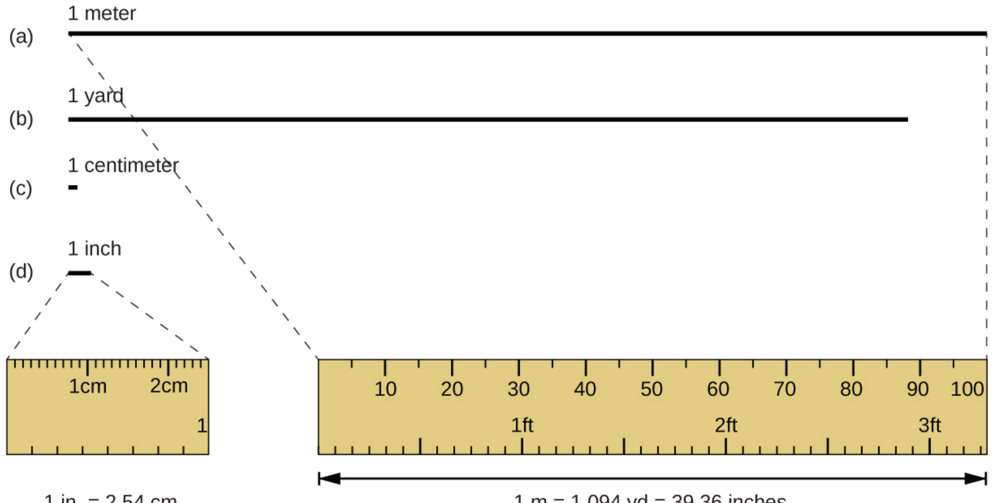


Figure 4.2 The relative lengths of 1 m, 1 yd, 1 cm, and 1 in. are shown (not actual size), as well as comparisons of 2.54 cm and 1 in., and of 1 m and 1.094 yd.

Mass

The standard unit of mass in the SI system is the **kilogram (kg)**. The kilogram was previously defined by the International Union of Pure and Applied Chemistry (IUPAC) as the mass of a specific reference object. This object was originally one liter of pure water, and more recently it was a metal cylinder made from a platinum-iridium alloy with a height and diameter of 39 mm (**Figure 4.3**). In May 2019, this definition was changed to one that is based instead on precisely measured values of several fundamental physical constants.[1]. One kilogram is about 2.2 pounds. The gram (g) is exactly equal to 1/1000 of the mass of the kilogram (10^{-3} kg).

1. For details see https://www.nist.gov/pml/weights-and-measures/si-units-mass

Figure 4.3 This replica prototype kilogram as previously defined is housed at the National Institute of Standards and Technology (NIST) in Maryland. (credit: National Institutes of Standards and Technology)

Temperature

Temperature is an intensive property. The SI unit of temperature is the **kelvin (K)**. The IUPAC convention is to use kelvin (all lowercase) for the word, K (uppercase) for the unit symbol, and neither the word "degree" nor the degree symbol (°). The degree **Celsius (°C)** is also allowed in the SI system, with both the word "degree" and the degree symbol used for Celsius measurements. Celsius degrees are the same magnitude as those of kelvin, but the two scales place their zeros in different places. Water freezes at 273.15 K (0 °C) and boils at 373.15 K (100 °C) by definition, and normal human body temperature is approximately 310 K (37 °C). The conversion between these two units and the Fahrenheit scale will be discussed later in this chapter.

Time

The SI base unit of time is the **second (s)**. Small and large time intervals can be expressed with the appropriate prefixes; for example, 3 microseconds = 0.000003 s = 3×10^{-6} and 5 megaseconds = 5,000,000 s = 5×10^{6} s. Alternatively, hours, days, and years can be used.

Derived SI Units

We can derive many units from the seven SI base units. For example, we can use the base unit of length to define a unit of volume, and the base units of mass and length to define a unit of density.

Volume

Volume is the measure of the amount of space occupied by an object. The standard SI unit of volume is defined by the base unit of length (Figure 4.4). The standard volume is a **cubic meter (m^3)**, a cube with an edge length of exactly one meter. To dispense a cubic meter of water, we could build a cubic box with edge lengths of exactly one meter. This box would hold a cubic meter of water or any other substance.

A more commonly used unit of volume is derived from the decimeter (0.1 m, or 10 cm). A cube with edge lengths

of exactly one decimeter contains a volume of one cubic decimeter (dm^3). A **liter (L)** is the more common name for the cubic decimeter. One liter is about 1.06 quarts.

A **cubic centimeter (cm^3)** is the volume of a cube with an edge length of exactly one centimeter. The abbreviation **cc** (for **c**ubic **c**entimeter) is often used by health professionals. A cubic centimeter is equivalent to a **milliliter (mL)** and is 1/1000 of a liter.

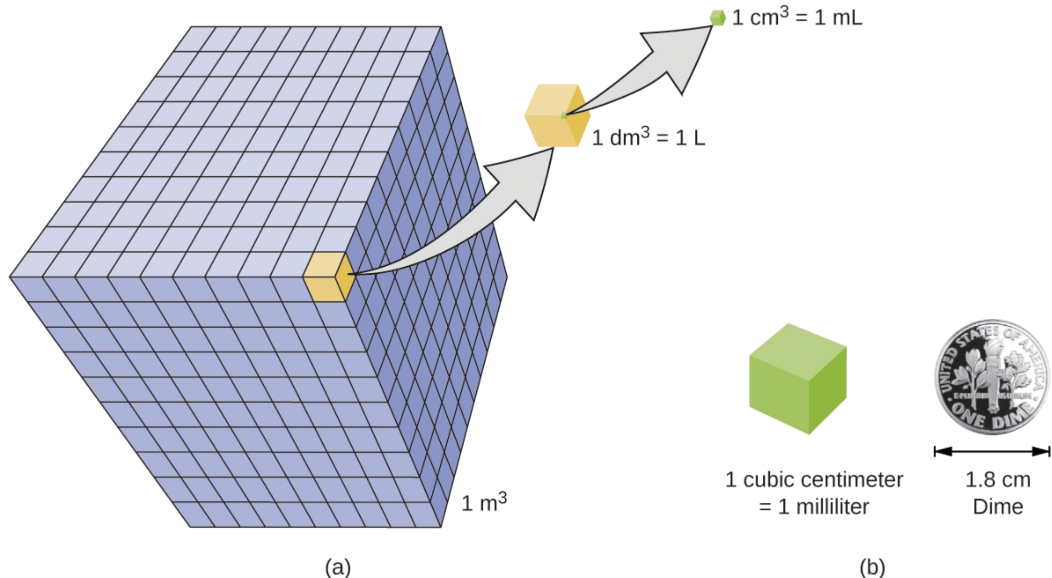


Figure 4.4 (a) The relative volumes are shown for cubes of 1 m^3, 1 dm^3 (1 L), and 1 cm^3 (1 mL) (not to scale). (b) The diameter of a dime is compared relative to the edge length of a 1-cm^3 (1-mL) cube.

Density

We use the mass and volume of a substance to determine its density. Thus, the units of density are defined by the base units of mass and length.

The **density** of a substance is the ratio of the mass of a sample of the substance to its volume. The SI unit for density is the kilogram per cubic meter (kg/m^3). For many situations, however, this as an inconvenient unit, and we often use grams per cubic centimeter (g/cm^3) for the densities of solids and liquids, and grams per liter (g/L) for gases. Although there are exceptions, most liquids and solids have densities that range from about 0.7 g/cm^3 (the density of gasoline) to 19 g/cm^3 (the density of gold). The density of air is about 1.2 g/L. **Table 4.3** shows the densities of some common substances.

Densities of Common Substances

Solids	Liquids	Gases (at 25 °C and 1 atm)
ice (at 0 °C) 0.92 g/cm^3	water 1.0 g/cm^3	dry air 1.20 g/L
oak (wood) 0.60–0.90 g/cm^3	ethanol 0.79 g/cm^3	oxygen 1.31 g/L
iron 7.9 g/cm^3	acetone 0.79 g/cm^3	nitrogen 1.14 g/L
copper 9.0 g/cm^3	glycerin 1.26 g/cm^3	carbon dioxide 1.80 g/L
lead 11.3 g/cm^3	olive oil 0.92 g/cm^3	helium 0.16 g/L
silver 10.5 g/cm^3	gasoline 0.70–0.77 g/cm^3	neon 0.83 g/L
gold 19.3 g/cm^3	mercury 13.6 g/cm^3	radon 9.1 g/L

Table 4.3

While there are many ways to determine the density of an object, perhaps the most straightforward method involves separately finding the mass and volume of the object, and then dividing the mass of the sample by its volume. In the following example, the mass is found directly by weighing, but the volume is found indirectly through length measurements.

$$\text{density} = \frac{\text{mass}}{\text{volume}}$$

Example 4.1

Calculation of Density

Gold—in bricks, bars, and coins—has been a form of currency for centuries. In order to swindle people into paying for a brick of gold without actually investing in a brick of gold, people have considered filling the centers of hollow gold bricks with lead to fool buyers into thinking that the entire brick is gold. It does not work: Lead is a dense substance, but its density is not as great as that of gold, 19.3 g/cm^3. What is the density of lead if a cube of lead has an edge length of 2.00 cm and a mass of 90.7 g?

Solution

The density of a substance can be calculated by dividing its mass by its volume. The volume of a cube is calculated by cubing the edge length.

$$\text{volume of lead cube} = 2.00 \text{ cm} \times 2.00 \text{ cm} \times 2.00 \text{ cm} = 8.00 \text{ cm}^3$$

$$\text{density} = \frac{\text{mass}}{\text{volume}} = \frac{90.7 \text{ g}}{8.00 \text{ cm}^3} = 11.3 \text{ g/cm}^3$$

(We will discuss the reason for rounding to the first decimal place in the next section.)

Check Your Learning

(a) To three decimal places, what is the volume of a cube (cm^3) with an edge length of 0.843 cm?

(b) If the cube in part (a) is copper and has a mass of 5.34 g, what is the density of copper to two decimal places?

Answer:

(a) 0.599 cm^3; (b) 8.91 g/cm^3

To learn more about the relationship between mass, volume, and density, use this **interactive simulator (http://openstax.org/l/16phetmasvolden)** to explore the density of different materials.

Example 4.2

Using Displacement of Water to Determine Density

This exercise uses a **simulation (http://openstax.org/l/16phetmasvolden)** to illustrate an alternative approach to the determination of density that involves measuring the object's volume via displacement of water. Use the simulator to determine the densities iron and wood.

Solution

Click the "turn fluid into water" button in the simulator to adjust the density of liquid in the beaker to 1.00 g/mL. Remove the red block from the beaker and note the volume of water is 25.5 mL. Select the iron sample by clicking "iron" in the table of materials at the bottom of the screen, place the iron block on the balance pan, and observe its mass is 31.48 g. Transfer the iron block to the beaker and notice that it sinks, displacing a volume of water equal to its own volume and causing the water level to rise to 29.5 mL. The volume of the iron block is therefore:

$$v_{\text{iron}} = 29.5 \text{ mL} - 25.5 \text{ mL} - 4.0 \text{ mL}$$

The density of the iron is then calculated to be:

$$\text{density} = \frac{\text{mass}}{\text{volume}} = \frac{31.48 \text{ g}}{4.0 \text{ mL}} = 7.9 \text{ g/mL}$$

Remove the iron block from the beaker, change the block material to wood, and then repeat the mass and volume measurements. Unlike iron, the wood block does not sink in the water but instead floats on the water's surface. To measure its volume, drag it beneath the water's surface so that it is fully submerged.

$$\text{density} = \frac{\text{mass}}{\text{volume}} = \frac{1.95\text{ g}}{3.0\text{ mL}} = 0.65\text{ g/mL}$$

Note: The sink versus float behavior illustrated in this example demonstrates the property of "buoyancy" (see end of chapter **Exercise 4.9** and **Exercise 4.10**).

Check Your Learning

Following the water displacement approach, use the simulator to measure the density of the foam sample.

Answer:

0.230 g/mL

Key Concepts and Summary

Measurements provide quantitative information that is critical in studying and practicing chemistry. Each measurement has an amount, a unit for comparison, and an uncertainty. Measurements can be represented in either decimal or scientific notation. Scientists primarily use SI (International System) units such as meters, seconds, and kilograms, as well as derived units, such as liters (for volume) and g/cm^3 (for density). In many cases, it is convenient to use prefixes that yield fractional and multiple units, such as microseconds (10^{-6} seconds) and megahertz (10^6 hertz), respectively.

Key Equations

$$\text{density} = \frac{\text{mass}}{\text{volume}}$$

Chemistry End of Chapter Exercises

Exercise 4.1

Is one liter about an ounce, a pint, a quart, or a gallon?

Exercise 4.2

Is a meter about an inch, a foot, a yard, or a mile?

Solution

about a yard

Exercise 4.3

Indicate the SI base units or derived units that are appropriate for the following measurements:

(a) the length of a marathon race (26 miles 385 yards)

(b) the mass of an automobile

(c) the volume of a swimming pool

(d) the speed of an airplane

(e) the density of gold

(f) the area of a football field

(g) the maximum temperature at the South Pole on April 1, 1913

Exercise 4.4

Indicate the SI base units or derived units that are appropriate for the following measurements:

(a) the mass of the moon

(b) the distance from Dallas to Oklahoma City

(c) the speed of sound

(d) the density of air

(e) the temperature at which alcohol boils

(f) the area of the state of Delaware

(g) the volume of a flu shot or a measles vaccination

Solution
(a) kilograms; (b) meters; (c) meters/second; (d) kilograms/cubic meter; (e) kelvin; (f) square meters; (g) cubic meters

Exercise 4.5

Give the name and symbol of the prefixes used with SI units to indicate multiplication by the following exact quantities.

(a) 10^3

(b) 10^{-2}

(c) 0.1

(d) 10^{-3}

(e) 1,000,000

(f) 0.000001

Exercise 4.6

Give the name of the prefix and the quantity indicated by the following symbols that are used with SI base units.

(a) c

(b) d

(c) G

(d) k

(e) m

(f) n

(g) p

(h) T

Solution
(a) centi-, $\times\ 10^{-2}$; (b) deci-, $\times\ 10^{-1}$; (c) Giga-, $\times\ 10^{9}$; (d) kilo-, $\times\ 10^{3}$; (e) milli-, $\times\ 10^{-3}$; (f) nano-, $\times\ 10^{-9}$; (g) pico-, $\times\ 10^{-12}$; (h) tera-, $\times\ 10^{12}$

Exercise 4.7

A large piece of jewelry has a mass of 132.6 g. A graduated cylinder initially contains 48.6 mL water. When the jewelry is submerged in the graduated cylinder, the total volume increases to 61.2 mL.

(a) Determine the density of this piece of jewelry.

(b) Assuming that the jewelry is made from only one substance, what substance is it likely to be? Explain.

Exercise 4.8

Visit this **density simulation (http://openstax.org/l/16phetmasvolden)** and click the "turn fluid into water" button to adjust the density of liquid in the beaker to 1.00 g/mL.

(a) Use the water displacement approach to measure the mass and volume of the unknown material (select the green block with question marks).

(b) Use the measured mass and volume data from step (a) to calculate the density of the unknown material.

(c) Link out to the link provided.

(d) Assuming this material is a copper-containing gemstone, identify its three most likely identities by comparing the measured density to the values tabulated at **this gemstone density guide (https://www.ajsgem.com/articles/gemstone-density-definitive-guide.html)** .

(e) How are mass and density related for blocks of the same volume?

Solution
(a) m = 18.58 g, V = 5.7 mL. (b) d = 3.3 g/mL (c) dioptase (copper cyclosilicate, d = 3.28—3.31 g/mL); malachite (basic copper carbonate, d = 3.25—4.10 g/mL); Paraiba tourmaline (sodium lithium boron silicate with copper, d = 2.82—3.32 g/mL)

Exercise 4.9

Visit this **density simulation (http://openstax.org/l/16phetmasvolden)** and click the "reset" button to ensure all simulator parameters are at their default values.

(a) Use the water displacement approach to measure the mass and volume of the red block.

(b) Use the measured mass and volume data from step (a) to calculate the density of the red block.

(c) Use the vertical green slide control to adjust the fluid density to values well above, then well below, and finally nearly equal to the density of the red block, reporting your observations.

Exercise 4.10

Visit this **density simulation (http://openstax.org/l/16phetmasvolden)** and click the “turn fluid into water” button to adjust the density of liquid in the beaker to 1.00 g/mL. Change the block material to foam, and then wait patiently until the foam block stops bobbing up and down in the water.

(a) The foam block should be floating on the surface of the water (that is, only partially submerged). What is the volume of water displaced?
(b) Use the water volume from part (a) and the density of water (1.00 g/mL) to calculate the mass of water displaced.
(c) Remove and weigh the foam block. How does the block’s mass compare to the mass of displaced water from part (b)?

Solution
(a) displaced water volume = 2.8 mL; (b) displaced water mass = 2.8 g; (c) The block mass is 2.76 g, essentially equal to the mass of displaced water (2.8 g) and consistent with Archimedes’ principle of buoyancy.

4.3 | Measurement Uncertainty, Accuracy, and Precision

By the end of this section, you will be able to:

- Define accuracy and precision
- Distinguish exact and uncertain numbers
- Correctly represent uncertainty in quantities using significant figures
- Apply proper rounding rules to computed quantities

Counting is the only type of measurement that is free from uncertainty, provided the number of objects being

counted does not change while the counting process is underway. The result of such a counting measurement is an example of an **exact number**. By counting the eggs in a carton, one can determine *exactly* how many eggs the carton contains. The numbers of defined quantities are also exact. By definition, 1 foot is exactly 12 inches, 1 inch is exactly 2.54 centimeters, and 1 gram is exactly 0.001 kilogram. Quantities derived from measurements other than counting, however, are uncertain to varying extents due to practical limitations of the measurement process used.

Significant Figures in Measurement

The numbers of measured quantities, unlike defined or directly counted quantities, are not exact. To measure the volume of liquid in a graduated cylinder, you should make a reading at the bottom of the meniscus, the lowest point on the curved surface of the liquid.

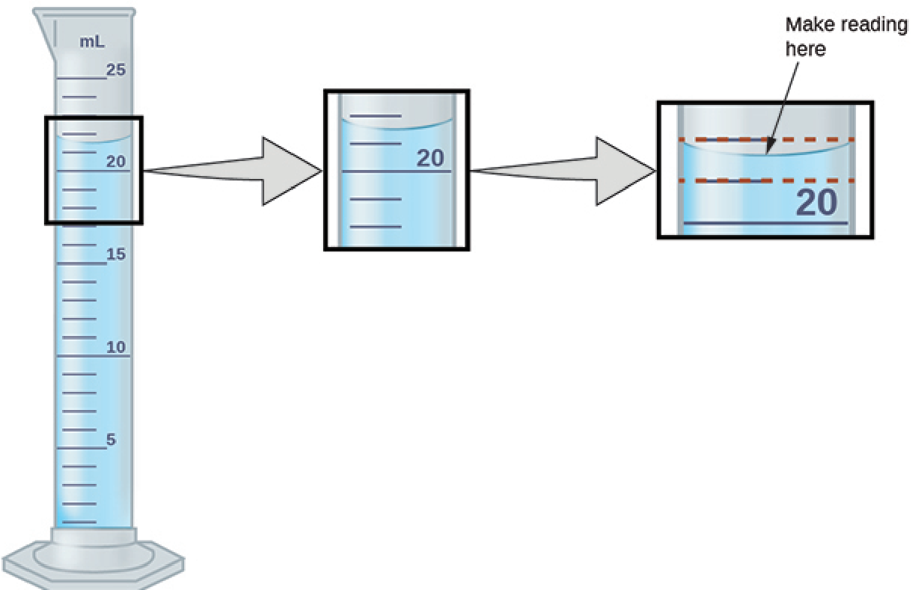


Figure 4.5 To measure the volume of liquid in this graduated cylinder, you must mentally subdivide the distance between the 21 and 22 mL marks into tenths of a milliliter, and then make a reading (estimate) at the bottom of the meniscus.

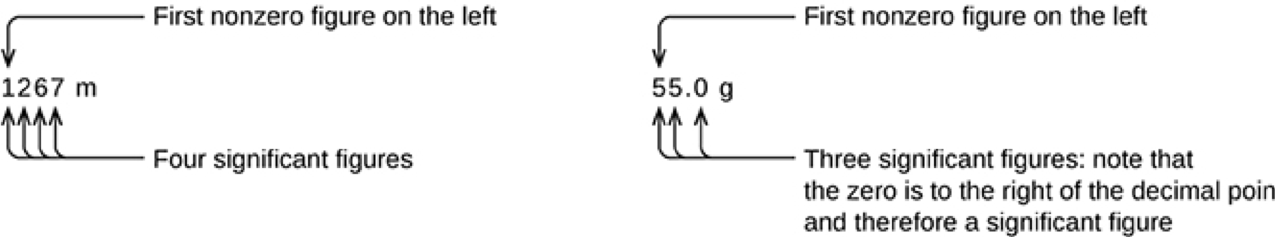
Refer to the illustration in **Figure 4.5**. The bottom of the meniscus in this case clearly lies between the 21 and 22 markings, meaning the liquid volume is *certainly* greater than 21 mL but less than 22 mL. The meniscus appears to be a bit closer to the 22-mL mark than to the 21-mL mark, and so a reasonable estimate of the liquid's volume would be 21.6 mL. In the number 21.6, then, the digits 2 and 1 are certain, but the 6 is an estimate. Some people might estimate the meniscus position to be equally distant from each of the markings and estimate the tenth-place digit as 5, while others may think it to be even closer to the 22-mL mark and estimate this digit to be 7. Note that it would be pointless to attempt to estimate a digit for the hundredths place, given that the tenths-place digit is uncertain. In general, numerical scales such as the one on this graduated cylinder will permit measurements to one-tenth of the smallest scale division. The scale in this case has 1-mL divisions, and so volumes may be measured to the nearest 0.1 mL.

This concept holds true for all measurements, even if you do not actively make an estimate. If you place a quarter on a standard electronic balance, you may obtain a reading of 6.72 g. The digits 6 and 7 are certain, and the 2 indicates that the mass of the quarter is likely between 6.71 and 6.73 grams. The quarter weighs *about* 6.72 grams, with a nominal uncertainty in the measurement of ± 0.01 gram. If the coin is weighed on a more sensitive balance, the mass might be 6.723 g. This means its mass lies between 6.722 and 6.724 grams, an uncertainty of 0.001 gram. Every measurement has some **uncertainty**, which depends on the device used (and the user's ability). All of the digits in a measurement, including the uncertain last digit, are called **significant figures** or **significant digits**. Note that zero may be a measured value; for example, if you stand on a scale that shows weight to the nearest pound and it shows "120," then the 1 (hundreds), 2 (tens) and 0 (ones) are all significant (measured) values.

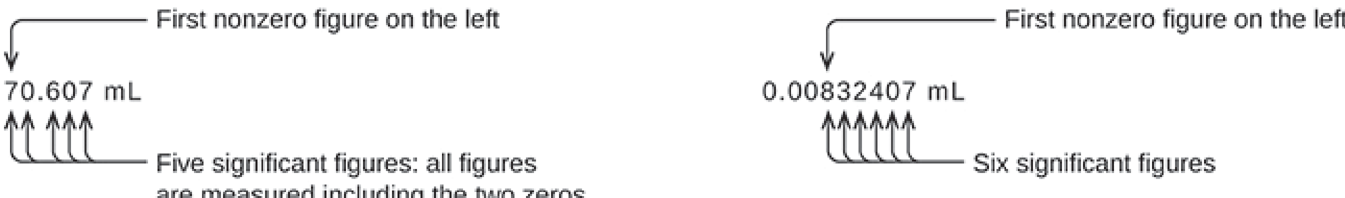
A measurement result is properly reported when its significant digits accurately represent the certainty of the measurement process. But what if you were analyzing a reported value and trying to determine what is

significant and what is not? Well, for starters, all nonzero digits are significant, and it is only zeros that require some thought. We will use the terms “leading,” “trailing,” and “captive” for the zeros and will consider how to deal with them.

Starting with the first nonzero digit on the left, count this digit and all remaining digits to the right. This is the number of significant figures in the measurement unless the last digit is a trailing zero lying to the left of the decimal point.



Captive zeros result from measurement and are therefore always significant. Leading zeros, however, are never significant—they merely tell us where the decimal point is located.



The leading zeros in this example are not significant. We could use exponential notation (as described in **Appendix B (https://legacy.cnx.org/content/m68860/latest/)**) and express the number as 8.32407×10^{-3}; then the number 8.32407 contains all of the significant figures, and 10^{-3} locates the decimal point.

The number of significant figures is uncertain in a number that ends with a zero to the left of the decimal point location. The zeros in the measurement 1,300 grams could be significant or they could simply indicate where the decimal point is located. The ambiguity can be resolved with the use of exponential notation: 1.3×10^3 (two significant figures), 1.30×10^3 (three significant figures, if the tens place was measured), or 1.300×10^3 (four significant figures, if the ones place was also measured). In cases where only the decimal-formatted number is available, it is prudent to assume that all trailing zeros are not significant.

Significant figures: clearly result of measurement

1300 g

These zeros could be significant (measured), or they could be placeholders

When determining significant figures, be sure to pay attention to reported values and think about the measurement and significant figures in terms of what is reasonable or likely when evaluating whether the value makes sense. For example, the official January 2014 census reported the resident population of the US as 317,297,725. Do you think the US population was correctly determined to the reported nine significant figures, that is, to the exact number of people? People are constantly being born, dying, or moving into or out of the country, and assumptions are made to account for the large number of people who are not actually counted. Because of these uncertainties, it might be more reasonable to expect that we know the population to within perhaps a million or so, in which case the population should be reported as 3.17×10^8 people.

Significant Figures in Calculations

A second important principle of uncertainty is that results calculated from a measurement are at least as uncertain as the measurement itself. Take the uncertainty in measurements into account to avoid misrepresenting the uncertainty in calculated results. One way to do this is to report the result of a calculation with the correct number of significant figures, which is determined by the following three rules for **rounding** numbers:

1. When adding or subtracting numbers, round the result to the same number of decimal places as the number with the least number of decimal places (the least certain value in terms of addition and subtraction).
2. When multiplying or dividing numbers, round the result to the same number of digits as the number with the least number of significant figures (the least certain value in terms of multiplication and division).
3. If the digit to be dropped (the one immediately to the right of the digit to be retained) is less than 5, “round down” and leave the retained digit unchanged; if it is more than 5, “round up” and increase the retained digit by 1. If the dropped digit is 5, and it’s either the last digit in the number or it’s followed only by zeros, round up or down, whichever yields an even value for the retained digit. If any nonzero digits follow the dropped 5, round up. (The last part of this rule may strike you as a bit odd, but it’s based on reliable statistics and is aimed at avoiding any bias when dropping the digit “5,” since it is equally close to both possible values of the retained digit.)

The following examples illustrate the application of this rule in rounding a few different numbers to three significant figures:

- 0.028675 rounds “up” to 0.0287 (the dropped digit, 7, is greater than 5)
- 18.3384 rounds “down” to 18.3 (the dropped digit, 3, is less than 5)
- 6.8752 rounds “up” to 6.88 (the dropped digit is 5, and a nonzero digit follows it)
- 92.85 rounds “down” to 92.8 (the dropped digit is 5, and the retained digit is even)

Let’s work through these rules with a few examples.

Example 4.3

Rounding Numbers

Round the following to the indicated number of significant figures:

(a) 31.57 (to two significant figures)

(b) 8.1649 (to three significant figures)

(c) 0.051065 (to four significant figures)

(d) 0.90275 (to four significant figures)

Solution

(a) 31.57 rounds “up” to 32 (the dropped digit is 5, and the retained digit is even)

(b) 8.1649 rounds “down” to 8.16 (the dropped digit, 4, is less than 5)

(c) 0.051065 rounds “down” to 0.05106 (the dropped digit is 5, and the retained digit is even)

(d) 0.90275 rounds “up” to 0.9028 (the dropped digit is 5, and the retained digit is even)

Check Your Learning

Round the following to the indicated number of significant figures:

(a) 0.424 (to two significant figures)

(b) 0.0038661 (to three significant figures)

(c) 421.25 (to four significant figures)

(d) 28,683.5 (to five significant figures)

Answer:

(a) 0.42; (b) 0.00387; (c) 421.2; (d) 28,684

Example 4.4

Addition and Subtraction with Significant Figures

Rule: When adding or subtracting numbers, round the result to the same number of decimal places as the number with the fewest decimal places (i.e., the least certain value in terms of addition and subtraction).

(a) Add 1.0023 g and 4.383 g.

(b) Subtract 421.23 g from 486 g.

Solution

(a) $$\begin{array}{r} 1.0023\text{ g} \\ +\,4.383\text{ g} \\ \hline 5.3853\text{ g} \end{array}$$

Answer is 5.385 g (round to the thousandths place; three decimal places)

(b) $$\begin{array}{r} 486\text{ g} \\ -421.23\text{ g} \\ \hline 64.77\text{ g} \end{array}$$

Answer is 65 g (round to the ones place; no decimal places)

1.0023 ← Ten thousandths place
+ 4.383 ← Thousandths place: least precise
5.3853
Round to thousandths
(a)

486 g
− 421.23 g
64.77 g → Answer is 65 g
Round to ones
(b)

Check Your Learning

(a) Add 2.334 mL and 0.31 mL.

(b) Subtract 55.8752 m from 56.533 m.

Answer:

(a) 2.64 mL; (b) 0.658 m

Example 4.5

Multiplication and Division with Significant Figures

Rule: When multiplying or dividing numbers, round the result to the same number of digits as the number with the fewest significant figures (the least certain value in terms of multiplication and division).

(a) Multiply 0.6238 cm by 6.6 cm.

(b) Divide 421.23 g by 486 mL.

Solution

(a)
$$\begin{array}{l} 0.6238 \text{ cm} \times 6.6 \text{ cm} = 4.11708 \text{ cm}^2 \longrightarrow \text{result is } 4.1 \text{ cm}^2 \text{ (round to two significant figures)} \\ \text{four significant figures} \times \text{two significant figures} \longrightarrow \text{two significant figures answer} \end{array}$$

(b)
$$\begin{array}{l} \dfrac{421.23 \text{ g}}{486 \text{ mL}} = 0.866728... \text{ g/mL} \longrightarrow \text{result is } 0.867 \text{ g/mL (round to three significant figures)} \\ \dfrac{\text{five significant figures}}{\text{three significant figures}} \longrightarrow \text{three significant figures answer} \end{array}$$

Check Your Learning

(a) Multiply 2.334 cm and 0.320 cm.

(b) Divide 55.8752 m by 56.53 s.

Answer:

(a) 0.747 cm^2 (b) 0.9884 m/s

In the midst of all these technicalities, it is important to keep in mind the reason for these rules about significant figures and rounding—to correctly represent the certainty of the values reported and to ensure that a calculated result is not represented as being more certain than the least certain value used in the calculation.

Example 4.6

Calculation with Significant Figures

One common bathtub is 13.44 dm long, 5.920 dm wide, and 2.54 dm deep. Assume that the tub is rectangular and calculate its approximate volume in liters.

Solution

$$\begin{aligned} V &= l \times w \times d \\ &= 13.44 \text{ dm} \times 5.920 \text{ dm} \times 2.54 \text{ dm} \\ &= 202.09459... \text{ dm}^3 \text{ (value from calculator)} \\ &= 202 \text{ dm}^3\text{, or } 202 \text{ L (answer rounded to three significant figures)} \end{aligned}$$

Check Your Learning

What is the density of a liquid with a mass of 31.1415 g and a volume of 30.13 cm^3?

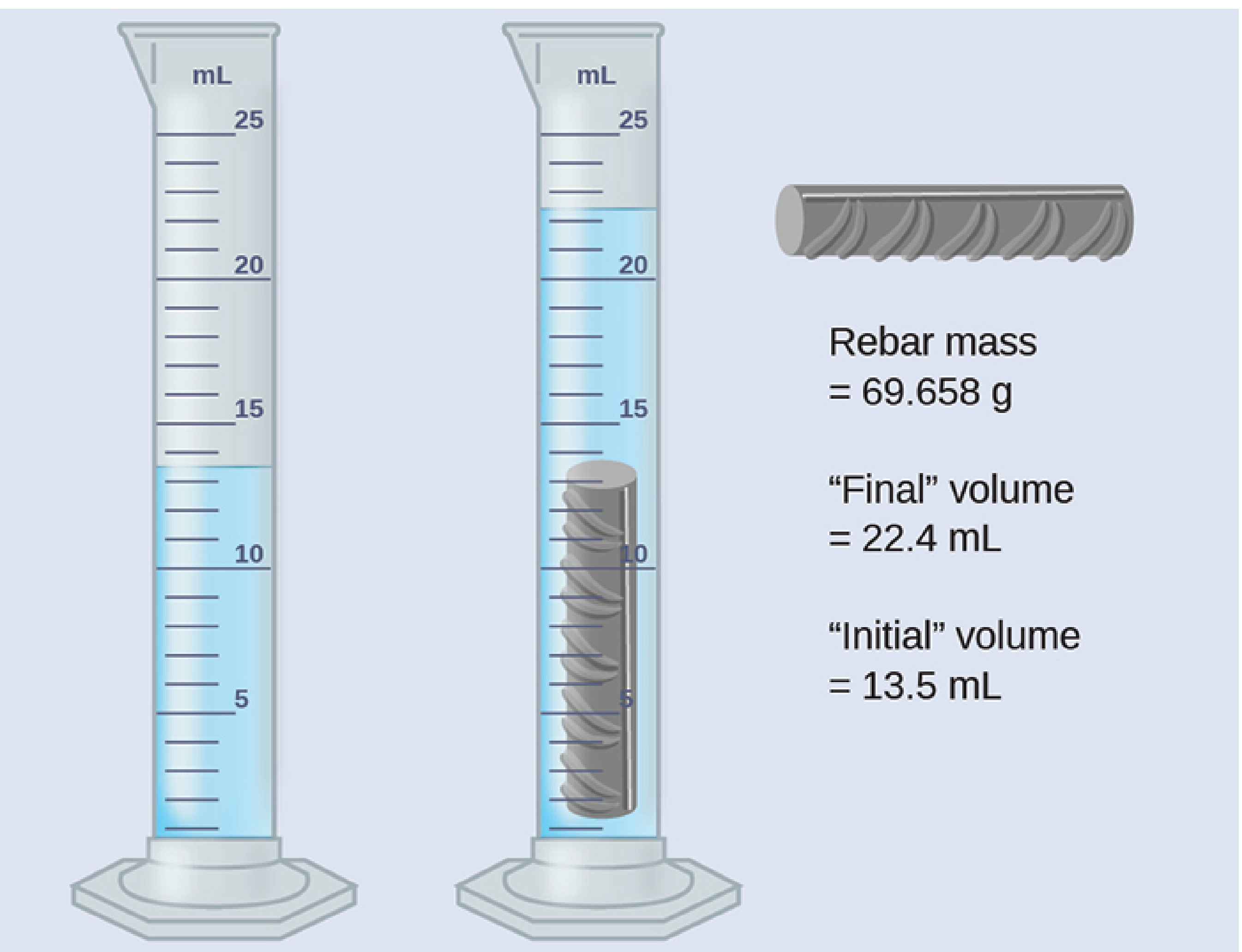
Answer:

1.034 g/mL

Example 4.7

Experimental Determination of Density Using Water Displacement

A piece of rebar is weighed and then submerged in a graduated cylinder partially filled with water, with results as shown.



(a) Use these values to determine the density of this piece of rebar.

(b) Rebar is mostly iron. Does your result in (a) support this statement? How?

Solution

The volume of the piece of rebar is equal to the volume of the water displaced:

$$\text{volume} = 22.4\text{ mL} - 13.5\text{ mL} = 8.9\text{ mL} = 8.9\text{ cm}^3$$

(rounded to the nearest 0.1 mL, per the rule for addition and subtraction)

The density is the mass-to-volume ratio:

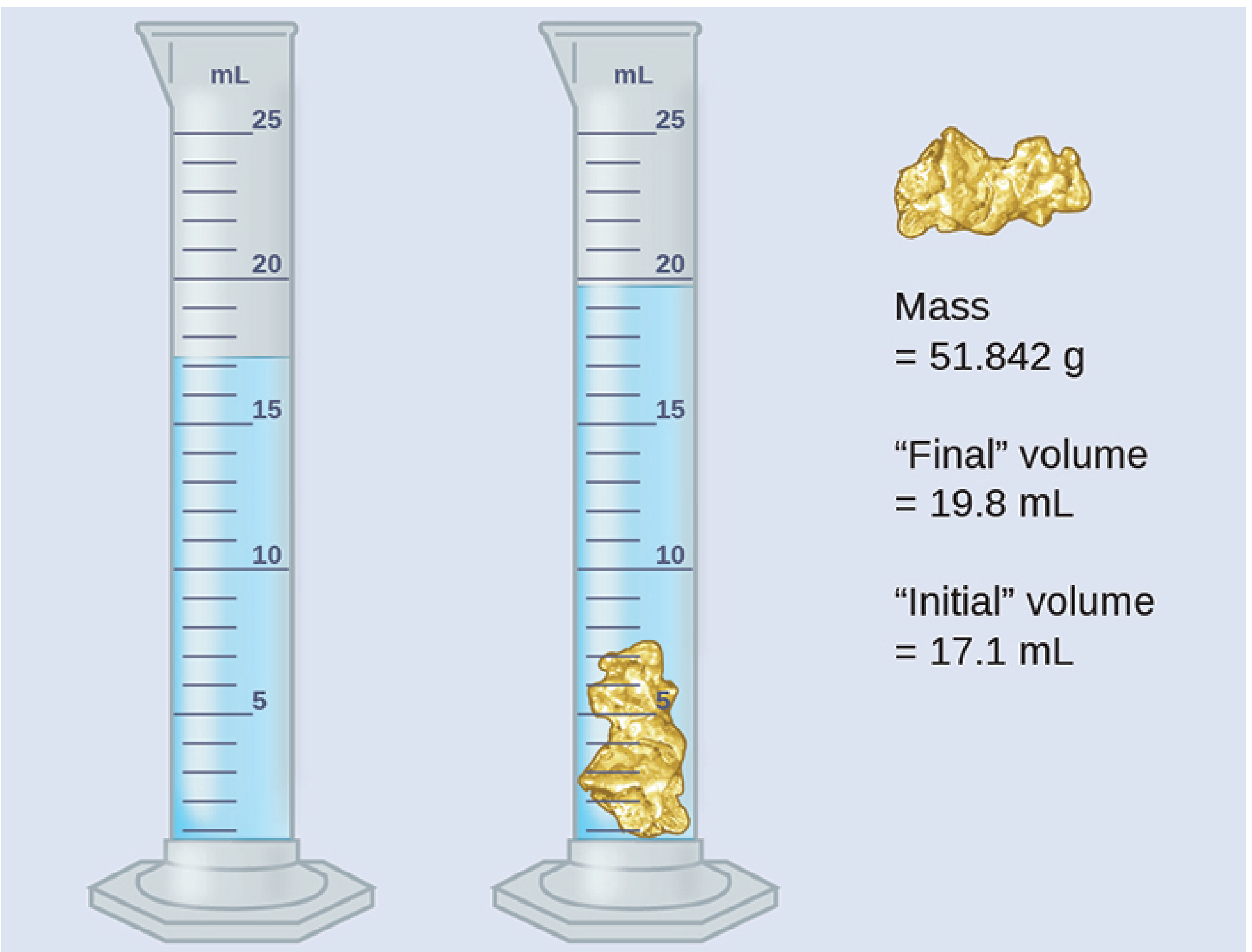
$$\text{density} = \frac{\text{mass}}{\text{volume}} = \frac{69.658\text{ g}}{8.9\text{ cm}^3} = 7.8\text{ g/cm}^3$$

(rounded to two significant figures, per the rule for multiplication and division)

From **Table 4.3**, the density of iron is 7.9 g/cm^3, very close to that of rebar, which lends some support to the fact that rebar is mostly iron.

Check Your Learning

An irregularly shaped piece of a shiny yellowish material is weighed and then submerged in a graduated cylinder, with results as shown.



(a) Use these values to determine the density of this material.

(b) Do you have any reasonable guesses as to the identity of this material? Explain your reasoning.

Answer:

(a) 19 g/cm^3; (b) It is likely gold; the right appearance for gold and very close to the density given for gold in Table 4.3.

Accuracy and Precision

Scientists typically make repeated measurements of a quantity to ensure the quality of their findings and to evaluate both the **precision** and the **accuracy** of their results. Measurements are said to be precise if they yield very similar results when repeated in the same manner. A measurement is considered accurate if it yields a result that is very close to the true or accepted value. Precise values agree with each other; accurate values agree with a true value. These characterizations can be extended to other contexts, such as the results of an archery competition (Figure 4.6).

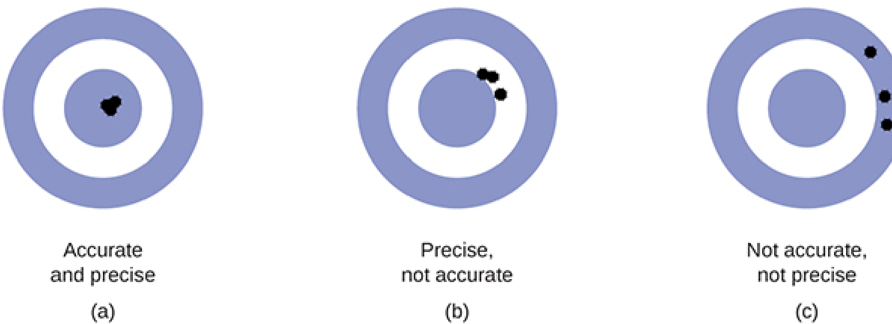


Figure 4.6 (a) These arrows are close to both the bull's eye and one another, so they are both accurate and precise. (b) These arrows are close to one another but not on target, so they are precise but not accurate. (c) These arrows are neither on target nor close to one another, so they are neither accurate nor precise.

Suppose a quality control chemist at a pharmaceutical company is tasked with checking the accuracy and precision of three different machines that are meant to dispense 10 ounces (296 mL) of cough syrup into storage bottles. She proceeds to use each machine to fill five bottles and then carefully determines the actual volume dispensed, obtaining the results tabulated in Table 4.4.

Volume (mL) of Cough Medicine Delivered by 10-oz (296 mL) Dispensers

Dispenser #1	Dispenser #2	Dispenser #3
283.3	298.3	296.1
284.1	294.2	295.9
283.9	296.0	296.1
284.0	297.8	296.0
284.1	293.9	296.1

Table 4.4

Considering these results, she will report that dispenser #1 is precise (values all close to one another, within a few tenths of a milliliter) but not accurate (none of the values are close to the target value of 296 mL, each being more than 10 mL too low). Results for dispenser #2 represent improved accuracy (each volume is less than 3 mL away from 296 mL) but worse precision (volumes vary by more than 4 mL). Finally, she can report that dispenser #3 is working well, dispensing cough syrup both accurately (all volumes within 0.1 mL of the target volume) and precisely (volumes differing from each other by no more than 0.2 mL).

Key Concepts and Summary

Quantities can be defined or measured. Measured quantities have an associated uncertainty that is represented by the number of significant figures in the quantity's number. The uncertainty of a calculated quantity depends on the uncertainties in the quantities used in the calculation and is reflected in how the value is rounded. Quantities are characterized with regard to accuracy (closeness to a true or accepted value) and precision (variation among replicate measurement results).

Chemistry End of Chapter Exercises

Exercise 4.11

Express each of the following numbers in scientific notation with correct significant figures:

(a) 711.0

(b) 0.239

(c) 90743

(d) 134.2

(e) 0.05499

(f) 10000.0

(g) 0.000000738592

Exercise 4.12

Express each of the following numbers in exponential notation with correct significant figures:

(a) 704

(b) 0.03344

(c) 547.9

(d) 22086

(e) 1000.00

(f) 0.0000000651

(g) 0.007157

Solution

(a) 7.04×10^{2}; (b) 3.344×10^{-2}; (c) 5.479×10^{2}; (d) 2.2086×10^{4}; (e) 1.00000×10^{3}; (f) 6.51×10^{-8}; (g) 7.157×10^{-3}

Exercise 4.13

Indicate whether each of the following can be determined exactly or must be measured with some degree of uncertainty:

(a) the number of eggs in a basket

(b) the mass of a dozen eggs

(c) the number of gallons of gasoline necessary to fill an automobile gas tank

(d) the number of cm in 2 m

(e) the mass of a textbook

(f) the time required to drive from San Francisco to Kansas City at an average speed of 53 mi/h

Exercise 4.14

Indicate whether each of the following can be determined exactly or must be measured with some degree of uncertainty:

(a) the number of seconds in an hour

(b) the number of pages in this book

(c) the number of grams in your weight

(d) the number of grams in 3 kilograms

(e) the volume of water you drink in one day

(f) the distance from San Francisco to Kansas City

Solution

(a) exact; (b) exact; (c) uncertain; (d) exact; (e) uncertain; (f) uncertain

Exercise 4.15

How many significant figures are contained in each of the following measurements?

(a) 38.7 g

(b) 2×10^{18} m

(c) 3,486,002 kg

(d) 9.74150×10^{-4} J

(e) 0.0613 cm^3

(f) 17.0 kg

(g) 0.01400 g/mL

Exercise 4.16

How many significant figures are contained in each of the following measurements?

(a) 53 cm

(b) 2.05×10^8 m

(c) 86,002 J

(d) 9.740×10^4 m/s

(e) 10.0613 m^3

(f) 0.17 g/mL

(g) 0.88400 s

Solution

(a) two; (b) three; (c) five; (d) four; (e) six; (f) two; (g) five

Exercise 4.17

The following quantities were reported on the labels of commercial products. Determine the number of significant figures in each.

(a) 0.0055 g active ingredients

(b) 12 tablets

(c) 3% hydrogen peroxide

(d) 5.5 ounces

(e) 473 mL

(f) 1.75% bismuth

(g) 0.001% phosphoric acid

(h) 99.80% inert ingredients

Exercise 4.18

Round off each of the following numbers to two significant figures:

(a) 0.436

(b) 9.000

(c) 27.2

(d) 135

(e) 1.497×10^{-3}

(f) 0.445

Solution

(a) 0.44; (b) 9.0; (c) 27; (d) 140; (e) 1.5×10^{-3}; (f) 0.44

Exercise 4.19

Round off each of the following numbers to two significant figures:

(a) 517

(b) 86.3

(c) 6.382×10^3

(d) 5.0008

(e) 22.497

(f) 0.885

Exercise 4.20

Perform the following calculations and report each answer with the correct number of significant figures.

(a) 628×342

(b) $(5.63 \times 10^2) \times (7.4 \times 10^3)$

(c) $\frac{28.0}{13.483}$

(d) 8119×0.000023

(e) 14.98 + 27,340 + 84.7593

(f) 42.7 + 0.259

Solution

(a) 2.15×10^5; (b) 4.2×10^6; (c) 2.08; (d) 0.19; (e) 27,440; (f) 43.0

Exercise 4.21

Perform the following calculations and report each answer with the correct number of significant figures.

(a) 62.8×34

(b) 0.147 + 0.0066 + 0.012

(c) $38 \times 95 \times 1.792$

(d) 15 – 0.15 – 0.6155

(e) $8.78 \times \left(\frac{0.0500}{0.478}\right)$

(f) 140 + 7.68 + 0.014

(g) 28.7 – 0.0483

(h) $\frac{(88.5 - 87.57)}{45.13}$

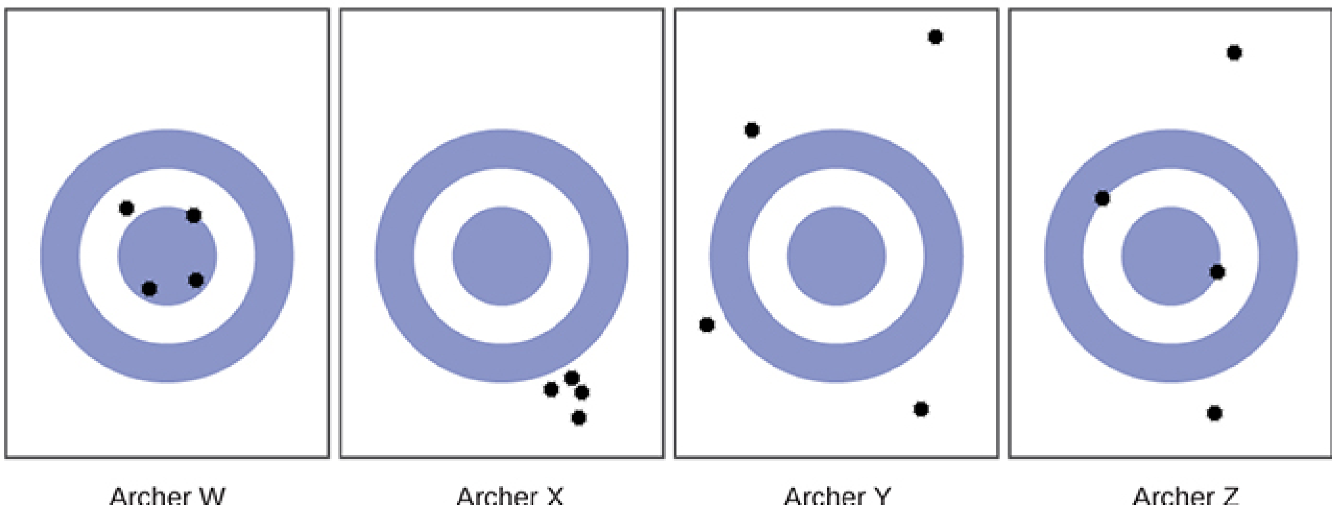
Exercise 4.22

Consider the results of the archery contest shown in this figure.

(a) Which archer is most precise?

(b) Which archer is most accurate?

(c) Who is both least precise and least accurate?



Solution

(a) Archer X; (b) Archer W; (c) Archer Y

Exercise 4.23

Classify the following sets of measurements as accurate, precise, both, or neither.

(a) Checking for consistency in the weight of chocolate chip cookies: 17.27 g, 13.05 g, 19.46 g, 16.92 g

(b) Testing the volume of a batch of 25-mL pipettes: 27.02 mL, 26.99 mL, 26.97 mL, 27.01 mL

(c) Determining the purity of gold: 99.9999%, 99.9998%, 99.9998%, 99.9999%

4.4 | Method Validation

Method validation is the process used to determine the conditions to obtain a result reliably, and the limits of that procedure. Formal validation of test methods is required in GMP compliant laboratories. However, the aspects of validation are appropriate for any research or testing laboratory.

METHOD VALIDATION

Method validation is the process used to determine the conditions to obtain a result reliably, and the limits of that procedure. Formal validation of test methods is required in GMP compliant laboratories. However, the aspects of validation are appropriate for any research or testing laboratory.

Accuracy

Accuracy is the closeness of a test result to the true or accepted value. Accuracy can be tested by using a reference standard value is known. If it is a method being tested, results are compared to those of a standardized assay. Tests for accuracy are also used to validate equipment. Accuracy is calculated by determining the percent error of the mean (PEM).

PEM = (calculated mean – true value) ÷ true value x 100%

Precision

Precision is the degree of agreement between individual test results when the procedure is applied repeatedly. Repeatability is the precision of measurements made under uniform conditions and reproducibility (ruggedness) is the precision of measurements made under non-uniform conditions such as in two different laboratories. When precision is high, there is a high degree of agreement in data. **Precision is calculated using standard deviation**.

Limit of Detection (LOD)

LOD is the lowest concentration of the analyte which can be detected above background by a method. There is always some error associated with any analytical measurement. An important determination that must be made is how significant a signal must be before it can be distinguishable from background. The accepted rule in analytical chemistry is that the signal must be at least three times greater than the background noise. It is important to note that an analytical method can never prove that a substance is not present; rather it can show only the limit which the sample cannot be detected. Interpolate from linear regression to determine the concentration value of LOD.

LOD= Instrument Output of the blank + 3xSD of the blank

Limit of Quantitation (LOQ)

LOQ is the lowest concentration of the analyte that the method can quantify with acceptable accuracy and precision. Depending on the application, this is 5-10X the standard deviation of the blank. Interpolate from linear regression to determine the concentration value of LOQ.

LOQ=Instrument Output of the blank + 10xSD of the blank

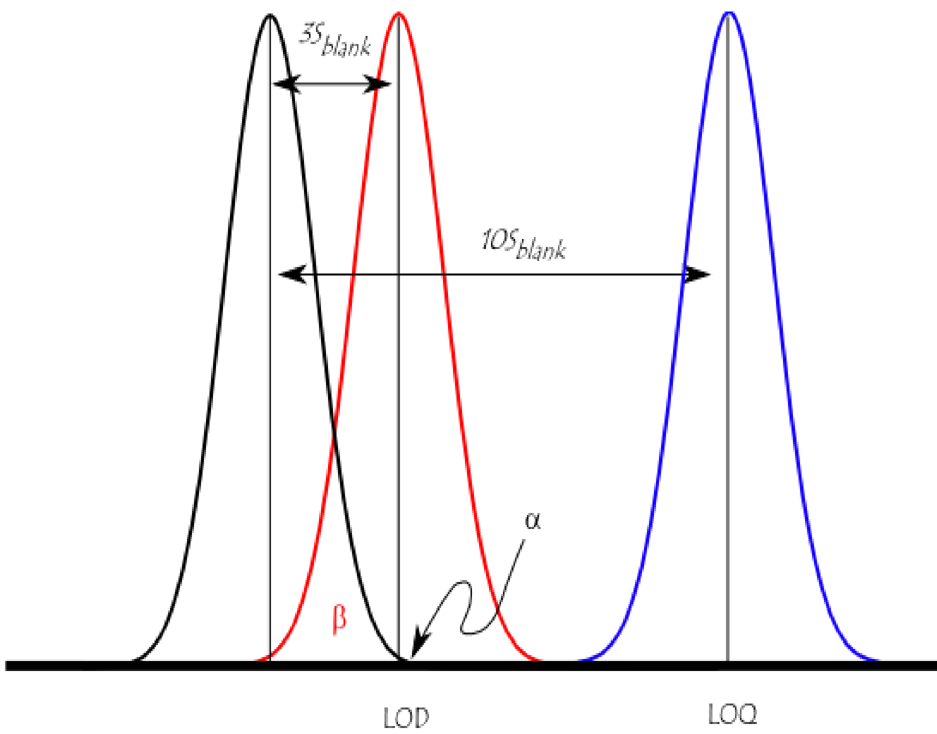


Figure 4.7 Illustration of the concept of detection limit and quantitation limit by showing the theoretical normal distributions associated with blank, detection limit, and quantification limit level samples. Image credit: **https://commons.wikimedia.org/wiki/File:LOD.png (https://commons.wikimedia.org/wiki/File:LOD.png)**

Specificity

Specificity is a measure of the extent to which a method can identify the presence of a compound in a sample without interference from other materials that are present. This is sometimes referred to as selectivity. A very selective test will only give a positive result for the compound of interest.

Linearity

Linearity is the ability of a method to give test results that are directly proportional to the concentration of the material of interest within a given concentration range. The range is defined as the limits of concentrations, from the lowest to the highest, that a method can measure with acceptable results. Tests and Assays have a range in which they exhibit linearity. For example, many assays for proteins display a standard curve that is linear at lower concentrations but flattens out at higher concentrations of the protein. Therefore, a test for protein will be accurate only in the range of lower concentrations when the curve is linear.

Robustness

Robustness is a measure of the capacity of a method to remain unaffected when there are small, deliberate

variations in method parameters. It indicates the method's reliability during normal use.

Ruggedness

Ruggedness is the degree of reproducibility of the results obtained by the analysis of the same samples under a variety of conditions. Ruggedness provides a measure of reproducibility across variations of conditions normally expected, such as, from lab to lab and analyst to analyst.

The Clinical Laboratory Improvement Amendments

Content credit: **https://en.wikipedia.org/wiki/Clinical_Laboratory_Improvement_Amendments (https://en.wikipedia.org/wiki/Clinical_Laboratory_Improvement_Amendments)** and **https://www.cms.gov/ (https://www.cms.gov/)**

The Clinical Laboratory Improvement Amendments (CLIA) of 1988 are United States federal regulatory standards that apply to all clinical laboratory testing performed on humans in the United States, except clinical trials and basic research. CLIA "defines quality standards for all laboratory testing to ensure accuracy, reliability, and timeliness of patient results regardless of where the test was performed" (CMS.gov).

FDA	Categorizes tests based on the complexity Reviews requests for Waiver by Application Develops rules/guidance for CLIA complexity categorization
CMS (http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/index.html?redirect=/clia/)	Issues laboratory certificates Collects user fees Conducts inspections and enforces regulatory compliance Monitors laboratory performance on Proficiency Testing Publishes CLIA rules and regulations
CDC (http://wwwn.cdc.gov/CLIA/Default.aspx)	Provides analysis, research, and technical assistance Develops technical standards and laboratory practice guidelines Conducts laboratory quality improvement studies Monitors proficiency testing practices Develops and distributes educational resources

Table 4.5 Three federal agencies are responsible for CLIA, each having a unique role in assuring quality standards: The Food and Drug Administration (FDA), Center for Medicaid Services (CMS) and the Center for Disease Control (CDC).

CLIA Program

In accord with the CLIA, the CLIA Program sets standards and issues certificates for clinical laboratory testing. CLIA defines a clinical laboratory as any facility which performs laboratory testing on specimens derived from humans to provide information for diagnosis, prevention, or treatment of disease or impairment, and health assessments.

An objective of the CLIA is to ensure the accuracy, reliability and timeliness of test results regardless of where

the test was performed. Most Laboratory Developed Tests have been regulated under this program. In 2014 the FDA started a public discussion about regulating some LDTs. Per CLIA, each specific laboratory system, assay, examination is graded for level of complexity by assigning scores of 1, 2, or 3 for each of the following seven criteria. A score 1 is the lowest level of complexity and a score of 3 indicates the highest level. Score 2 is assigned when the characteristics for a test are intermediate between the descriptions listed for scores of 1 and 3.

Criteria for categorization:

1. Knowledge
2. Training and experience
3. Reagents and materials preparation
4. Characteristics of operational steps
5. Calibration, quality control, and proficiency testing materials
6. Test system troubleshooting and equipment maintenance
7. Interpretation and judgment

Centers for Medicare and Medicaid Services (CMS) has the primary responsibility for the operation of the CLIA Program. Within CMS, the program is implemented by the Center for Medicaid and State Operations, Survey and Certification Group, and the Division of Laboratory Services.

CLIA-waived tests

Under CLIA, tests and test systems that meet risk, error, and complexity requirements are issued a CLIA certificate of waiver. In November 2007, the CLIA waiver provisions were revised by the United States Congress to make it clear that tests approved by the FDA for home use automatically qualify for CLIA waiver, although many waived tests are not done according to designed protocols – more than 50% of such tests are done incorrectly – and result in medical errors, some with fatal consequences.

KEY TERMS

accuracy how closely a measurement aligns with a correct value

Celsius (°C) unit of temperature; water freezes at 0 °C and boils at 100 °C on this scale

cubic centimeter (cm^3 or cc) volume of a cube with an edge length of exactly 1 cm

cubic meter (m^3) SI unit of volume

density ratio of mass to volume for a substance or object

exact number number derived by counting or by definition

kelvin (K) SI unit of temperature; 273.15 K = 0 ºC

kilogram (kg) standard SI unit of mass; 1 kg = approximately 2.2 pounds

length measure of one dimension of an object

liter (L) (also, cubic decimeter) unit of volume; 1 L = 1,000 cm^3

meter (m) standard metric and SI unit of length; 1 m = approximately 1.094 yards

milliliter (mL) 1/1,000 of a liter; equal to 1 cm^3

precision how closely a measurement matches the same measurement when repeated

rounding procedure used to ensure that calculated results properly reflect the uncertainty in the measurements used in the calculation

second (s) SI unit of time

SI units (International System of Units) standards fixed by international agreement in the International System of Units (*Le Système International d'Unités*)

significant figures (also, significant digits) all of the measured digits in a determination, including the uncertain last digit

uncertainty estimate of amount by which measurement differs from true value

unit standard of comparison for measurements

volume amount of space occupied by an object

5 | BIOTECHNICIAN TOOLS: PREPARING SOLUTIONS

Figure 5.1 Chemical substances and processes are essential for our existence, providing sustenance, keeping us clean and healthy, fabricating electronic devices, enabling transportation, and much more. (credit "left": modification of work by "vxla"/Flickr; credit "left middle": modification of work by "the Italian voice"/Flickr; credit "right middle": modification of work by Jason Trim; credit "right": modification of work by "gosheshe"/Flickr)

Chapter Outline

5.1: Formula Mass and the Mole Concept
5.2: Molarity
5.3: Other Units for Solution Concentrations
5.4: Buffers
5.5: Water Standards
5.6: Water Purification

Introduction

Your alarm goes off and, after hitting "snooze" once or twice, you pry yourself out of bed. You make a cup of coffee to help you get going, and then you shower, get dressed, eat breakfast, and check your phone for messages. On your way to school, you stop to fill your car's gas tank, almost making you late for the first day of chemistry class. As you find a seat in the classroom, you read the question projected on the screen: "Welcome to class! Why should we study chemistry?"

Do you have an answer? You may be studying chemistry because it fulfills an academic requirement, but if you consider your daily activities, you might find chemistry interesting for other reasons. Most everything you do and encounter during your day involves chemistry. Making coffee, cooking eggs, and toasting bread involve chemistry. The products you use—like soap and shampoo, the fabrics you wear, the electronics that keep you connected to your world, the gasoline that propels your car—all of these and more involve chemical substances and processes. Whether you are aware or not, chemistry is part of your everyday world. In this course, you will learn many of the essential principles underlying the chemistry of modern-day life.

5.1 | Formula Mass and the Mole Concept

By the end of this section, you will be able to:

- Calculate formula masses for covalent and ionic compounds
- Define the amount unit mole and the related quantity Avogadro's number Explain the relation between mass, moles, and numbers of atoms or molecules, and perform calculations deriving these quantities from one another

Many argue that modern chemical science began when scientists started exploring the quantitative as well as the qualitative aspects of chemistry. For example, Dalton's atomic theory was an attempt to explain the results of measurements that allowed him to calculate the relative masses of elements combined in various compounds. Understanding the relationship between the masses of atoms and the chemical formulas of compounds allows us to quantitatively describe the composition of substances.

Formula Mass

An earlier chapter of this text described the development of the atomic mass unit, the concept of average atomic masses, and the use of chemical formulas to represent the elemental makeup of substances. These ideas can be extended to calculate the **formula mass** of a substance by summing the average atomic masses of all the atoms represented in the substance's formula.

Formula Mass for Covalent Substances

For covalent substances, the formula represents the numbers and types of atoms composing a single molecule of the substance; therefore, the formula mass may be correctly referred to as a molecular mass. Consider chloroform ($CHCl_3$), a covalent compound once used as a surgical anesthetic and now primarily used in the production of tetrafluoroethylene, the building block for the "anti-stick" polymer, Teflon. The molecular formula of chloroform indicates that a single molecule contains one carbon atom, one hydrogen atom, and three chlorine atoms. The average molecular mass of a chloroform molecule is therefore equal to the sum of the average atomic masses of these atoms. Figure 5.2 outlines the calculations used to derive the molecular mass of chloroform, which is 119.37 amu.

Element	Quantity		Average atomic mass (amu)		Subtotal (amu)
C	1	×	12.01	=	12.01
H	1	×	1.008	=	1.008
Cl	3	×	35.45	=	106.35
				Molecular mass	119.37

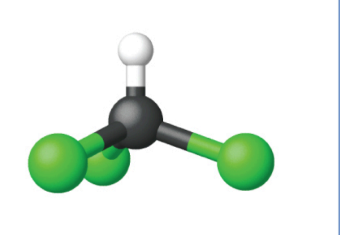


Figure 5.2 The average mass of a chloroform molecule, $CHCl_3$, is 119.37 amu, which is the sum of the average atomic masses of each of its constituent atoms. The model shows the molecular structure of chloroform.

Likewise, the molecular mass of an aspirin molecule, $C_9H_8O_4$, is the sum of the atomic masses of nine carbon atoms, eight hydrogen atoms, and four oxygen atoms, which amounts to 180.15 amu (Figure 5.3).

Element	Quantity		Average atomic mass (amu)		Subtotal (amu)
C	9	×	12.01	=	108.09
H	8	×	1.008	=	8.064
O	4	×	16.00	=	64.00
			Molecular mass		180.15

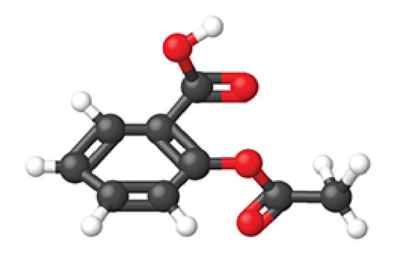


Figure 5.3 The average mass of an aspirin molecule is 180.15 amu. The model shows the molecular structure of aspirin, $C_9H_8O_4$.

Example 5.1

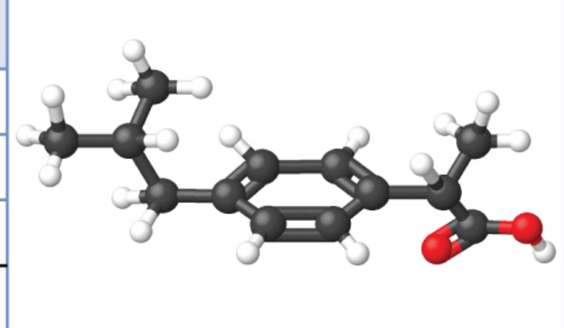
Computing Molecular Mass for a Covalent Compound

Ibuprofen, $C_{13}H_{18}O_2$, is a covalent compound and the active ingredient in several popular nonprescription pain medications, such as Advil and Motrin. What is the molecular mass (amu) for this compound?

Solution

Molecules of this compound are composed of 13 carbon atoms, 18 hydrogen atoms, and 2 oxygen atoms. Following the approach described above, the average molecular mass for this compound is therefore:

Element	Quantity		Average atomic mass (amu)		Subtotal (amu)
C	13	×	12.01	=	156.13
H	18	×	1.008	=	18.144
O	2	×	16.00	=	32.00
			Molecular mass		206.27



Check Your Learning

Acetaminophen, $C_8H_9NO_2$, is a covalent compound and the active ingredient in several popular nonprescription pain medications, such as Tylenol. What is the molecular mass (amu) for this compound?

Answer:

151.16 amu

Formula Mass for Ionic Compounds

Ionic compounds are composed of discrete cations and anions combined in ratios to yield electrically neutral bulk matter. The formula mass for an ionic compound is calculated in the same way as the formula mass for covalent compounds: by summing the average atomic masses of all the atoms in the compound's formula. Keep in mind, however, that the formula for an ionic compound does not represent the composition of a discrete molecule, so it may not correctly be referred to as the "molecular mass."

As an example, consider sodium chloride, NaCl, the chemical name for common table salt. Sodium chloride is an ionic compound composed of sodium cations, Na^+, and chloride anions, Cl^-, combined in a 1:1 ratio. The formula mass for this compound is computed as 58.44 amu (see **Figure 5.4**).

Element	Quantity		Average atomic mass (amu)		Subtotal
Na	1	×	22.99	=	22.99
Cl	1	×	35.45	=	35.45
				Formula mass	58.44

Figure 5.4 Table salt, NaCl, contains an array of sodium and chloride ions combined in a 1:1 ratio. Its formula mass is 58.44 amu.

Note that the average masses of neutral sodium and chlorine atoms were used in this computation, rather than the masses for sodium cations and chlorine anions. This approach is perfectly acceptable when computing the formula mass of an ionic compound. Even though a sodium cation has a slightly smaller mass than a sodium atom (since it is missing an electron), this difference will be offset by the fact that a chloride anion is slightly more massive than a chloride atom (due to the extra electron). Moreover, the mass of an electron is negligibly small with respect to the mass of a typical atom. Even when calculating the mass of an isolated ion, the missing or additional electrons can generally be ignored, since their contribution to the overall mass is negligible, reflected only in the nonsignificant digits that will be lost when the computed mass is properly rounded. The few exceptions to this guideline are very light ions derived from elements with precisely known atomic masses.

Example 5.2

Computing Formula Mass for an Ionic Compound

Aluminum sulfate, $Al_2(SO_4)_3$, is an ionic compound that is used in the manufacture of paper and in various water purification processes. What is the formula mass (amu) of this compound?

Solution

The formula for this compound indicates it contains Al^{3+} and $SO_4{}^{2-}$ ions combined in a 2:3 ratio. For purposes of computing a formula mass, it is helpful to rewrite the formula in the simpler format, $Al_2S_3O_{12}$. Following the approach outlined above, the formula mass for this compound is calculated as follows:

Element	Quantity		Average atomic mass (amu)		Subtotal (amu)
Al	2	×	26.98	=	53.96
S	3	×	32.06	=	96.18
O	12	×	16.00	=	192.00
				Molecular mass	342.14

Check Your Learning

Calcium phosphate, $Ca_3(PO_4)_2$, is an ionic compound and a common anti-caking agent added to food products. What is the formula mass (amu) of calcium phosphate?

Answer:

310.18 amu

The Mole

The identity of a substance is defined not only by the types of atoms or ions it contains, but by the quantity of each type of atom or ion. For example, water, H_2O, and hydrogen peroxide, H_2O_2, are alike in that their respective molecules are composed of hydrogen and oxygen atoms. However, because a hydrogen peroxide molecule contains two oxygen atoms, as opposed to the water molecule, which has only one, the two substances

exhibit very different properties. Today, sophisticated instruments allow the direct measurement of these defining microscopic traits; however, the same traits were originally derived from the measurement of macroscopic properties (the masses and volumes of bulk quantities of matter) using relatively simple tools (balances and volumetric glassware). This experimental approach required the introduction of a new unit for amount of substances, the *mole*, which remains indispensable in modern chemical science.

The *mole* is an amount unit similar to familiar units like pair, dozen, gross, etc. It provides a specific measure of *the number* of atoms or molecules in a sample of matter. One Latin connotation for the word “mole” is “large mass” or “bulk,” which is consistent with its use as the name for this unit. The mole provides a link between an easily measured macroscopic property, bulk mass, and an extremely important fundamental property, number of atoms, molecules, and so forth. A **mole** of substance is that amount in which there are $6.02214076 \times 10^{23}$ discrete entities (atoms or molecules). This large number is a fundamental constant known as **Avogadro’s number (N_A)** or the Avogadro constant in honor of Italian scientist Amedeo Avogadro. This constant is properly reported with an explicit unit of “per mole,” a conveniently rounded version being 6.022×10^{23}/mol.

Consistent with its definition as an amount unit, 1 mole of any element contains the same number of atoms as 1 mole of any other element. The masses of 1 mole of different elements, however, are different, since the masses of the individual atoms are drastically different. The **molar mass** of an element (or compound) is the mass in grams of 1 mole of that substance, a property expressed in units of grams per mole (g/mol) (see **Figure 5.5**).

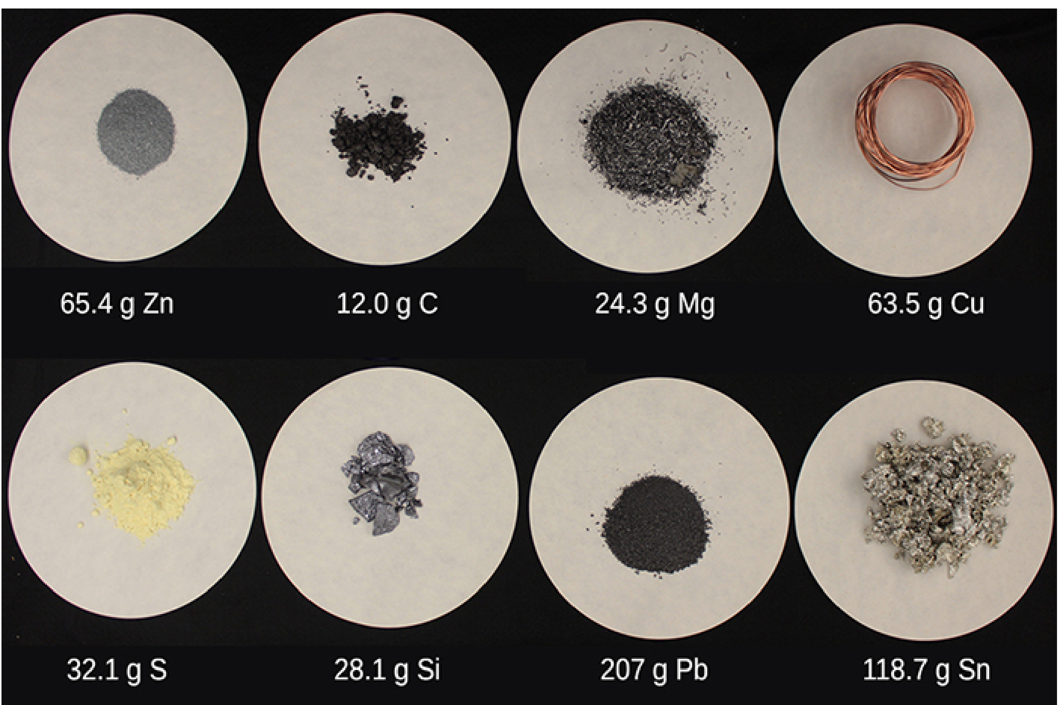


Figure 5.5 Each sample contains 6.022×10^{23} atoms —1.00 mol of atoms. From left to right (top row): 65.4 g zinc, 12.0 g carbon, 24.3 g magnesium, and 63.5 g copper. From left to right (bottom row): 32.1 g sulfur, 28.1 g silicon, 207 g lead, and 118.7 g tin. (credit: modification of work by Mark Ott)

The molar mass of any substance is numerically equivalent to its atomic or formula weight in amu. Per the amu definition, a single ^{12}C atom weighs 12 amu (its atomic mass is 12 amu). A mole of ^{12}C weighs 12 g (its molar mass is 12 g/mol). This relationship holds for all elements, since their atomic masses are measured relative to that of the amu-reference substance, ^{12}C. Extending this principle, the molar mass of a compound in grams is likewise numerically equivalent to its formula mass in amu (**Figure 5.6**).

Figure 5.6 Each sample contains 6.02×10^{23} molecules or formula units—1.00 mol of the compound or element. Clock-wise from the upper left: 130.2 g of $C_8H_{17}OH$ (1-octanol, formula mass 130.2 amu), 454.4 g of HgI_2 (mercury(II) iodide, formula mass 454.4 amu), 32.0 g of CH_3OH (methanol, formula mass 32.0 amu) and 256.5 g of S_8 (sulfur, formula mass 256.5 amu). (credit: Sahar Atwa)

Element	Average Atomic Mass (amu)	Molar Mass (g/mol)	Atoms/Mole
C	12.01	12.01	6.022×10^{23}
H	1.008	1.008	6.022×10^{23}
O	16.00	16.00	6.022×10^{23}
Na	22.99	22.99	6.022×10^{23}
Cl	35.45	35.45	6.022×10^{23}

While atomic mass and molar mass are numerically equivalent, keep in mind that they are vastly different in terms of scale, as represented by the vast difference in the magnitudes of their respective units (amu versus g). To appreciate the enormity of the mole, consider a small drop of water weighing about 0.03 g (see **Figure 5.7**). Although this represents just a tiny fraction of 1 mole of water (~18 g), it contains more water molecules than can be clearly imagined. If the molecules were distributed equally among the roughly seven billion people on earth, each person would receive more than 100 billion molecules.

Figure 5.7 The number of molecules in a single droplet of water is roughly 100 billion times greater than the number of people on earth. (credit: “tanakawho”/Wikimedia commons)

The mole is used in chemistry to represent 6.022×10^{23} of something, but it can be difficult to conceptualize such a large number. Watch this **video (http://openstax.org/l/16molevideo)** and then complete the “Think” questions that follow. Explore more about the mole by reviewing the information under “Dig Deeper.”

The relationships between formula mass, the mole, and Avogadro’s number can be applied to compute various quantities that describe the composition of substances and compounds, as demonstrated in the next several example problems.

Example 5.3

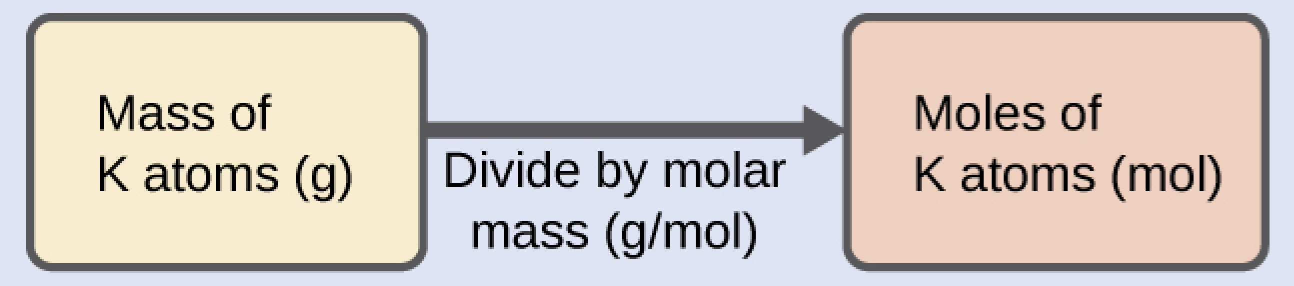
Deriving Moles from Grams for an Element

According to nutritional guidelines from the US Department of Agriculture, the estimated average requirement for dietary potassium is 4.7 g. What is the estimated average requirement of potassium in moles?

Solution

The mass of K is provided, and the corresponding amount of K in moles is requested. Referring to the periodic table, the atomic mass of K is 39.10 amu, and so its molar mass is 39.10 g/mol. The given mass of K (4.7 g) is a bit more than one-tenth the molar mass (39.10 g), so a reasonable “ballpark” estimate of the number of moles would be slightly greater than 0.1 mol.

The molar amount of a substance may be calculated by dividing its mass (g) by its molar mass (g/mol):



The factor-label method supports this mathematical approach since the unit “g” cancels and the answer has units of “mol:”

$$4.7\ \cancel{\text{g K}}\left(\frac{\text{mol K}}{39.10\ \cancel{\text{g K}}}\right) = 0.12\ \text{mol K}$$

The calculated magnitude (0.12 mol K) is consistent with our ballpark expectation, since it is a bit greater than 0.1 mol.

Check Your Learning

Beryllium is a light metal used to fabricate transparent X-ray windows for medical imaging instruments. How many moles of Be are in a thin-foil window weighing 3.24 g?

Answer:

0.360 mol

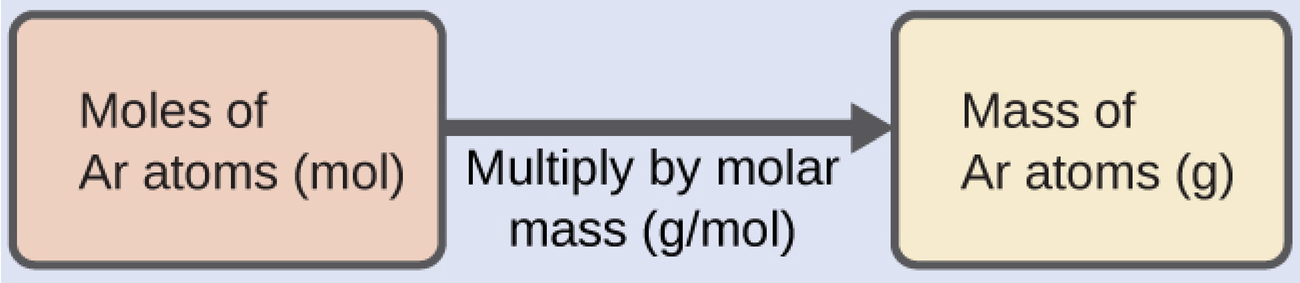
Example 5.4

Deriving Grams from Moles for an Element

A liter of air contains 9.2×10^{-4} mol argon. What is the mass of Ar in a liter of air?

Solution

The molar amount of Ar is provided and must be used to derive the corresponding mass in grams. Since the amount of Ar is less than 1 mole, the mass will be less than the mass of 1 mole of Ar, approximately 40 g. The molar amount in question is approximately one-one thousandth ($\sim 10^{-3}$) of a mole, and so the corresponding mass should be roughly one-one thousandth of the molar mass (~0.04 g):



In this case, logic dictates (and the factor-label method supports) multiplying the provided amount (mol) by the molar mass (g/mol):

$$9.2 \times 10^{-4}\ \cancel{\text{mol Ar}}\left(\frac{39.95\ \text{g Ar}}{\cancel{\text{mol Ar}}}\right) = 0.037\ \text{g Ar}$$

The result is in agreement with our expectations, around 0.04 g Ar.

Check Your Learning

What is the mass of 2.561 mol of gold?

Answer:

504.4 g

Example 5.5

Deriving Number of Atoms from Mass for an Element

Copper is commonly used to fabricate electrical wire (**Figure 5.8**). How many copper atoms are in 5.00 g of copper wire?

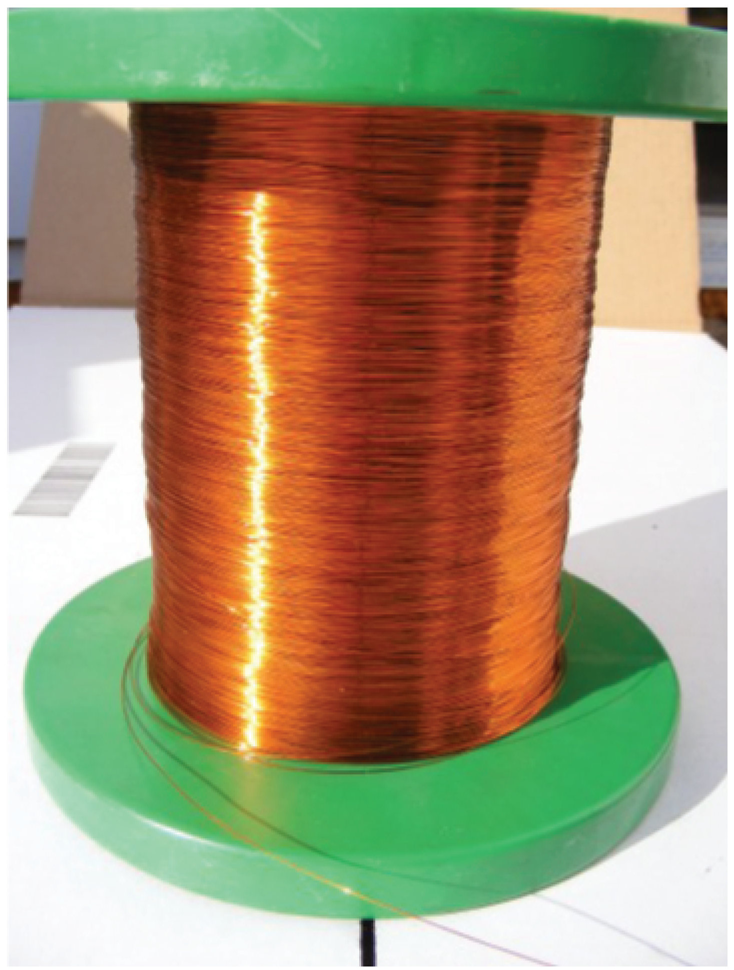
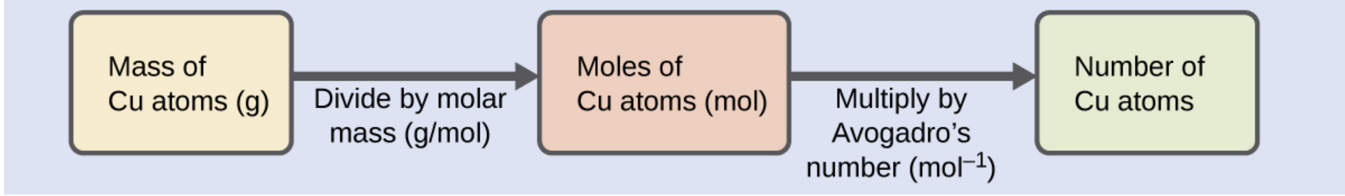


Figure 5.8 Copper wire is composed of many, many atoms of Cu. (credit: Emilian Robert Vicol)

Solution

The number of Cu atoms in the wire may be conveniently derived from its mass by a two-step computation: first calculating the molar amount of Cu, and then using Avogadro's number (N_A) to convert this molar amount to number of Cu atoms:



Considering that the provided sample mass (5.00 g) is a little less than one-tenth the mass of 1 mole of Cu

(~64 g), a reasonable estimate for the number of atoms in the sample would be on the order of one-tenth N_A, or approximately 10^{22} Cu atoms. Carrying out the two-step computation yields:

$$5.00\ \cancel{\text{g Cu}}\left(\frac{\cancel{\text{mol Cu}}}{63.55\ \cancel{\text{g Cu}}}\right)\left(\frac{6.022 \times 10^{23}\ \text{Cu atoms}}{\cancel{\text{mol Cu}}}\right) = 4.74 \times 10^{22}\ \text{Cu atoms}$$

The factor-label method yields the desired cancellation of units, and the computed result is on the order of 10^{22} as expected.

Check Your Learning

A prospector panning for gold in a river collects 15.00 g of pure gold. How many Au atoms are in this quantity of gold?

Answer:

4.586×10^{22} Au atoms

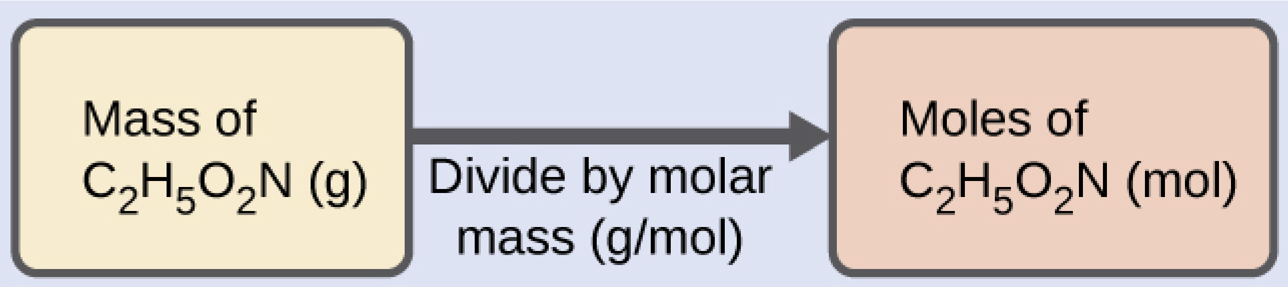
Example 5.6

Deriving Moles from Grams for a Compound

Our bodies synthesize protein from amino acids. One of these amino acids is glycine, which has the molecular formula $C_2H_5O_2N$. How many moles of glycine molecules are contained in 28.35 g of glycine?

Solution

Derive the number of moles of a compound from its mass following the same procedure used for an element in Example 5.3:



The molar mass of glycine is required for this calculation, and it is computed in the same fashion as its molecular mass. One mole of glycine, $C_2H_5O_2N$, contains 2 moles of carbon, 5 moles of hydrogen, 2 moles of oxygen, and 1 mole of nitrogen:

Element	Quantity (mol element/ mol compound)		Molar mass (g/mol element)		Subtotal (g/mol compound)
C	2	×	12.01	=	24.02
H	5	×	1.008	=	5.040
O	2	×	16.00	=	32.00
N	1	×	14.007	=	14.007
			Molecular mass (g/mol compound)		75.07

The provided mass of glycine (~28 g) is a bit more than one-third the molar mass (~75 g/mol), so the computed

result is expected to be a bit greater than one-third of a mole (~0.33 mol). Dividing the compound's mass by its molar mass yields:

$$28.35\ \cancel{\text{g glycine}}\left(\frac{\text{mol glycine}}{75.07\ \cancel{\text{g glycine}}}\right) = 0.378\ \text{mol glycine}$$

This result is consistent with the rough estimate.

Check Your Learning

How many moles of sucrose, $C_{12}H_{22}O_{11}$, are in a 25-g sample of sucrose?

Answer:

0.073 mol

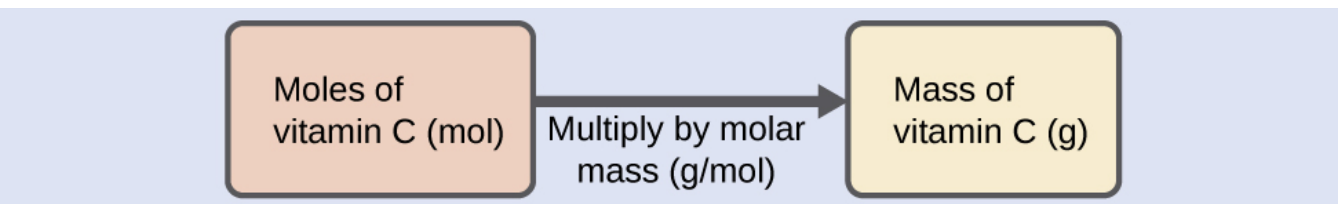
Example 5.7

Deriving Grams from Moles for a Compound

Vitamin C is a covalent compound with the molecular formula $C_6H_8O_6$. The recommended daily dietary allowance of vitamin C for children aged 4–8 years is 1.42×10^{-4} mol. What is the mass of this allowance in grams?

Solution

As for elements, the mass of a compound can be derived from its molar amount as shown:



The molar mass for this compound is computed to be 176.124 g/mol. The given number of moles is a very small fraction of a mole (~10^{-4} or one-ten thousandth); therefore, the corresponding mass is expected to be about one-ten thousandth of the molar mass (~0.02 g). Performing the calculation yields:

$$1.42 \times 10^{-4}\ \cancel{\text{mol vitamin C}}\left(\frac{176.124\ \text{g vitamin C}}{\cancel{\text{mol vitamin C}}}\right) = 0.0250\ \text{g vitamin C}$$

This is consistent with the anticipated result.

Check Your Learning

What is the mass of 0.443 mol of hydrazine, N_2H_4?

Answer:

14.2 g

Example 5.8

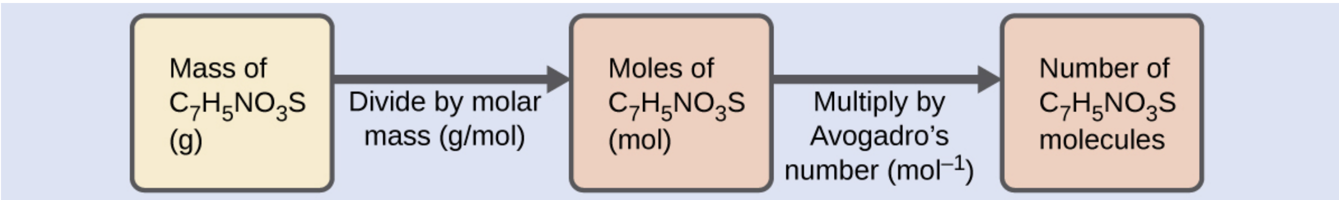
Deriving the Number of Atoms and Molecules from the Mass of a Compound

A packet of an artificial sweetener contains 40.0 mg of saccharin ($C_7H_5NO_3S$), which has the structural formula:

Given that saccharin has a molar mass of 183.18 g/mol, how many saccharin molecules are in a 40.0-mg (0.0400-g) sample of saccharin? How many carbon atoms are in the same sample?

Solution

The number of molecules in a given mass of compound is computed by first deriving the number of moles, as demonstrated in **Example 5.6**, and then multiplying by Avogadro's number:



Using the provided mass and molar mass for saccharin yields:

$$0.0400 \, \cancel{g\, C_7H_5NO_3S} \left(\frac{mol\, C_7H_5NO_3S}{183.18 \, \cancel{g\, C_7H_5NO_3S}}\right)\left(\frac{6.022 \times 10^{23} \, C_7H_5NO_3S \text{ molecules}}{1 \, \cancel{mol\, C_7H_5NO_3S}}\right)$$

$$= 1.31 \times 10^{20} \, C_7H_5NO_3S \text{ molecules}$$

The compound's formula shows that each molecule contains seven carbon atoms, and so the number of C atoms in the provided sample is:

$$1.31 \times 10^{20} \, C_7H_5NO_3S \text{ molecules} \left(\frac{7 \text{ C atoms}}{1 \, C_7H_5NO_3S \text{ molecule}}\right) = 9.17 \times 10^{20} \text{ C atoms}$$

Check Your Learning

How many C_4H_{10} molecules are contained in 9.213 g of this compound? How many hydrogen atoms?

Answer:

9.545×10^{22} molecules C_4H_{10}; 9.545×10^{23} atoms H

Counting Neurotransmitter Molecules in the Brain

The brain is the control center of the central nervous system (**Figure 5.9**). It sends and receives signals to and from muscles and other internal organs to monitor and control their functions; it processes stimuli detected by sensory organs to guide interactions with the external world; and it houses the complex physiological processes that give rise to our intellect and emotions. The broad field of neuroscience spans all aspects of the structure and function of the central nervous system, including research on the anatomy

and physiology of the brain. Great progress has been made in brain research over the past few decades, and the BRAIN Initiative, a federal initiative announced in 2013, aims to accelerate and capitalize on these advances through the concerted efforts of various industrial, academic, and government agencies (more details available at www.whitehouse.gov/share/brain-initiative).

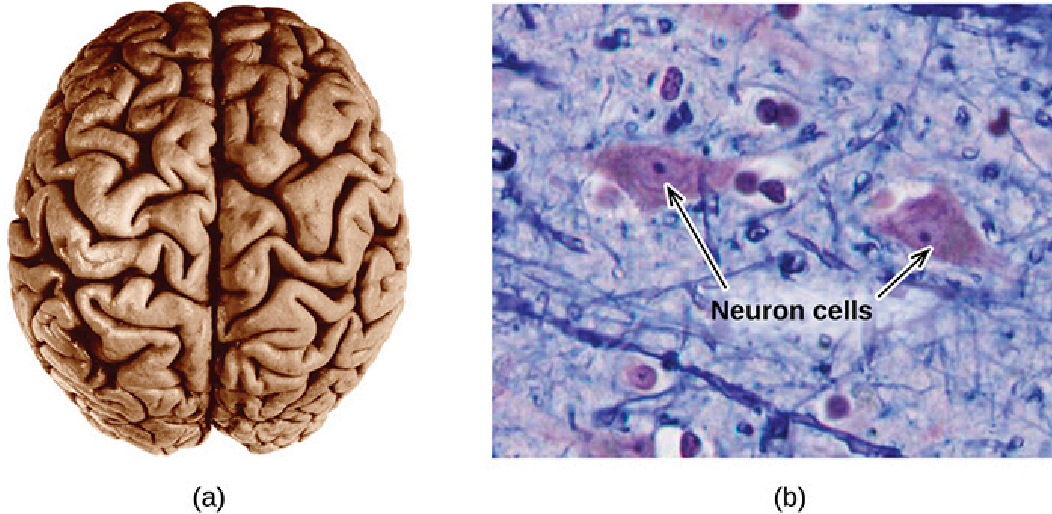


Figure 5.9 (a) A typical human brain weighs about 1.5 kg and occupies a volume of roughly 1.1 L. (b) Information is transmitted in brain tissue and throughout the central nervous system by specialized cells called neurons (micrograph shows cells at 1600× magnification).

Specialized cells called neurons transmit information between different parts of the central nervous system by way of electrical and chemical signals. Chemical signaling occurs at the interface between different neurons when one of the cells releases molecules (called neurotransmitters) that diffuse across the small gap between the cells (called the synapse) and bind to the surface of the other cell. These neurotransmitter molecules are stored in small intracellular structures called vesicles that fuse to the cell wall and then break open to release their contents when the neuron is appropriately stimulated. This process is called exocytosis (see **Figure 5.10**). One neurotransmitter that has been very extensively studied is dopamine, $C_8H_{11}NO_2$. Dopamine is involved in various neurological processes that impact a wide variety of human behaviors. Dysfunctions in the dopamine systems of the brain underlie serious neurological diseases such as Parkinson’s and schizophrenia.

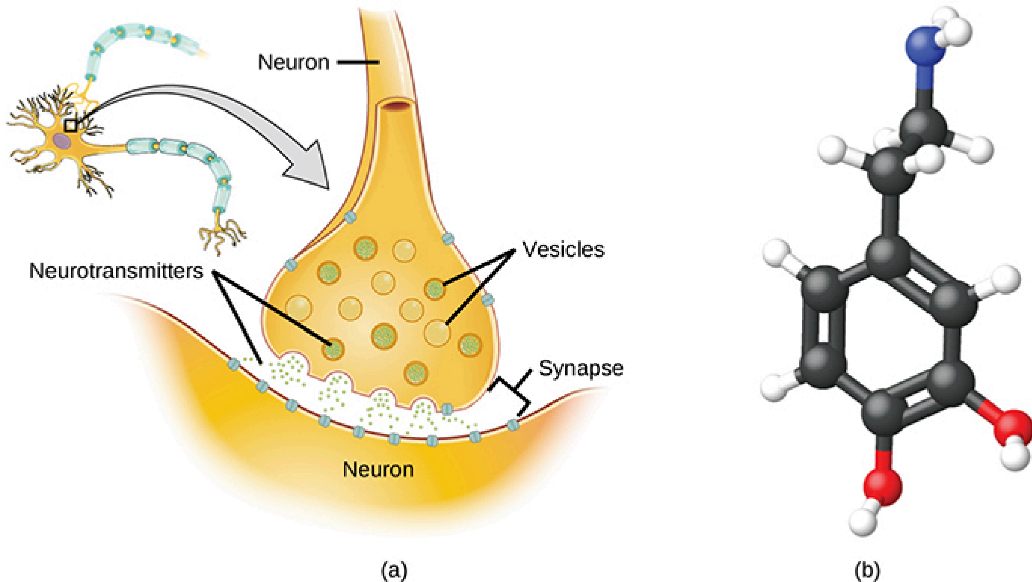


Figure 5.10 (a) Chemical signals are transmitted from neurons to other cells by the release of neurotransmitter molecules into the small gaps (synapses) between the cells. (b) Dopamine, $C_8H_{11}NO_2$, is a neurotransmitter involved in a number of neurological processes.

One important aspect of the complex processes related to dopamine signaling is the number of neurotransmitter molecules released during exocytosis. Since this number is a central factor in determining neurological response (and subsequent human thought and action), it is important to know how this number changes with certain controlled stimulations, such as the administration of drugs. It is also important to understand the mechanism responsible for any changes in the number of neurotransmitter molecules released—for example, some dysfunction in exocytosis, a change in the number of vesicles in the neuron, or a change in the number of neurotransmitter molecules in each vesicle.

Significant progress has been made recently in directly measuring the number of dopamine molecules stored in individual vesicles and the amount actually released when the vesicle undergoes exocytosis. Using miniaturized probes that can selectively detect dopamine molecules in very small amounts, scientists have determined that the vesicles of a certain type of mouse brain neuron contain an average of 30,000 dopamine molecules per vesicle (about 5×10^{-20} mol or 50 zmol). Analysis of these neurons from mice subjected to various drug therapies shows significant changes in the average number of dopamine molecules contained in individual vesicles, increasing or decreasing by up to three-fold, depending on the specific drug used. These studies also indicate that not all of the dopamine in a given vesicle is released during exocytosis, suggesting that it may be possible to regulate the fraction released using pharmaceutical therapies.[1]

5.2 | Molarity

By the end of this section, you will be able to:

- Describe the fundamental properties of solutions
- Calculate solution concentrations using molarity
- Perform dilution calculations using the dilution equation

Preceding sections of this chapter focused on the composition of substances: samples of matter that contain only one type of element or compound. However, mixtures—samples of matter containing two or more substances

1. Omiatek, Donna M., Amanda J. Bressler, Ann-Sofie Cans, Anne M. Andrews, Michael L. Heien, and Andrew G. Ewing. “The Real Catecholamine Content of Secretory Vesicles in the CNS Revealed by Electrochemical Cytometry.” *Scientific Report* 3 (2013): 1447, accessed January 14, 2015, doi:10.1038/srep01447.

physically combined—are more commonly encountered in nature than are pure substances. Similar to a pure substance, the relative composition of a mixture plays an important role in determining its properties. The relative amount of oxygen in a planet's atmosphere determines its ability to sustain aerobic life. The relative amounts of iron, carbon, nickel, and other elements in steel (a mixture known as an "alloy") determine its physical strength and resistance to corrosion. The relative amount of the active ingredient in a medicine determines its effectiveness in achieving the desired pharmacological effect. The relative amount of sugar in a beverage determines its sweetness (see **Figure 5.11**). This section will describe one of the most common ways in which the relative compositions of mixtures may be quantified.

Figure 5.11 Sugar is one of many components in the complex mixture known as coffee. The amount of sugar in a given amount of coffee is an important determinant of the beverage's sweetness. (credit: Jane Whitney)

Solutions

Solutions have previously been defined as homogeneous mixtures, meaning that the composition of the mixture (and therefore its properties) is uniform throughout its entire volume. Solutions occur frequently in nature and have also been implemented in many forms of manmade technology. A more thorough treatment of solution properties is provided in the chapter on solutions and colloids, but provided here is an introduction to some of the basic properties of solutions.

The relative amount of a given solution component is known as its **concentration**. Often, though not always, a solution contains one component with a concentration that is significantly greater than that of all other components. This component is called the **solvent** and may be viewed as the medium in which the other components are dispersed, or **dissolved**. Solutions in which water is the solvent are, of course, very common on our planet. A solution in which water is the solvent is called an **aqueous solution**.

A **solute** is a component of a solution that is typically present at a much lower concentration than the solvent. Solute concentrations are often described with qualitative terms such as **dilute** (of relatively low concentration) and **concentrated** (of relatively high concentration).

Concentrations may be quantitatively assessed using a wide variety of measurement units, each convenient for

particular applications. **Molarity (*M*)** is a useful concentration unit for many applications in chemistry. Molarity is defined as the number of moles of solute in exactly 1 liter (1 L) of the solution:

$$M = \frac{\text{mol solute}}{\text{L solution}}$$

Example 5.9

Calculating Molar Concentrations

A 355-mL soft drink sample contains 0.133 mol of sucrose (table sugar). What is the molar concentration of sucrose in the beverage?

Solution

Since the molar amount of solute and the volume of solution are both given, the molarity can be calculated using the definition of molarity. Per this definition, the solution volume must be converted from mL to L:

$$M = \frac{\text{mol solute}}{\text{L solution}} = \frac{0.133\text{ mol}}{355\text{ mL} \times \frac{1\text{ L}}{1000\text{ mL}}} = 0.375\ M$$

Check Your Learning

A teaspoon of table sugar contains about 0.01 mol sucrose. What is the molarity of sucrose if a teaspoon of sugar has been dissolved in a cup of tea with a volume of 200 mL?

Answer:

0.05 *M*

Example 5.10

Deriving Moles and Volumes from Molar Concentrations

How much sugar (mol) is contained in a modest sip (~10 mL) of the soft drink from **Example 5.9**?

Solution

Rearrange the definition of molarity to isolate the quantity sought, moles of sugar, then substitute the value for molarity derived in **Example 5.9**, 0.375 *M*:

$$M = \frac{\text{mol solute}}{\text{L solution}}$$

$$\text{mol solute} = M \times \text{L solution}$$

$$\text{mol solute} = 0.375\ \frac{\text{mol sugar}}{\text{L}} \times \left(10\text{ mL} \times \frac{1\text{ L}}{1000\text{ mL}}\right) = 0.004\text{ mol sugar}$$

Check Your Learning

What volume (mL) of the sweetened tea described in **Example 5.9** contains the same amount of sugar (mol) as 10 mL of the soft drink in this example?

Answer:

80 mL

Example 5.11

Calculating Molar Concentrations from the Mass of Solute

Distilled white vinegar (**Figure 5.12**) is a solution of acetic acid, CH_3CO_2H, in water. A 0.500-L vinegar solution contains 25.2 g of acetic acid. What is the concentration of the acetic acid solution in units of molarity?

Figure 5.12 Distilled white vinegar is a solution of acetic acid in water.

Solution

As in previous examples, the definition of molarity is the primary equation used to calculate the quantity sought. Since the mass of solute is provided instead of its molar amount, use the solute's molar mass to obtain the amount of solute in moles:

$$M = \frac{\text{mol solute}}{\text{L solution}} = \frac{25.2 \text{ g CH}_3\text{CO}_2\text{H} \times \frac{1 \text{ mol CH}_3\text{CO}_2\text{H}}{60.052 \text{ g CH}_3\text{CO}_2\text{H}}}{0.500 \text{ L solution}} = 0.839\,M$$

$$M = \frac{\text{mol solute}}{\text{L solution}} = 0.839\,M$$

$$M = \frac{0.839 \text{ mol solute}}{1.00 \text{ L solution}}$$

Check Your Learning

Calculate the molarity of 6.52 g of $CoCl_2$ (128.9 g/mol) dissolved in an aqueous solution with a total volume of 75.0 mL.

Answer:

0.674 *M*

Example 5.12

Determining the Mass of Solute in a Given Volume of Solution

How many grams of NaCl are contained in 0.250 L of a 5.30-*M* solution?

Solution

The volume and molarity of the solution are specified, so the amount (mol) of solute is easily computed as demonstrated in **Example 5.10**:

$$M = \frac{\text{mol solute}}{\text{L solution}}$$

$$\text{mol solute} = M \times \text{L solution}$$

$$\text{mol solute} = 5.30\,\frac{\text{mol NaCl}}{\text{L}} \times 0.250\,\text{L} = 1.325\text{ mol NaCl}$$

Finally, this molar amount is used to derive the mass of NaCl:

$$1.325\text{ mol NaCl} \times \frac{58.44\text{ g NaCl}}{\text{mol NaCl}} = 77.4\text{ g NaCl}$$

Check Your Learning

How many grams of $CaCl_2$ (110.98 g/mol) are contained in 250.0 mL of a 0.200-*M* solution of calcium chloride?

Answer:

5.55 g $CaCl_2$

When performing calculations stepwise, as in **Example 5.12**, it is important to refrain from rounding any intermediate calculation results, which can lead to rounding errors in the final result. In **Example 5.12**, the molar amount of NaCl computed in the first step, 1.325 mol, would be properly rounded to 1.32 mol if it were to be reported; however, although the last digit (5) is not significant, it must be retained as a guard digit in the intermediate calculation. If the guard digit had not been retained, the final calculation for the mass of NaCl would have been 77.1 g, a difference of 0.3 g.

In addition to retaining a guard digit for intermediate calculations, rounding errors may also be avoided by performing computations in a single step (see **Example 5.13**). This eliminates intermediate steps so that only the final result is rounded.

Example 5.13

Determining the Volume of Solution Containing a Given Mass of Solute

In **Example 5.11**, the concentration of acetic acid in white vinegar was determined to be 0.839 *M*. What volume of vinegar contains 75.6 g of acetic acid?

Solution

First, use the molar mass to calculate moles of acetic acid from the given mass:

$$\text{g solute} \times \frac{\text{mol solute}}{\text{g solute}} = \text{mol solute}$$

Then, use the molarity of the solution to calculate the volume of solution containing this molar amount of solute:

$$\text{mol solute} \times \frac{\text{L solution}}{\text{mol solute}} = \text{L solution}$$

Combining these two steps into one yields:

$$\text{g solute} \times \frac{\text{mol solute}}{\text{g solute}} \times \frac{\text{L solution}}{\text{mol solute}} = \text{L solution}$$

$$75.6\text{ g CH}_3\text{CO}_2\text{H}\left(\frac{\text{mol CH}_3\text{CO}_2\text{H}}{60.05\text{ g}}\right)\left(\frac{\text{L solution}}{0.839\text{ mol CH}_3\text{CO}_2\text{H}}\right) = 1.50\text{ L solution}$$

Check Your Learning

What volume of a 1.50-*M* KBr solution contains 66.0 g KBr?

Answer:

0.370 L

Dilution of Solutions

Dilution is the process whereby the concentration of a solution is lessened by the addition of solvent. For example, a glass of iced tea becomes increasingly diluted as the ice melts. The water from the melting ice increases the volume of the solvent (water) and the overall volume of the solution (iced tea), thereby reducing the relative concentrations of the solutes that give the beverage its taste (Figure 5.13).

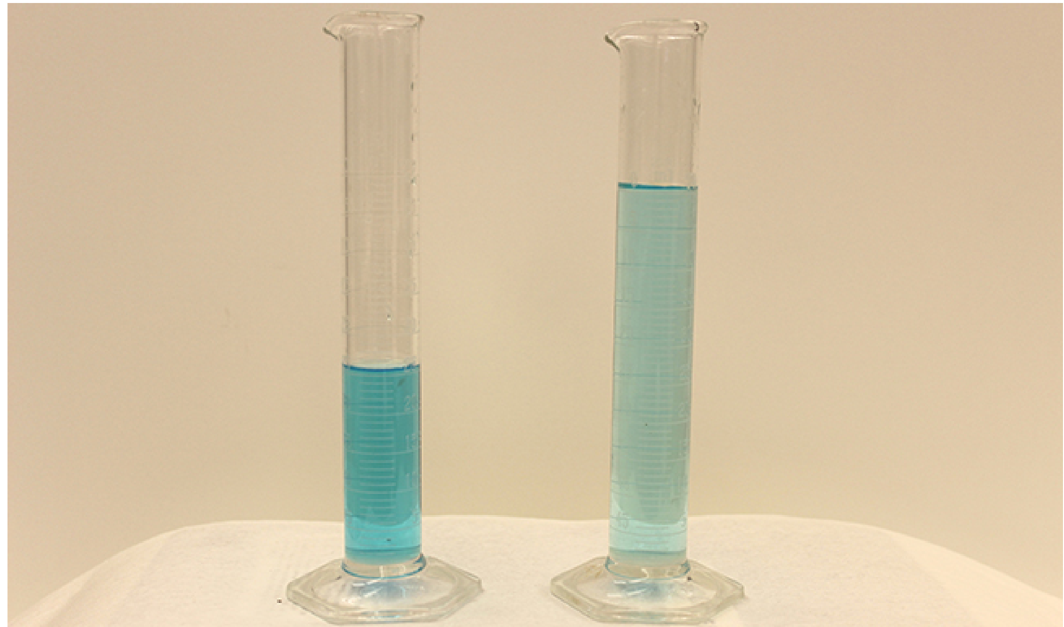


Figure 5.13 Both solutions contain the same mass of copper nitrate. The solution on the right is more dilute because the copper nitrate is dissolved in more solvent. (credit: Mark Ott)

Dilution is also a common means of preparing solutions of a desired concentration. By adding solvent to a measured portion of a more concentrated *stock solution*, a solution of lesser concentration may be prepared. For example, commercial pesticides are typically sold as solutions in which the active ingredients are far more concentrated than is appropriate for their application. Before they can be used on crops, the pesticides must be diluted. This is also a very common practice for the preparation of a number of common laboratory reagents.

A simple mathematical relationship can be used to relate the volumes and concentrations of a solution before and after the dilution process. According to the definition of molarity, the number of moles of solute in a solution (n) is equal to the product of the solution's molarity (M) and its volume in liters (L):

$$n = ML$$

Expressions like these may be written for a solution before and after it is diluted:

$$n_1 = M_1 L_1$$
$$n_2 = M_2 L_2$$

where the subscripts "1" and "2" refer to the solution before and after the dilution, respectively. Since the dilution process *does not change the amount of solute in the solution,* $n_1 = n_2$. Thus, these two equations may be set equal to one another:

$$M_1 L_1 = M_2 L_2$$

This relation is commonly referred to as the dilution equation. Although this equation uses molarity as the unit of concentration and liters as the unit of volume, other units of concentration and volume may be used as long as the units properly cancel per the factor-label method. Reflecting this versatility, the dilution equation is often written in the more general form:

$$C_1 V_1 = C_2 V_2$$

where *C* and *V* are concentration and volume, respectively.

Use the **simulation (http://openstax.org/l/16Phetsolvents)** to explore the relations between solute amount, solution volume, and concentration and to confirm the dilution equation.

Example 5.14

Determining the Concentration of a Diluted Solution

If 0.850 L of a 5.00-*M* solution of copper nitrate, $Cu(NO_3)_2$, is diluted to a volume of 1.80 L by the addition of water, what is the molarity of the diluted solution?

Solution

The stock concentration, C_1, and volume, V_1, are provided as well as the volume of the diluted solution, V_2. Rearrange the dilution equation to isolate the unknown property, the concentration of the diluted solution, C_2:

$$C_1 V_1 = C_2 V_2$$
$$C_2 = \frac{C_1 V_1}{V_2}$$

Since the stock solution is being diluted by more than two-fold (volume is increased from 0.85 L to 1.80 L), the diluted solution's concentration is expected to be less than one-half 5 *M*. This ballpark estimate will be compared to the calculated result to check for any gross errors in computation (for example, such as an improper substitution of the given quantities). Substituting the given values for the terms on the right side of this equation yields:

$$C_2 = \frac{0.850 \text{ L} \times 5.00 \frac{\text{mol}}{\text{L}}}{1.80 \text{ L}} = 2.36 \, M$$

This result compares well to our ballpark estimate (it's a bit less than one-half the stock concentration, 5 *M*).

Check Your Learning

What is the concentration of the solution that results from diluting 25.0 mL of a 2.04-*M* solution of CH_3OH to 500.0 mL?

Answer:

0.102 *M* CH_3OH

Example 5.15

Volume of a Diluted Solution

What volume of 0.12 *M* HBr can be prepared from 11 mL (0.011 L) of 0.45 *M* HBr?

Solution

Provided are the volume and concentration of a stock solution, V_1 and C_1, and the concentration of the resultant diluted solution, C_2. Find the volume of the diluted solution, V_2 by rearranging the dilution equation to isolate V_2:

$$C_1 V_1 = C_2 V_2$$
$$V_2 = \frac{C_1 V_1}{C_2}$$

Since the diluted concentration (0.12 *M*) is slightly more than one-fourth the original concentration (0.45 *M*), the volume of the diluted solution is expected to be roughly four times the original volume, or around 44 mL. Substituting the given values and solving for the unknown volume yields:

$$V_2 = \frac{(0.45\ M)(0.011\ \text{L})}{(0.12\ M)}$$

$$V_2 = 0.041\ \text{L}$$

The volume of the 0.12-*M* solution is 0.041 L (41 mL). The result is reasonable and compares well with the rough estimate.

Check Your Learning

A laboratory experiment calls for 0.125 *M* HNO_3. What volume of 0.125 *M* HNO_3 can be prepared from 0.250 L of 1.88 *M* HNO_3?

Answer:

3.76 L

Example 5.16

Volume of a Concentrated Solution Needed for Dilution

What volume of 1.59 *M* KOH is required to prepare 5.00 L of 0.100 *M* KOH?

Solution

Given are the concentration of a stock solution, C_1, and the volume and concentration of the resultant diluted solution, V_2 and C_2. Find the volume of the stock solution, V_1 by rearranging the dilution equation to isolate V_1:

$$C_1 V_1 = C_2 V_2$$
$$V_1 = \frac{C_2 V_2}{C_1}$$

Since the concentration of the diluted solution 0.100 *M* is roughly one-sixteenth that of the stock solution (1.59 *M*), the volume of the stock solution is expected to be about one-sixteenth that of the diluted solution, or around 0.3 liters. Substituting the given values and solving for the unknown volume yields:

$$V_1 = \frac{(0.100\ M)(5.00\ \text{L})}{1.59\ M}$$

$$V_1 = 0.314\ \text{L}$$

Thus, 0.314 L of the 1.59-*M* solution is needed to prepare the desired solution. This result is consistent with the rough estimate.

Check Your Learning

What volume of a 0.575-*M* solution of glucose, $C_6H_{12}O_6$, can be prepared from 50.00 mL of a 3.00-*M* glucose solution?

Answer:

0.261 L

5.3 | Other Units for Solution Concentrations

By the end of this section, you will be able to:

- Define the concentration units of mass percentage, volume percentage, mass-volume percentage, parts-per-million (ppm), and parts-per-billion (ppb)
- Perform computations relating a solution's concentration and its components' volumes and/or masses using these units

The previous section introduced molarity, a very useful measurement unit for evaluating the concentration of

solutions. However, molarity is only one measure of concentration. This section will describe some other units of concentration that are commonly used in various applications, either for convenience or by convention.

Mass Percentage

Earlier in this chapter, percent composition was introduced as a measure of the relative amount of a given element in a compound. Percentages are also commonly used to express the composition of mixtures, including solutions. The **mass percentage** of a solution component is defined as the ratio of the component's mass to the solution's mass, expressed as a percentage:

$$\text{mass percentage} = \frac{\text{mass of component}}{\text{mass of solution}} \times 100\%$$

Mass percentage is also referred to by similar names such as *percent mass, percent weight, weight/weight percent*, and other variations on this theme. The most common symbol for mass percentage is simply the percent sign, %, although more detailed symbols are often used including %mass, %weight, and (w/w)%. Use of these more detailed symbols can prevent confusion of mass percentages with other types of percentages, such as volume percentages (to be discussed later in this section).

Mass percentages are popular concentration units for consumer products. The label of a typical liquid bleach bottle (**Figure 5.14**) cites the concentration of its active ingredient, sodium hypochlorite (NaOCl), as being 7.4%. A 100.0-g sample of bleach would therefore contain 7.4 g of NaOCl.

Figure 5.14 Liquid bleach is an aqueous solution of sodium hypochlorite (NaOCl). This brand has a concentration of 7.4% NaOCl by mass.

Example 5.17

Calculation of Percent by Mass

A 5.0-g sample of spinal fluid contains 3.75 mg (0.00375 g) of glucose. What is the percent by mass of glucose in spinal fluid?

Solution

The spinal fluid sample contains roughly 4 mg of glucose in 5000 mg of fluid, so the mass fraction of glucose should be a bit less than one part in 1000, or about 0.1%. Substituting the given masses into the equation defining mass percentage yields:

$$\% \text{ glucose} = \frac{3.75 \text{ mg glucose} \times \frac{1 \text{ g}}{1000 \text{ mg}}}{5.0 \text{ g spinal fluid}} = 0.075\%$$

The computed mass percentage agrees with our rough estimate (it's a bit less than 0.1%).

Note that while any mass unit may be used to compute a mass percentage (mg, g, kg, oz, and so on), the same unit must be used for both the solute and the solution so that the mass units cancel, yielding a dimensionless ratio. In this case, the solute mass unit in the numerator was converted from mg to g to match the units in the denominator. Alternatively, the spinal fluid mass unit in the denominator could have been converted from g to mg instead. As long as identical mass units are used for both solute and solution, the computed mass percentage will be correct.

Check Your Learning

A bottle of a tile cleanser contains 135 g of HCl and 775 g of water. What is the percent by mass of HCl in this cleanser?

Answer:

14.8%

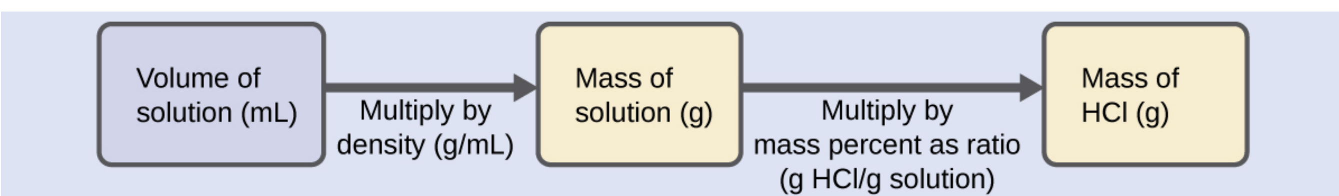
Example 5.18

Calculations using Mass Percentage

"Concentrated" hydrochloric acid is an aqueous solution of 37.2% HCl that is commonly used as a laboratory reagent. The density of this solution is 1.19 g/mL. What mass of HCl is contained in 0.500 L of this solution?

Solution

The HCl concentration is near 40%, so a 100-g portion of this solution would contain about 40 g of HCl. Since the solution density isn't greatly different from that of water (1 g/mL), a reasonable estimate of the HCl mass in 500 g (0.5 L) of the solution is about five times greater than that in a 100 g portion, or $5 \times 40 = 200$ g. In order to derive the mass of solute in a solution from its mass percentage, the mass of the solution must be known. Using the solution density given, convert the solution's volume to mass, and then use the given mass percentage to calculate the solute mass. This mathematical approach is outlined in this flowchart:



For proper unit cancellation, the 0.500-L volume is converted into 500 mL, and the mass percentage is expressed as a ratio, 37.2 g HCl/g solution:

$$500 \text{ mL solution} \left(\frac{1.19 \text{ g solution}}{\text{mL solution}}\right)\left(\frac{37.2 \text{ g HCl}}{100 \text{ g solution}}\right) = 221 \text{ g HCl}$$

This mass of HCl is consistent with our rough estimate of approximately 200 g.

Check Your Learning

What volume of concentrated HCl solution contains 125 g of HCl?

Answer:

282 mL

Volume Percentage

Liquid volumes over a wide range of magnitudes are conveniently measured using common and relatively inexpensive laboratory equipment. The concentration of a solution formed by dissolving a liquid solute in a liquid

solvent is therefore often expressed as a **volume percentage**, %vol or (v/v)%:

$$\text{volume percentage} = \frac{\text{volume solute}}{\text{volume solution}} \times 100\%$$

Example 5.19

Calculations using Volume Percentage

Rubbing alcohol (isopropanol) is usually sold as a 70%vol aqueous solution. If the density of isopropyl alcohol is 0.785 g/mL, how many grams of isopropyl alcohol are present in a 355 mL bottle of rubbing alcohol?

Solution

Per the definition of volume percentage, the isopropanol volume is 70% of the total solution volume. Multiplying the isopropanol volume by its density yields the requested mass:

$$(355\text{ mL solution})\left(\frac{70\text{ mL isopropyl alcohol}}{100\text{ mL solution}}\right)\left(\frac{0.785\text{ g isopropyl alcohol}}{1\text{ mL isopropyl alcohol}}\right) = 195\text{ g isopropyl alchol}$$

Check Your Learning

Wine is approximately 12% ethanol (CH_3CH_2OH) by volume. Ethanol has a molar mass of 46.06 g/mol and a density 0.789 g/mL. How many moles of ethanol are present in a 750-mL bottle of wine?

Answer:

1.5 mol ethanol

Mass-Volume Percentage

"Mixed" percentage units, derived from the mass of solute and the volume of solution, are popular for certain biochemical and medical applications. A **mass-volume percent** is a ratio of a solute's mass to the solution's volume expressed as a percentage. The specific units used for solute mass and solution volume may vary, depending on the solution. For example, physiological saline solution, used to prepare intravenous fluids, has a concentration of 0.9% mass/volume (m/v), indicating that the composition is 0.9 g of solute per 100 mL of solution. The concentration of glucose in blood (commonly referred to as "blood sugar") is also typically expressed in terms of a mass-volume ratio. Though not expressed explicitly as a percentage, its concentration is usually given in milligrams of glucose per deciliter (100 mL) of blood (**Figure 5.15**).

(a)

(b)

Figure 5.15 "Mixed" mass-volume units are commonly encountered in medical settings. (a) The NaCl concentration of physiological saline is 0.9% (m/v). (b) This device measures glucose levels in a sample of blood. The normal range for glucose concentration in blood (fasting) is around 70–100 mg/dL. (credit a: modification of work by "The National Guard"/Flickr; credit b: modification of work by Biswarup Ganguly)

Parts per Million and Parts per Billion

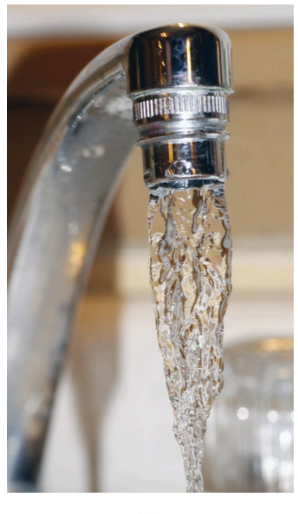
Very low solute concentrations are often expressed using appropriately small units such as **parts per million (ppm)** or **parts per billion (ppb)**. Like percentage ("part per hundred") units, ppm and ppb may be defined in terms of masses, volumes, or mixed mass-volume units. There are also ppm and ppb units defined with respect to numbers of atoms and molecules.

The mass-based definitions of ppm and ppb are given here:

$$\text{ppm} = \frac{\text{mass solute}}{\text{mass solution}} \times 10^6 \text{ ppm}$$

$$\text{ppb} = \frac{\text{mass solute}}{\text{mass solution}} \times 10^9 \text{ ppb}$$

Both ppm and ppb are convenient units for reporting the concentrations of pollutants and other trace contaminants in water. Concentrations of these contaminants are typically very low in treated and natural waters, and their levels cannot exceed relatively low concentration thresholds without causing adverse effects on health and wildlife. For example, the EPA has identified the maximum safe level of fluoride ion in tap water to be 4 ppm. Inline water filters are designed to reduce the concentration of fluoride and several other trace-level contaminants in tap water (**Figure 5.16**).



(a)

(b)

Figure 5.16 (a) In some areas, trace-level concentrations of contaminants can render unfiltered tap water unsafe for drinking and cooking. (b) Inline water filters reduce the concentration of solutes in tap water. (credit a: modification of work by Jenn Durfey; credit b: modification of work by "vastateparkstaff"/Wikimedia commons)

Example 5.20

Calculation of Parts per Million and Parts per Billion Concentrations

According to the EPA, when the concentration of lead in tap water reaches 15 ppb, certain remedial actions must be taken. What is this concentration in ppm? At this concentration, what mass of lead (μg) would be contained in a typical glass of water (300 mL)?

Solution

The definitions of the ppm and ppb units may be used to convert the given concentration from ppb to ppm. Comparing these two unit definitions shows that ppm is 1000 times greater than ppb (1 ppm = 10^3 ppb). Thus:

$$15 \text{ ppb} \times \frac{1 \text{ ppm}}{10^3 \text{ ppb}} = 0.015 \text{ ppm}$$

The definition of the ppb unit may be used to calculate the requested mass if the mass of the solution is provided. Since the volume of solution (300 mL) is given, its density must be used to derive the corresponding mass. Assume the density of tap water to be roughly the same as that of pure water (~1.00 g/mL), since the concentrations of any dissolved substances should not be very large. Rearranging the equation defining the ppb

unit and substituting the given quantities yields:

$$\text{ppb} = \frac{\text{mass solute}}{\text{mass solution}} \times 10^9\ \text{ppb}$$

$$\text{mass solute} = \frac{\text{ppb} \times \text{mass solution}}{10^9\ \text{ppb}}$$

$$\text{mass solute} = \frac{15\ \text{ppb} \times 300\ \text{mL} \times \frac{1.00\ \text{g}}{\text{mL}}}{10^9\ \text{ppb}} = 4.5 \times 10^{-6}\ \text{g}$$

Finally, convert this mass to the requested unit of micrograms:

$$4.5 \times 10^{-6}\ \text{g} \times \frac{1\ \mu\text{g}}{10^{-6}\ \text{g}} = 4.5\ \mu\text{g}$$

Check Your Learning

A 50.0-g sample of industrial wastewater was determined to contain 0.48 mg of mercury. Express the mercury concentration of the wastewater in ppm and ppb units.

Answer:

9.6 ppm, 9600 ppb

5.4 | Buffers

Buffer solutions help a biological system maintain its proper pH.

Buffers:

A buffer solution is an aqueous solution consisting of a mixture of a weak acid and its conjugate base, or vice versa. Within a buffer solution, pH changes very little when adding a small amount of strong acid or base. In nature, many systems use buffering for pH regulation. For example, the bicarbonate buffering system is used to regulate the pH of blood. Buffer solutions help maintain a biological system at its proper pH.

It is essential when working with protein (and other biomolecular) solutions that you pay attention to pH. The pH determines not only the overall native conformation (and proper functioning) of the protein it can also determine how the protein interacts with other biomolecules and how the protein interacts with non-biological surfaces such as an ion exchange column. When a solution is buffered, it resists a change in pH, even when H+ ions are added or lost from the system. Many chemicals can act as a buffer and can work at a wide range of pH.

Factors Impacting Buffer Selection:

- The pKa of a buffer is the pH at which the buffer experiences little change in pH with the addition of acids and bases. When choosing a buffer, select one with a pKa one unit above or below the pH of the solution. Buffers are effective in resisting a change in pH within a range of approximately one pH unit. For example, an acetate buffer has a pKa of 4.8 and is, therefore, effective in a range of 3.8-5.8 pH units.
- Not all buffering systems are compatible with all biochemistry procedures, and technicians may need to research the appropriate buffer to use in a biochemical application. For example, although Tris is the most common buffer used in a biotechnology lab, it is inappropriate for some protein assays because it tends to react with the assay components resulting in erroneous data.
- Two factors that influence the pH of a buffer: concentration and temperature. Although it is convenient to prepare a buffer as a concentrated stock, buffer pH can vary when diluted. Always check the final pH of a buffered solution if a concentrated stock was used to prepare it. Temperature also affects the pH of a buffer. Always pH a buffer at the temperature it will be used.

Buffer Preparation by Titration:

Sodium phosphate buffers are commonly used for physiological applications since it mimics certain components of extracellular fluids and is non-toxic to cells. Unlike Tris buffer, it is not sensitive to temperature changes but is sensitive to dilution; therefore, it is best to prepare stock solutions at or close to the final concentration. It is also highly susceptible to microorganism contamination and therefore, stored at 4°C.

A sodium phosphate buffer is a useful solution in a biosciences lab because the phosphoric acid has multiple dissociation constants, permitting the preparation of phosphate buffers near any of the three pKa values (pH values); 2.15, 6.86 and 12.32. This buffer is most commonly prepared at pH 7.0 using monosodium phosphate and its conjugate base, disodium phosphate. Phosphate buffers are prepared by mixing stock solutions of the desired concentration of dibasic and monobasic in known ratios, or through titration. To prepare a sodium phosphate buffer, first determine the concentration and working pH of the buffer. Using Henderson-Hassel Bach equation, calculate how much acid and base is required. Alternatively, use one of the many published tables available, such as the one below.

TABLE:

To prepare 1 L of 0.1 M sodium phosphate buffer of the desired pH, dilute the following mixtures should to 1 L (final volume) with H2O. Reference: Cold Springs Harbor Protocols, **http://cshprotocols.cshlp.org (http://cshprotocols.cshlp.org)**

pH	Volume (mL) of 1 M Na 2 HPO 4	Volume (mL) of 1 M NaH 2 PO 4
5.8	7.9	92.1
6.0	12.0	88.0
6.2	17.8	82.2
6.4	25.5	74.5
6.6	35.2	64.8
6.8	46.3	53.7
7.0	57.7	42.3
7.2	68.4	31.6
7.4	77.4	22.6
7.6	84.5	15.5
7.8	89.6	10.4
8.0	93.2	6.8

Table 5.1

5.5 | Water Standards

In a pharmaceutical company, water can be used for cleaning, raw material, formulation, and as a solvent. Therefore it is critical to use the highest quality water and comply with water standards.

Water Standards:

Water is the primary solvent used in a biosciences lab. In a pharmaceutical company, water can be used for cleaning, raw material, formulation, and as a solvent. Therefore it is critical to use the highest quality water and comply with water standards. However, what does ‘high quality’ mean? How do we qualify water? Each application requires different quality standards, and therefore, water quality is one of the most important considerations for a biotechnology lab. It is also one of the most costly expenditures!

The USP

Several professional societies publish standards, which contain specifications for relative water purity. The U.S. Pharmacopeia Convention (USP) is a nonprofit organization that works with scientists world-wide to protect public health by establishing standards for pharmaceuticals including identity, purity and quality, and the FDA oversees the enforcement of these standards. To learn more about what the USP does watch this video: **https://youtu.be/ZGEZTSz3amU (https://youtu.be/ZGEZTSz3amU)**

Interesting pharmaceutical facts are here: **http://www.usp.org/frequently-asked-questions/water-pharmaceutical-and-analytical-purposes (http://www.usp.org/frequently-asked-questions/water-pharmaceutical-and-analytical-purposes)**

TYPES OF WATER

The FDA & USP recognize eight types of water:

1. Non-potable
2. Potable (drinkable) water
3. USP purified water
4. USP water for injection (WFI)
5. USP sterile water for injection
6. LUSP sterile water for inhalation
7. USP bacteriostatic water for injection
8. USP sterile water for irrigation

Source water:

Source water, such as municipal water, can only be used for non-contact with product uses, such as cooling systems. Water for pharmaceutical manufacturing and testing purposes must be highly purified. Tap water can have high levels of chlorine and other ions, particulate matter and non-indicator microorganisms and viruses not tested for, by drinking quality standards.

Ultra-Pure Water:

The United States Pharmacopoeia (USP) distinguishes between water for injections (WFI) and purified water. WFI water must be produced by distillation or reverse osmosis and must have extremely low endotoxin levels. It is also the water of choice for maintenance of mammalian and insect cell lines, as they are sensitive to the presence of endotoxin. Endotoxins are pyrogens, which induce fever in humans. They are part of the cell wall in gram-negative bacteria and are lipopolysaccharides—molecules containing lipids and polysaccharides. A person with a systemic gram-negative infection runs the risk of dying from septic shock when treated with an antibiotic that kills the bacterial infection. The killed bacteria release endotoxin triggering the person's immune system resulting in septic shock. Tap water is contaminated with endotoxin and therefore NOT appropriate for injection. The concentration of endotoxin is endotoxin units per milliliter (EU/mL). A common water quality test performed on biopharmaceuticals is Limulus Amebocyte Lysate Test or LAL test, to assess the pyrogen (endotoxin) content in water.

Table 1: Pharmacopeia specifications for water for injection (Wikipedia.org)

Properties	European Pharmacopoeia	United States Pharmacopeia
Conductivity**[B] (https://en.wikipedia.org/wiki/Ultrapure_water#cite_note-14)**	<1.3 μS/cm at 25 °C	<1.3 μS/cm at 25 °C
Total Organic Carbon (https://en.wikipedia.org/wiki/Total_organic_carbon) (TOC)	<0.5 mg/L	<0.50 mg/L

Table 5.2

Properties	European Pharmacopoeia	United States Pharmacopeia
Bacteria (guideline)	<10 CFU (https://en.wikipedia.org/wiki/Colony-forming_unit) /100 mL	<10 CFU (https://en.wikipedia.org/wiki/Colony-forming_unit) /100 mL
Endotoxin	<0.25 IU/mL	<0.25 EU/mL [C] (https://en.wikipedia.org/wiki/Ultrapure_water#cite_note-15)
Nitrates	<0.2 ppm	N/A
Aluminum	<10 ppb	N/A

Table 5.2

ISO Standards:

ISO also publishes and recognizes water standards. They have three grades of water with Grade I, as the purest. Their summary guidance can be found here, and provide international harmonization of water standards: **https://www.iso.org/files/live/sites/isoorg/files/archive/pdf/en/iso_and_water.pdf (https://www.iso.org/files/live/sites/isoorg/files/archive/pdf/en/iso_and_water.pdf)**

ASTM Standards:

The American Society for Testing and Materials (ASTM) publishes water standards as well (D1193-06). They have four grades of water, with Type I being the purest. Type I water is typically used for cell culture, and HPLC applications and is generated using a reverse osmosis system.

- ***Type I water*** is the highest class of purity and is used for most analytical procedures, tissue culture and instrumentation since it has very low levels of contaminants. It is routinely prepared by reverse osmosis (RO) in combination with distillation and deionization.
- ***Type II water*** is suitable for most routine lab work and is prepared by distillation or RO. Although it is not as reactive as type I, it should also be stored in non-reactive containers.
- ***Type III & IV water***, such as tap water, is useful for some applications such as rinsing glassware or preparing microscope slides.

ASTM further classifies water according to grade: A, B and C that are applied to these four types of water. These grades vary in the levels of bacterial and endotoxin contamination permitted. It's important to note, water used for making pharmaceuticals requires even stricter standards than Type I water.

5.6 | Water Purification

There are many different types of contaminants found in water and a variety of methods used to remove these contaminants. Typically, many methods are used in tandem to achieve the highest quality needed.

Water Purification

There are many different types of contaminants found in water and may include dissolved inorganic, dissolved organics, suspended particles, dissolved gasses, microorganisms, and most importantly for pharmaceutical companies, pyrogen, and endotoxins. There are a variety of methods used to remove these contaminants from water, and frequently many methods are used together to achieve the highest quality needed. It is important to note that 100% pure water does not exist. Although the techniques described below do an excellent job of removing contaminants, water is never truly free of all contaminants.

Source water

Most labs and biotechnology industries begin with partially purified tap (municipal) water, the water we drink. The quality of municipal water varies depending on geographical location and its influence on the water. You may

have noticed how water tastes different in different cities. Due to the high cost of making ultrapure water, some biopharmaceutical industries choose their site depending on the quality of the local water. Although municipal water is drinkable, it needs to be further purified for lab work.

There are five major ways water is purified:

Distillation is often used to purify water for the lab. Water is heated to boiling; the steam travels through a cooled condenser coil, where it condenses back to liquid form and is collected in a different container. Although distillation removes many contaminants, it is not effective at removing dissolved ionized gasses. One of the main disadvantages of distillation is that it is a relatively expensive and slow process.

In **ion exchange,**water passes through filters with bead-shaped resins, which remove ions. Cationic resins remove positive ions, and anionic resins remove negative ions. It is essential that lab water be deionized. Ions are highly reactive and can ruin experiments, interfere in the production process and contaminate the final product. In Central Texas, we have hard water, water in which many ions, such as, calcium, are dissolved. We can see this hard water in the low sudsing of our soaps and shampoos as well as the tough-to-remove ring around the tub. Water softeners are cationic resins with Na+ loosely attached to them. As our hard water is passed over the water softener, the hard water Ca^{++} ions are exchanged for the Na^{+} ions. Many homes use these water softeners. However, because of the presence of Na+, they are not sufficient for lab purification.

Deionization is accomplished using both a cationic exchange column with H+ (instead of Na+) ions as well as an anionic exchange column with OH-. The positive contaminants are removed by the first column and exchanged with the H+, and the negative contaminants exchanged for the OH-. The resulting ions combine to form more water molecules, and the water is purified. It is purer than tap or softened water but still contains many contaminants such as dissolved organics, bacteria, and pyrogens.

Carbon Adsorption is very effective in removing dissolved organic compounds from water. The water is passed over activated charcoal (carbon) made traditionally by burning wood. Most activated carbon is made from styrene beads today since this produces a purer carbon. The organic contaminants stick to the activated carbon and are removed. Organic removal is usually the preliminary step before deionization.

There are many types of **filtration methods** used in treating water:

- The first of these are **depth filters** made of sand or matted fibers. They are often used at the beginning of the filtration system to remove large debris.
- **Microfiltration membrane filters** are filters with a pore size of 0.20 um, which filter out bacteria but not smaller dissolved molecules.
- Small dissolved molecules, including most organics, are filtered out of the water with an **ultrafiltration membrane** whose pore sizes are smaller than the microfilters. Molecules smaller than most proteins can be separated!
- **Reverse osmosis (RO)** filters are more restrictive than ultrafiltration membranes and with a range of 0.0001 um in diameter and smaller, removes molecules less than 300 Daltons. RO is successful at removing viruses, bacteria, and pyrogens. They also reject ions and polar molecules such as sugars. In RO, water passes through a thin membrane, which retains material based both on their size and on ionic charge. Water is usually under pressure to speed up this slow process. Reverse osmosis is often used to make Type I water.
- **Nanofiltration** is a similar process to RO and can also remove viruses and pyrogens; however, its range is a little smaller at 0.001 um.

There are several other methods used to purify water. Two worth mentioning are **ultraviolet (UV) oxidation** and **ozone sterilization**. In UV oxidation, water is passed for about 30 minutes over a UV lamp with a wavelength of 185 nm. The organic compounds are oxidized to simple compounds such as carbon dioxide. A wavelength of 245 nm will kill bacteria and is sometimes used to sterilize water. In ozone sterilization, ozone kills bacteria by rupturing their membranes. Ozone is highly reactive and therefore must be removed in subsequent purification steps.

WATER PURIFICATION SUMMARY

Each one of these purification methods has their advantages, limitations, and specific applications. It is important to note that a combination of these purification procedures is often employed in most labs. For example, Type I water may first be passed through an activated carbon filter and then RO filter to a storage tank. Finally, a series of ion exchange columns may be used before a final ultrafiltration step. Different labs have different water needs and will choose the standard that is recommended and used in their industry based on their product. Water

usage in a biotechnology company follows strict adherence to Standard Operating Procedures (SOPs). SOPs should explicitly state the source of water required, how it is purified, how the water is monitored and how the purification equipment is to be maintained.

WATER MONITORING

Labs must monitor their water purification systems to ensure that the systems are working properly. Labs usually keep logs of the date, the monitoring system(s) used and the results. The **maintenance** of the water purification systems includes cleaning (usually daily for distillation systems), sanitation and regeneration of ion exchange systems, sanitation and checking for holes of filtration systems, and cleaning and recharging of activated carbon.

Five quality parameters are typically used to monitor the water system.

1. **Resistance** is a measurement of the electrical current through the water. Since ions carry the current, water without ion contaminants should have a high resistance. There is usually an attached meter to read the water resistance of water purification systems. The acceptable value for Type I water is 17.0 megohm-cm. Resistance is only useful for monitoring ions in water.

1. **Bacterialcounts** are used to track levels of microorganisms in water. There are typically three different ways a **Heterotrophic Plate Count** is performed: the pour plate technique, the spread plate technique, and the membrane filtration technique. In all three methods, water samples using various techniques are incubated 25-35°C for 48-72 hours on agar plate media. The numbers of colonies growing are counted to give the number of Colony Forming Units (CFU) per mL of water. Different standards provide the maximum CFU permitted.

1. As mentioned in the introduction, **pyrogens** are tested with a Limulus Amebocyte Lysate (LAL) test. An extract from the blood of a horseshoe crab is mixed with different dilutions of water. Pyrogens will cause the blood extract to clot. The results are given in endotoxin units per milliliter (EU/ml).

1. **Organic carbon contaminants** are monitored with instruments such as the mass spectrometer, which analyze the carbon compounds.

1. The **pH** of ultrapure water exposed to the carbon dioxide in the air is about 5.7. In a covered container, water has a pH of about 6.0. The pH is easily monitored with a pH meter.

KEY TERMS

aqueous solution solution for which water is the solvent

Avogadro's number (N_A) experimentally determined value of the number of entities comprising 1 mole of substance, equal to 6.022×10^{23} mol^{-1}

concentrated qualitative term for a solution containing solute at a relatively high concentration

concentration quantitative measure of the relative amounts of solute and solvent present in a solution

dilute qualitative term for a solution containing solute at a relatively low concentration

dilution process of adding solvent to a solution in order to lower the concentration of solutes

dissolved describes the process by which solute components are dispersed in a solvent

formula mass sum of the average masses for all atoms represented in a chemical formula; for covalent compounds, this is also the molecular mass

mass percentage ratio of solute-to-solution mass expressed as a percentage

mass-volume percent ratio of solute mass to solution volume, expressed as a percentage

molar mass mass in grams of 1 mole of a substance

molarity (M) unit of concentration, defined as the number of moles of solute dissolved in 1 liter of solution

mole amount of substance containing the same number of atoms, molecules, ions, or other entities as the number of atoms in exactly 12 grams of ^{12}C

parts per billion (ppb) ratio of solute-to-solution mass multiplied by 10^9

parts per million (ppm) ratio of solute-to-solution mass multiplied by 10^6

solute solution component present in a concentration less than that of the solvent

solvent solution component present in a concentration that is higher relative to other components

volume percentage ratio of solute-to-solution volume expressed as a percentage

KEY-EQUATIONS

5.2 Molarity

$$M = \frac{\text{mol solute}}{\text{L solution}}$$

$$C_1V_1 = C_2V_2$$

5.3 Other Units for Solution Concentrations

$$\text{Percent by mass} = \frac{\text{mass of solute}}{\text{mass of solution}} \times 100$$

$$\text{ppm} = \frac{\text{mass solute}}{\text{mass solution}} \times 10^6 \text{ ppm}$$

$$\text{ppb} = \frac{\text{mass solute}}{\text{mass solution}} \times 10^9 \text{ ppb}$$

CHAPTER SUMMARY

5.1 Formula Mass and the Mole Concept

The formula mass of a substance is the sum of the average atomic masses of each atom represented in the chemical formula and is expressed in atomic mass units. The formula mass of a covalent compound is also called the molecular mass. A convenient amount unit for expressing very large numbers of atoms or molecules

is the mole. Experimental measurements have determined the number of entities composing 1 mole of substance to be 6.022×10^{23}, a quantity called Avogadro's number. The mass in grams of 1 mole of substance is its molar mass. Due to the use of the same reference substance in defining the atomic mass unit and the mole, the formula mass (amu) and molar mass (g/mol) for any substance are numerically equivalent (for example, one H_2O molecule weighs approximately18 amu and 1 mole of H_2O molecules weighs approximately 18 g).

5.2 Molarity

Solutions are homogeneous mixtures. Many solutions contain one component, called the solvent, in which other components, called solutes, are dissolved. An aqueous solution is one for which the solvent is water. The concentration of a solution is a measure of the relative amount of solute in a given amount of solution. Concentrations may be measured using various units, with one very useful unit being molarity, defined as the number of moles of solute per liter of solution. The solute concentration of a solution may be decreased by adding solvent, a process referred to as dilution. The dilution equation is a simple relation between concentrations and volumes of a solution before and after dilution.

5.3 Other Units for Solution Concentrations

In addition to molarity, a number of other solution concentration units are used in various applications. Percentage concentrations based on the solution components' masses, volumes, or both are useful for expressing relatively high concentrations, whereas lower concentrations are conveniently expressed using ppm or ppb units. These units are popular in environmental, medical, and other fields where mole-based units such as molarity are not as commonly used.

EXERCISES

5.1 Formula Mass and the Mole Concept

1. What is the total mass (amu) of carbon in each of the following molecules?

(a) CH_4

(b) $CHCl_3$

(c) $C_{12}H_{10}O_6$

(d) $CH_3CH_2CH_2CH_2CH_3$

2. What is the total mass of hydrogen in each of the molecules?

(a) CH_4

(b) $CHCl_3$

(c) $C_{12}H_{10}O_6$

(d) $CH_3CH_2CH_2CH_2CH_3$

3. Calculate the molecular or formula mass of each of the following:

(a) P_4

(b) H_2O

(c) $Ca(NO_3)_2$

(d) CH_3CO_2H (acetic acid)

(e) $C_{12}H_{22}O_{11}$ (sucrose, cane sugar)

4. Determine the molecular mass of the following compounds:

(a)

(b)

(c)

(d)

5. Determine the molecular mass of the following compounds:

(a)

CH_2CH_3

(b)

(c)

(d)

6. Which molecule has a molecular mass of 28.05 amu?

(a)

(b)

(c)

7. Write a sentence that describes how to determine the number of moles of a compound in a known mass of the compound using its molecular formula.

8. Compare 1 mole of H_2, 1 mole of O_2, and 1 mole of F_2.

(a) Which has the largest number of molecules? Explain why.

(b) Which has the greatest mass? Explain why.

9. Which contains the greatest mass of oxygen: 0.75 mol of ethanol (C_2H_5OH), 0.60 mol of formic acid (HCO_2H), or 1.0 mol of water (H_2O)? Explain why.

10. Which contains the greatest number of moles of oxygen atoms: 1 mol of ethanol (C_2H_5OH), 1 mol of formic acid (HCO_2H), or 1 mol of water (H_2O)? Explain why.

11. How are the molecular mass and the molar mass of a compound similar and how are they different?

12. Calculate the molar mass of each of the following compounds:

(a) hydrogen fluoride, HF

(b) ammonia, NH_3

(c) nitric acid, HNO_3

(d) silver sulfate, Ag_2SO_4

(e) boric acid, $B(OH)_3$

13. Calculate the molar mass of each of the following:

(a) S_8

(b) C_5H_{12}

(c) $Sc_2(SO_4)_3$

(d) CH_3COCH_3 (acetone)

(e) $C_6H_{12}O_6$ (glucose)

14. Calculate the empirical or molecular formula mass and the molar mass of each of the following minerals:

(a) limestone, $CaCO_3$

(b) halite, NaCl

(c) beryl, $Be_3Al_2Si_6O_{18}$

(d) malachite, $Cu_2(OH)_2CO_3$

(e) turquoise, $CuAl_6(PO_4)_4(OH)_8(H_2O)_4$

15. Calculate the molar mass of each of the following:

(a) the anesthetic halothane, $C_2HBrClF_3$

(b) the herbicide paraquat, $C_{12}H_{14}N_2Cl_2$

(c) caffeine, $C_8H_{10}N_4O_2$

(d) urea, $CO(NH_2)_2$

(e) a typical soap, $C_{17}H_{35}CO_2Na$

16. Determine the number of moles of compound and the number of moles of each type of atom in each of the following:

(a) 25.0 g of propylene, C_3H_6

(b) 3.06×10^{-3} g of the amino acid glycine, $C_2H_5NO_2$

(c) 25 lb of the herbicide Treflan, $C_{13}H_{16}N_2O_4F$ (1 lb = 454 g)

(d) 0.125 kg of the insecticide Paris Green, $Cu_4(AsO_3)_2(CH_3CO_2)_2$

(e) 325 mg of aspirin, $C_6H_4(CO_2H)(CO_2CH_3)$

17. Determine the mass of each of the following:

(a) 0.0146 mol KOH

(b) 10.2 mol ethane, C_2H_6

(c) 1.6×10^{-3} mol Na_2SO_4

(d) 6.854×10^3 mol glucose, $C_6H_{12}O_6$

(e) 2.86 mol $Co(NH_3)_6Cl_3$

18. Determine the number of moles of the compound and determine the number of moles of each type of atom in each of the following:

(a) 2.12 g of potassium bromide, KBr

(b) 0.1488 g of phosphoric acid, H_3PO_4

(c) 23 kg of calcium carbonate, $CaCO_3$

(d) 78.452 g of aluminum sulfate, $Al_2(SO_4)_3$

(e) 0.1250 mg of caffeine, $C_8H_{10}N_4O_2$

19. Determine the mass of each of the following:

(a) 2.345 mol LiCl

(b) 0.0872 mol acetylene, C_2H_2

(c) 3.3×10^{-2} mol Na_2CO_3

(d) 1.23×10^3 mol fructose, $C_6H_{12}O_6$

(e) 0.5758 mol $FeSO_4(H_2O)_7$

20. The approximate minimum daily dietary requirement of the amino acid leucine, $C_6H_{13}NO_2$, is 1.1 g. What is this requirement in moles?

21. Determine the mass in grams of each of the following:

(a) 0.600 mol of oxygen atoms

(b) 0.600 mol of oxygen molecules, O_2

(c) 0.600 mol of ozone molecules, O_3

22. A 55-kg woman has 7.5×10^{-3} mol of hemoglobin (molar mass = 64,456 g/mol) in her blood. How many hemoglobin molecules is this? What is this quantity in grams?

23. Determine the number of atoms and the mass of zirconium, silicon, and oxygen found in 0.3384 mol of zircon, $ZrSiO_4$, a semiprecious stone.

24. Determine which of the following contains the greatest mass of hydrogen: 1 mol of CH_4, 0.6 mol of C_6H_6, or 0.4 mol of C_3H_8.

25. Determine which of the following contains the greatest mass of aluminum: 122 g of $AlPO_4$, 266 g of Al_2Cl_6, or 225 g of Al_2S_3.

26. Diamond is one form of elemental carbon. An engagement ring contains a diamond weighing 1.25 carats (1 carat = 200 mg). How many atoms are present in the diamond?

27. The Cullinan diamond was the largest natural diamond ever found (January 25, 1905). It weighed 3104 carats (1 carat = 200 mg). How many carbon atoms were present in the stone?

28. One 55-gram serving of a particular cereal supplies 270 mg of sodium, 11% of the recommended daily allowance. How many moles and atoms of sodium are in the recommended daily allowance?

29. A certain nut crunch cereal contains 11.0 grams of sugar (sucrose, $C_{12}H_{22}O_{11}$) per serving size of 60.0 grams. How many servings of this cereal must be eaten to consume 0.0278 moles of sugar?

30. A tube of toothpaste contains 0.76 g of sodium monofluorophosphate (Na_2PO_3F) in 100 mL.

(a) What mass of fluorine atoms in mg was present?

(b) How many fluorine atoms were present?

31. Which of the following represents the least number of molecules?

(a) 20.0 g of H_2O (18.02 g/mol)

(b) 77.0 g of CH_4 (16.06 g/mol)

(c) 68.0 g of CaH_2 (42.09 g/mol)

(d) 100.0 g of N_2O (44.02 g/mol)

(e) 84.0 g of HF (20.01 g/mol)

5.2 Molarity

32. Explain what changes and what stays the same when 1.00 L of a solution of NaCl is diluted to 1.80 L.

33. What information is needed to calculate the molarity of a sulfuric acid solution?

34. A 200-mL sample and a 400-mL sample of a solution of salt have the same molarity. In what ways are the two samples identical? In what ways are these two samples different?

35. Determine the molarity for each of the following solutions:

(a) 0.444 mol of $CoCl_2$ in 0.654 L of solution

(b) 98.0 g of phosphoric acid, H_3PO_4, in 1.00 L of solution

(c) 0.2074 g of calcium hydroxide, $Ca(OH)_2$, in 40.00 mL of solution

(d) 10.5 kg of $Na_2SO_4{\cdot}10H_2O$ in 18.60 L of solution

(e) 7.0×10^{-3} mol of I_2 in 100.0 mL of solution

(f) 1.8×10^{4} mg of HCl in 0.075 L of solution

36. Determine the molarity of each of the following solutions:

(a) 1.457 mol KCl in 1.500 L of solution

(b) 0.515 g of H_2SO_4 in 1.00 L of solution

(c) 20.54 g of $Al(NO_3)_3$ in 1575 mL of solution

(d) 2.76 kg of $CuSO_4{\cdot}5H_2O$ in 1.45 L of solution

(e) 0.005653 mol of Br_2 in 10.00 mL of solution

(f) 0.000889 g of glycine, $C_2H_5NO_2$, in 1.05 mL of solution

37. Consider this question: What is the mass of the solute in 0.500 L of 0.30 *M* glucose, $C_6H_{12}O_6$, used for intravenous injection?

(a) Outline the steps necessary to answer the question.

(b) Answer the question.

38. Consider this question: What is the mass of solute in 200.0 L of a 1.556-*M* solution of KBr?

(a) Outline the steps necessary to answer the question.

(b) Answer the question.

39. Calculate the number of moles and the mass of the solute in each of the following solutions:

(a) 2.00 L of 18.5 *M* H_2SO_4, concentrated sulfuric acid

(b) 100.0 mL of 3.8×10^{-6} *M* NaCN, the minimum lethal concentration of sodium cyanide in blood serum

(c) 5.50 L of 13.3 *M* H_2CO, the formaldehyde used to “fix” tissue samples

(d) 325 mL of 1.8×10^{-6} *M* $FeSO_4$, the minimum concentration of iron sulfate detectable by taste in drinking water

40. Calculate the number of moles and the mass of the solute in each of the following solutions:

(a) 325 mL of 8.23×10^{-5} *M* KI, a source of iodine in the diet

(b) 75.0 mL of 2.2×10^{-5} *M* H_2SO_4, a sample of acid rain

(c) 0.2500 L of 0.1135 *M* K_2CrO_4, an analytical reagent used in iron assays

(d) 10.5 L of 3.716 *M* $(NH_4)_2SO_4$, a liquid fertilizer

41. Consider this question: What is the molarity of $KMnO_4$ in a solution of 0.0908 g of $KMnO_4$ in 0.500 L of solution?

(a) Outline the steps necessary to answer the question.

(b) Answer the question.

42. Consider this question: What is the molarity of HCl if 35.23 mL of a solution of HCl contain 0.3366 g of HCl?

(a) Outline the steps necessary to answer the question.

(b) Answer the question.

43. Calculate the molarity of each of the following solutions:

(a) 0.195 g of cholesterol, $C_{27}H_{46}O$, in 0.100 L of serum, the average concentration of cholesterol in human serum

(b) 4.25 g of NH_3 in 0.500 L of solution, the concentration of NH_3 in household ammonia

(c) 1.49 kg of isopropyl alcohol, C_3H_7OH, in 2.50 L of solution, the concentration of isopropyl alcohol in rubbing alcohol

(d) 0.029 g of I_2 in 0.100 L of solution, the solubility of I_2 in water at 20 °C

44. Calculate the molarity of each of the following solutions:

(a) 293 g HCl in 666 mL of solution, a concentrated HCl solution

(b) 2.026 g $FeCl_3$ in 0.1250 L of a solution used as an unknown in general chemistry laboratories

(c) 0.001 mg Cd^{2+} in 0.100 L, the maximum permissible concentration of cadmium in drinking water

(d) 0.0079 g $C_7H_5SNO_3$ in one ounce (29.6 mL), the concentration of saccharin in a diet soft drink.

45. There is about 1.0 g of calcium, as Ca^{2+}, in 1.0 L of milk. What is the molarity of Ca^{2+} in milk?

46. What volume of a 1.00-*M* $Fe(NO_3)_3$ solution can be diluted to prepare 1.00 L of a solution with a concentration of 0.250 *M*?

47. If 0.1718 L of a 0.3556-*M* C_3H_7OH solution is diluted to a concentration of 0.1222 *M*, what is the volume of the resulting solution?

48. If 4.12 L of a 0.850 *M*-H_3PO_4 solution is be diluted to a volume of 10.00 L, what is the concentration of the resulting solution?

49. What volume of a 0.33-*M* $C_{12}H_{22}O_{11}$ solution can be diluted to prepare 25 mL of a solution with a concentration of 0.025 *M*?

50. What is the concentration of the NaCl solution that results when 0.150 L of a 0.556-*M* solution is allowed to evaporate until the volume is reduced to 0.105 L?

51. What is the molarity of the diluted solution when each of the following solutions is diluted to the given final volume?

(a) 1.00 L of a 0.250-*M* solution of $Fe(NO_3)_3$ is diluted to a final volume of 2.00 L

(b) 0.5000 L of a 0.1222-*M* solution of C_3H_7OH is diluted to a final volume of 1.250 L

(c) 2.35 L of a 0.350-*M* solution of H_3PO_4 is diluted to a final volume of 4.00 L

(d) 22.50 mL of a 0.025-*M* solution of $C_{12}H_{22}O_{11}$ is diluted to 100.0 mL

52. What is the final concentration of the solution produced when 225.5 mL of a 0.09988-*M* solution of Na_2CO_3 is allowed to evaporate until the solution volume is reduced to 45.00 mL?

53. A 2.00-L bottle of a solution of concentrated HCl was purchased for the general chemistry laboratory. The solution contained 868.8 g of HCl. What is the molarity of the solution?

54. An experiment in a general chemistry laboratory calls for a 2.00-*M* solution of HCl. How many mL of 11.9 *M* HCl would be required to make 250 mL of 2.00 *M* HCl?

55. What volume of a 0.20-*M* K_2SO_4 solution contains 57 g of K_2SO_4?

56. The US Environmental Protection Agency (EPA) places limits on the quantities of toxic substances that may be discharged into the sewer system. Limits have been established for a variety of substances, including hexavalent chromium, which is limited to 0.50 mg/L. If an industry is discharging hexavalent chromium as potassium dichromate ($K_2Cr_2O_7$), what is the maximum permissible molarity of that substance?

5.3 Other Units for Solution Concentrations

57. Consider this question: What mass of a concentrated solution of nitric acid (68.0% HNO_3 by mass) is needed to prepare 400.0 g of a 10.0% solution of HNO_3 by mass?

(a) Outline the steps necessary to answer the question.

(b) Answer the question.

58. What mass of a 4.00% NaOH solution by mass contains 15.0 g of NaOH?

59. What mass of solid NaOH (97.0% NaOH by mass) is required to prepare 1.00 L of a 10.0% solution of NaOH by mass? The density of the 10.0% solution is 1.109 g/mL.

60. What mass of HCl is contained in 45.0 mL of an aqueous HCl solution that has a density of 1.19 g cm^{-3} and contains 37.21% HCl by mass?

61. The hardness of water (hardness count) is usually expressed in parts per million (by mass) of $CaCO_3$, which is equivalent to milligrams of $CaCO_3$ per liter of water. What is the molar concentration of Ca^{2+} ions in a water sample with a hardness count of 175 mg $CaCO_3$/L?

62. The level of mercury in a stream was suspected to be above the minimum considered safe (1 part per billion by weight). An analysis indicated that the concentration was 0.68 parts per billion. Assume a density of 1.0 g/mL and calculate the molarity of mercury in the stream.

63. In Canada and the United Kingdom, devices that measure blood glucose levels provide a reading in millimoles per liter. If a measurement of 5.3 m*M* is observed, what is the concentration of glucose ($C_6H_{12}O_6$) in mg/dL?

64. A throat spray is 1.40% by mass phenol, C_6H_5OH, in water. If the solution has a density of 0.9956 g/mL, calculate the molarity of the solution.

65. Copper(I) iodide (CuI) is often added to table salt as a dietary source of iodine. How many moles of CuI are contained in 1.00 lb (454 g) of table salt containing 0.0100% CuI by mass?

66. A cough syrup contains 5.0% ethyl alcohol, C_2H_5OH, by mass. If the density of the solution is 0.9928 g/mL, determine the molarity of the alcohol in the cough syrup.

67. D5W is a solution used as an intravenous fluid. It is a 5.0% by mass solution of dextrose ($C_6H_{12}O_6$) in water. If the density of D5W is 1.029 g/mL, calculate the molarity of dextrose in the solution.

68. Find the molarity of a 40.0% by mass aqueous solution of sulfuric acid, H_2SO_4, for which the density is 1.3057 g/mL.

6 | BIOTECHNICIAN TOOLS: BASIC LABORATORY EQUIPMENT

6.1 | Introduction

Introduction to Equipment chapter

INTRODUCTION

Figure 6.1 Biotechnology program students learn how to operate basic laboratory equipment. Image Credit: Austin Community College

Learning how to operate correctly and care for basic lab equipment is a vital laboratory technician skill. Lab technicians frequently use electronic scales, balances, pH meters, centrifuges, and micropipettes to prepare a variety of chemical and biological reagents. Moreover, ensuring equipment is working properly is vital to preparing accurate solutions and performing meaningful experiments. Laboratory equipment should be high quality, properly installed, used correctly and for the appropriate application. In this chapter, basic lab equipment and validation is explored.

6.2 | Basic Lab Equipment

Basic lab equipment biotechnicians may use to prepare biological and chemical reagents.

LABORATORY EQUIPMENT

Calibration and Operation of a pH Meter

Most solutions prepared in the biological laboratory must have a carefully controlled pH. The pH measurement is a log of the hydrogen ion (H+) concentration of a solution. By definition, the pH value of neutral solutions is 7.0. Any solution with a pH < 7 is considered acidic, and any solution with a pH > 7 is considered basic. There are several effective means a biotechnician monitor's pH in solutions: pH indicator, pH paper, and a pH meter.

A pH indicator is a chemical added to a solution that changes color when it experiences different pH conditions. A chemical pH indicator is frequently used in cell culture media to provide biotechnicians quick assessment of cell growth. For example, using phenol red, when cells have grown rapidly and spent the media, the pH becomes acidic, changing the media from a bright red to deep yellow color.

Figure 6.2 Plastic box of pH indicator paper. A single pH paper strip with four colored squares sits on top of the box. By Michael Krahe - CC BY-SA 3.0, **https://en.wikipedia.org/wiki/PH_indicator#/media/File:PH_indicator_paper.jpg (https://en.wikipedia.org/wiki/PH_indicator#/media/File:PH_indicator_paper.jpg)** .

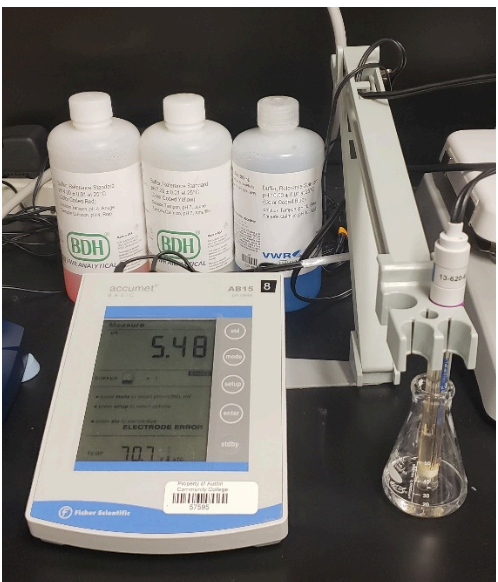


Figure 6.3 Digital pH meter. Photo credit: Jack O'Grady, Austin Community College

When looking for an estimate of pH, ***indicator paper*** can also be used. The indicator paper has an array of chemicals that exhibit a specific color when exposed to a particular pH (see figure). However, if a precise pH measurement is required, **a pH meter (https://en.wikipedia.org/wiki/PH_meter)** can be calibrated and used.

A ***pH meter*** is a scientific instrument that measures the hydrogen-ion activity in aqueous solutions, indicating its acidity or alkalinity expressed as pH. The pH meter measures the difference in electrical potential between a pH electrode and a reference electrode, and so the pH meter is sometimes referred to as a "potentiometric pH meter." The difference in electrical potential relates to the acidity or pH of the solution.

Very precise measurements require that the pH meter is calibrated frequently. Depending on the validation protocols, this may mean before each use, or once per day when turned on. Calibration is a critical process for pH meters, as the glass electrode does not provide reproducible electrostatic potentials over longer periods.

Consistent with principles of ***good laboratory practice***, calibration is performed with at least two standard buffer solutions that span the range of pH values to be measured. For general purposes, buffers at pH 4.00 and pH 10.00 are suitable. The pH meter has one calibration control to set the meter reading equal to the value of the first standard buffer and a second control to adjust the meter reading to the value of the second buffer. A third control allows the temperature to be set. More precise measurements sometimes require calibration at three different pH values. Some pH meters provide built-in temperature-coefficient correction, with temperature thermocouples in the electrode probes. The calibration process correlates the voltage produced by the probe (approximately 0.06 volts per pH unit) with the pH scale.

Good laboratory practice dictates that, after each measurement, the probes are rinsed with distilled water or deionized water to remove any traces of the solution being measured, blotted with a scientific wipe to absorb any remaining water, which could dilute the sample and thus alter the reading, and then immersed in a storage solution suitable for the probe type.

Watch the following video to learn how to calibrate a typical pH meter. (https://youtu.be/UfdKhja6u2I)

Electronic Balances

Instruments for weighing materials are called ***balances***, and most laboratories have more than one type of balance, depending on the amount of material being measured and the degree of accuracy required. ***Electronic balances*** usually have a digital readout and weighing dishes can be tarred to read zero mass before using. *Top loading electronic balances* used for the preparation of solutions have a sensitivity of +/- 0.01 g, but *analytical balances* (see figure) can be sensitive to +/- 0.01mg or less. Electronic balances require routine maintenance and recalibration.

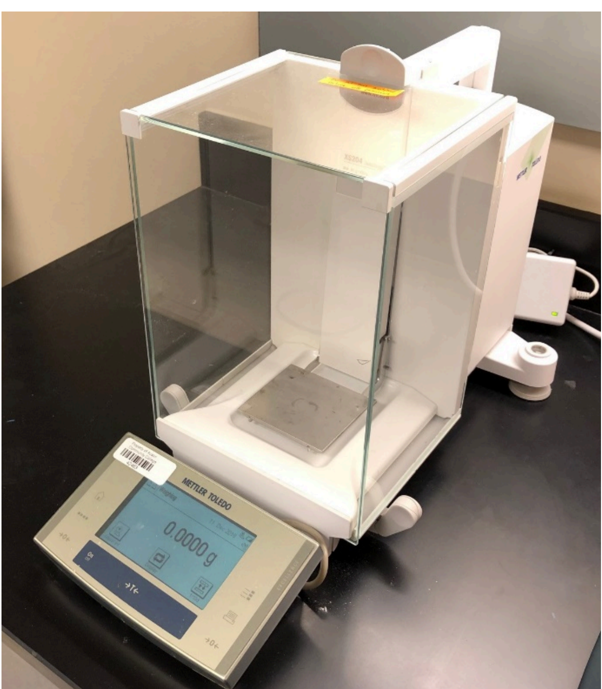


Figure 6.4 Mettler Analytical balance Photo credit: Jeremy Garza, Austin Community College.

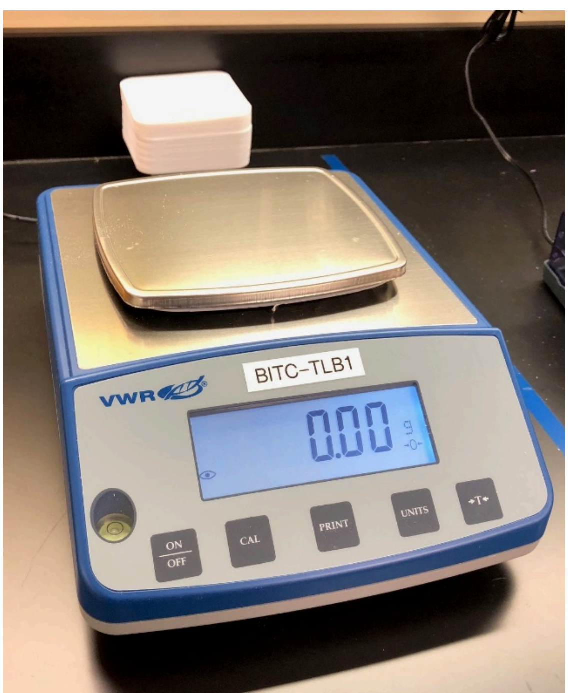


Figure 6.5 VWR top loading electronic balance. Photo credit: Jeremy Garza, Austin Community College.

Centrifugation

Many pieces of equipment are used to centrifuge biological samples for analysis. Centrifugation means to apply a centrifugal force to separate molecules by size (or shape, or density). Biotechnicians select the appropriate equipment for a task.

A ***preparative centrifuge*** has a balanced rotor that holds vessels and spins at high speed, up to 20,000 rpm. High-speed centrifugation will cause insoluble particles such as cells, and sometimes subcellular components, to form a pellet at the bottom of the vessel. Rotors are available that hold vessels as small as a few milliliters to as large as a liter. These centrifuges are often refrigerated so that heat-sensitive material, such as cells and proteins, are not damaged due to the high heat generated during centrifugation.

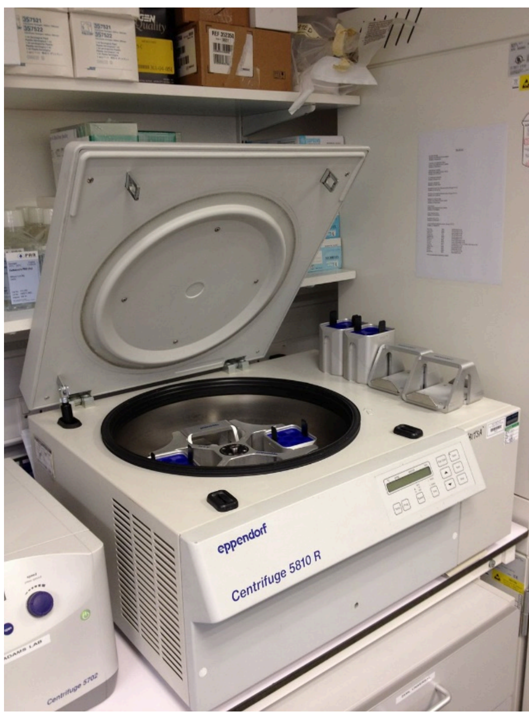


Figure 6.6 Tabletop refrigerated preparative centrifuge. Photo credits: **https://en.wikipedia.org/wiki/Laboratory_centrifuge (https://en.wikipedia.org/wiki/Laboratory_centrifuge)**

Figure 6.7 Refrigerated microcentrifuge. Photo credits: **https://en.wikipedia.org/wiki/Laboratory_centrifuge (https://en.wikipedia.org/wiki/Laboratory_centrifuge)**

A tabletop or ***clinical centrifuge*** is not refrigerated and spins at a much slower speed than a preparative centrifuge. Rotors for clinical centrifuges hold tubes with a capacity of 15 mL or less and a ‘swing-bucket’ rotor. These are frequently found in medical laboratory testing labs.

A ***microcentrifuge*** holds 2.0 mL (or smaller) microcentrifuge tubes with liquid and can centrifuge at high speeds (up to 14,000 rpm) to separate liquids and particulates in solutions. There are a variety of rotor sizes available, and there are refrigerated centrifuges available as well.

A ***picofuge*** is a fixed low-speed microcentrifuge, which spins much slower than a microcentrifuge and functions mostly to move liquid from the sides and top of a microcentrifuge tube to the bottom.

6.3 | Equipment to Measure Volume

Biotechnicians must select appropriate size measuring tool depending on the task. In this module is a summary of common laboratory tools for measuring volume including their relative accuracy.

MEASUREMENT OF VOLUME

There are many different types of glassware used in a biotechnology lab. The glassware selected will depend on the purpose of its usage as different glassware have various levels of accuracy of measurement.

Erlenmeyer flasks are primarily for the preparation or storage of solutions, not an accurate volume adjustment. Although there are volume markings on these flasks, they are not calibrated and should not be relied upon for exact volume measurements.

Beakers are used for preparing solutions, such as dissolving powdered reagents in water. Beakers are useful when a pH adjustment requires access to the solution by a pH probe. The volumetric markings on beakers are not reliable – and is not an accurate measuring tool.

Graduated cylinders are calibrated with enough accuracy for most volume measurements when preparing solutions. For example, the calibration of most 100 mL graduated cylinder can be relied upon to measure to within +/- 0.6 mL accurately. Graduated cylinders are most frequently used to bring solutions to a final volume. When pouring from graduated cylinders, some error and volume is lost as liquid remains in the cylinder.

Volumetric flasks are used to measure one volume with the highest degree of accuracy and are used to make standard solutions for analytical assays. For example, the calibration of a 100 mL volumetric flask can have an accuracy of +/- 0.1 mL. Note, there are no graduated markings in a volumetric flask – you can only bring solutions to one final volume in that flask.

Figure 6.8 Various types of glassware; beakers, Erlenmeyer flask, graduated cylinder, and volumetric flask. ***Image credit:*** **https://upload.wikimedia.org/wikipedia/commons/f/fc/Lab_glassware.jpg (https://upload.wikimedia.org/wikipedia/commons/f/fc/Lab_glassware.jpg)**

Pipets

Pipets are glass or plastic devices that are routinely used to measure and transfer liquids by drawing the liquid into the tube with a mechanical device.

- ***Pasteur pipets*** are small glass tubes used with a bulb to transfer volumes as little as a single drop and as large as a few milliliters. They are not graduated and are not used to measure volumes.
- ***Beral pipets*** (transfer pipets) are plastic pipettes with a bulb at one end used for transfer of liquids. Sometimes they have calibration marks, which have a low level of accuracy. They are often disposable, sterile and individually wrapped.
- ***Serological pipets*** are graduated tubes used to measure anywhere from 1 to 50 mL. When the liquid has drained from this pipet, the final drop in the tip is transferred with a puff of air. These are known as TC or *to contain* pipets. Serological pipets are most frequently used in bioscience labs with pipet controller (or pipet-aid), mechanical devices that aspirate liquid into and out of the pipet.
- ***Mohr pipets***, or "to deliver," pipets are like blowout pipets, but do not require a puff of air to deliver the desired volume accurately. They can be identified by the label "TD" on the top. These are sometimes called TD serological pipets.
- ***Volumetric pipets*** are not graduated but are calibrated to deliver a single, highly accurate, volume.
- ***Micropipettes*** are mechanical devices with disposable plastic tips, which deliver with a high degree of accuracy adjustable microliter volumes of liquid. There are several micropipette devices available of varying sizes, such as a 0.5-10ul, 2-20ul, 20-200ul, and 100-1000ul.
- ***Repeater Pipettes*** are mechanical devices that can be set to deliver, repeatedly, a precise microliter volume. The liquid is aspirated and dispensed from a disposable tip.
- ***Multichannel micropipettes*** can deliver the same volume from as many as 12 tips simultaneously.

SEROLOGICAL PIPETS

Serological, or "blowout," pipets are graduated tubes used to measure anywhere from 0.1 to 50 mL. They are typically made of single-use plastic and have the top end plugged with cotton to prevent contamination and overflow. When filling a ***serological pipet***, bring the container with the liquid and the pipet to eye level. Hold the tapered end beneath the surface of the liquid. Draw the liquid up the pipet by suction until the level is just above the desired, then bring the level down to the meniscus of the volume you want. When reading the volume, ALWAYS view the pipet at eye level with the pipet held vertically, perpendicular to the ground. Pipets are operated with a hand-held electronic pump (pipet-aid) or bulb, of which there are many varieties.

Watch the following **video on how to use a serological pipet (https://youtu.be/aei-tU1ZIkE)** correctly.

Play this fun pipetting game! (https://www.ncbionetwork.org/iet/pipetting/)

MICROPIPETTES

The micropipette is one of the biotechnician's most frequently used tools. There are different brands of micropipette but all of them work in the same way. All micropipettes are essentially long tubes with a handle and an adjustable piston inside. A disposable tip is placed on the bottom of the micropipette. This tip is the only piece that is inserted into the liquid. In or near the handle is a screw/knob/button that adjusts the volume of the micropipette by moving the piston up and down. On top, there is a plunger button for filling and for dispensing the liquid. There is often a second button on top for ejecting the tip. Micropipettes come in a variety of sizes; for example, 1-20 uL, 20-200 uL, and 100-1000 uL. When choosing which micropipette to use, the rule of thumb is to ***select the smallest size micropipette that can deliver the desired volume.***

Figure 6.9 Various micropipetting devices. From left to right: Set of 6 micropipettes measuring capacities from 2 microliters to 5 mL; 1 mL ergonomic digital micropipette; electronic pipet-aid (to draw liquid into serological pipet). Image Credit: Jack O'Grady, Austin Community College

To learn more about selecting and using a micropipette, watch this video! (https://youtu.be/uEy_NGDfo_8)

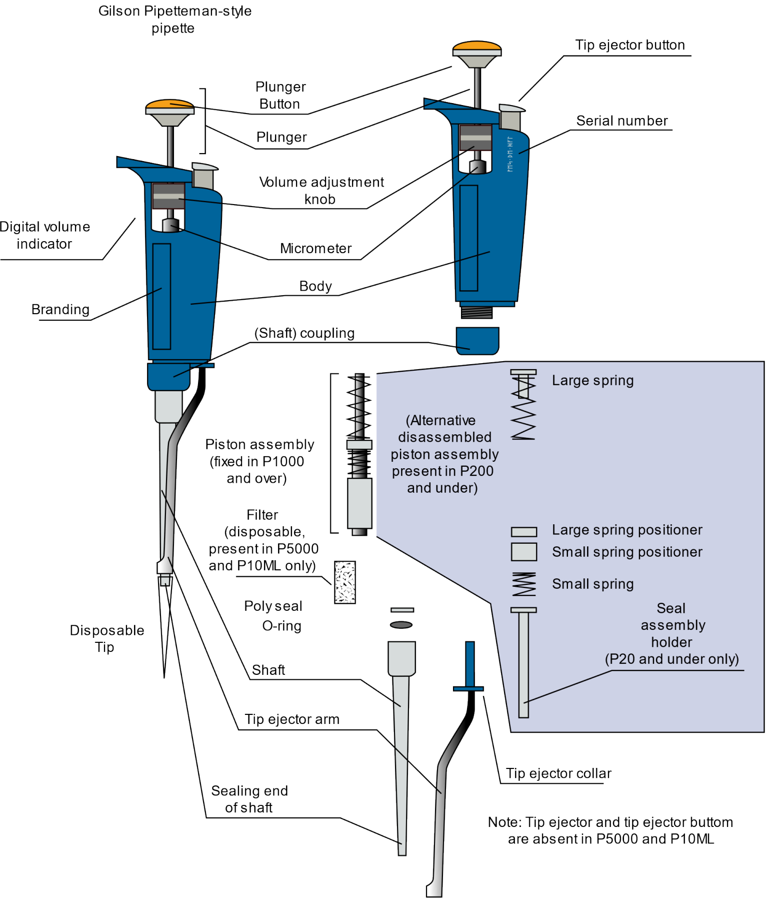


Figure 6.10 Labeled diagram of a Gilson brand micropipette. Note the plunger, tip ejector button, volume adjustment knob, digital volume indicator, shaft, and disposable tip. Image credit: https://commons.wikimedia.org/wiki/File:Gilson_schematic.svg

Micropipetting Technique

Of all the factors contributing to the performance of a micropipette, the skill of the operator is the most critical! Here are some tips to help improve your micropipette technique. **These tips are demonstrated in an excellent video. (https://www.artel-usa.com/resource-library/10-tips-to-improve-your-pipetting-technique/)**

1. Pre-wetting the tip by aspirating and expelling liquids three times increases the humidity within the tip and reduces sample loss due to evaporation.
2. Allow liquids to ambient temperature before dispensing. Humidity and pressure are temperature-dependent and therefore will affect the volume dispensed from the tip.
3. Examine the tip before and after dispensing. Remove droplets from the side of the tip and ensure there are no air bubbles in the tip before dispensing.
4. Use standard mode. Depress the plunger to the first stop, immerse into liquid, aspirate by releasing the plunger slowly. Remove tip from the liquid and depress the plunger to the second stop to dispense the entire contents.
5. Pause consistently after aspiration. Pause for one second before removing from the liquid. If aspirating a viscous liquid, pause for at least 3 seconds or until you can see no further liquid moving up the tip.
6. Pull the pipette straight out. This is particularly important for small volumes. If you hold your tip at an angle, it can alter the volume aspirated.
7. Minimize handling of the pipette and the tip with fingers. Touching the tip and tube with a gloved hand will warm them up resulting in inaccurate aspiration. Touching the tip with a bare hand can lead to contamination.

8. Immerse the tip to the proper depth. Immerse a p1000 5-6mm, and smaller micropipettes 2-3mm only. Too little immersion will lead to aspiration of air, too much can result in liquids clinging to the side of the tip, or touching the bottom causing incorrect aspiration volumes.
9. Use the correct pipette tip. Use the manufacturer’s recommended tip for the micropipette. Remember to use barrier tips when using biohazards or to avoid cross-contamination of your experiment is necessary (such as working with human blood samples).
10. Use consistent plunger pressure and speed. Depress and release the plunger smoothly, slowly, and consistently.

6.4 | Equipment Validation

In a typical biotechnology company bound by GMP or ISO9000 regulations, Equipment validation is part of routine Quality Control procedures, usually performed in a committed period (weekly, daily, or monthly). The technician follows a Standard Operating Procedure (SOP) and fills out the associated form documenting they have performed the task correctly and what the outcome of the task was. If the equipment fails the validation, the quality department must be notified through a deviation report. This report outlines the parameters of the equipment that did not meet the outlined specifications on the form. The equipment may need to be recalibrated or repaired, reinstalled or revalidated, or even replaced.

EQUIPMENT VALIDATION

Process validation is the method by which companies demonstrate that their activities, procedures, and processes consistently produce a quality result. Processes almost always involve equipment, and therefore, ***equipment validation*** is a critical process where a specified set of procedures are used to check if the equipment meets requirements. Different pieces of lab equipment are designed to operate within varying degrees of accuracy.

Each step in a biomanufacturing process must be controlled to ensure that the finished product meets all quality and design specifications. The actual requirement for process validation comes from the text of the GMPs, Section 211.100 which states that **“*there shall be written procedures for production and process control designed to assure that the drug products have the identity, strength, quality, and purity they purport or are represented to possess*.”** **CFR - Code of Federal Regulations Title 21 (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=21)** .

CALIBRATION, VERIFICATION & VALIDATION

GMP and ISO certified laboratories have written policies for equipment calibration, verification and validation for all equipment used in a cGMP lab.

- ***Calibration*** is a process that compares a known (the “standard” device) against an unknown (the target device in question). During the calibration process, the offset between these two devices is quantified, and the target device is adjusted back into tolerance (if possible). A calibration report usually contains both “as found” and “as left” data.
- ***Verification*** is simply the process of “verifying” that a device is within tolerance (within an acceptable range). Verification usually results in “as found” data. If the instrument is not within tolerance, it is sent for recalibration.
- ***Validation*** is a detailed process of confirming that the device is installed correctly, that it is operating effectively, and that it is performing without error. Validation in the pharmaceutical industry emerged from problems in the 1960s and 1970s and went together with the QA/QC philosophy that quality is built into the product not tested into the product. The FDA states that quality, safety, and effectiveness are designed and built into the product.

For example, micropipettes are validated by the manufacturer before shipping. However, they become less accurate the more they are used by biotechnicians. Therefore, the performance of a micropipette must be verified periodically and calibrated regularly.

EQUIPMENT VALIDATION

For a biomanufacturing process to proceed correctly, the equipment must be validated or qualified to ensure that

it will function reliably under all the conditions that may occur during production. Equipment qualification may be performed separately from process validation, but it is also a requirement for process validation. Equipment Validation is a detailed process of confirming that the instrument is installed correctly, that it is operating effectively, and that it is performing without error.

Validation of equipment is a vital link in the quality chain. Equipment should be adequately inspected, cleaned, and maintained as well as, tested, calibrated, and standardized at an appropriate frequency. Laboratories usually establish schedules for such activities based on manufacturer's recommendations, and frequency of use. Equipment is one of the major areas of focus for FDA (or any other regulatory body) audits. All equipment must meet manufacturer or preset standards for operation and performance.

Every biotechnology company approaches equipment validation with a different set of evaluation tools. What remains constant is that validation of equipment occurs *before* the equipment is used to support an associated process validation. The level of detail associated with each step of equipment validation depends on the usage of equipment. It is important, though that each step of the validation, *Installation Qualification (IQ)*, *Operational Qualification (OQ)*, and *Process or Performance Qualification (PQ)* must pass before proceeding to the next. If a piece of equipment is not passing the requirements written in the validation, remove the equipment from use and documentation of these deviations is included with the records.

The basic principles of equipment validation include:

The equipment is installed by vendor requirements, intended use, and regulations.

Requirements for calibration, maintenance, and cleaning have been developed as SOPs.

Operating requirements are established, and tests are conducted to ensure equipment is functioning correctly, under ideal, and challenged conditions.

Operator training requirements are established, completed, and recorded.

Equipment Validation is divided into three processes:

Installation Qualification (IQ). First, the equipment item is checked to be sure that it meets its design and purchase specifications and is properly installed; this is called installation qualification**.** Installation qualification includes, for example, checking that instruction manuals, schematic diagrams, and spare parts lists are present; checking that all parts of the device are installed; checking that the materials used in construction were those specified; and making sure that fittings, attachments, cables, plumbing, and wiring are properly connected. IQ is documented proof that the building, wiring, installation and calibration of equipment, utilities, SOPs, spare parts, and specifications meet the design intention.

Operational Qualification (OQ). After installation, the equipment can be tested to verify that it performs within acceptable limits. For example, an autoclave might be tested to see that it reaches the proper temperature, plus or minus certain limits, in a set period; that it reaches the proper pressure, plus or minus certain limits, etc. The penetration of steam to all parts of the chamber, the pressure achieved at various settings, and so forth, would all be tested in the context of the operational qualification of an autoclave. OQ is documented proof that the equipment performs as specified.

Performance Qualification (PQ). Once all measuring instruments are calibrated, and all equipment is validated, process validation (or qualification) can be performed. The validation of the process involves assessing the process under all the conditions that can be expected to occur during production. Testing includes checking the process endpoint(s) under these circumstances and establishing that the process consistently meets its specifications. PQ is documented proof the equipment or systems operate as intended under challenge conditions.

PQ also involves challenging the system with unusual circumstances. FDA speaks of the "worst case" situation(s) that might be encountered during production. For example, a sterilization process might be challenged by placing large numbers of the heat-resistant bacterium in the corner of the autoclave known to be least accessible to steam. The effectiveness of bacterial killing under these "worst case" conditions must meet the specifications for the process.

VALIDATION OF EQUIPMENT: CONSIDERATIONS

It is crucial when validating laboratory equipment to do so with calibrated and certified equipment and standards. Examples of certified equipment include a certified timer, certified weights, a certified thermometer and a certified tachometer which accurately measures rotor speed.

Figure 6.11 Electronic balance calibration weight set. Image credit: Dean Calma / IAEA **https://www.flickr.com/photos/iaea_imagebank/40785322521 (https://www.flickr.com/photos/iaea_imagebank/40785322521)**

Validation of Temperature: Temperature is a key element of many biomanufacturing processes, from solution preparation to growing microorganisms. Therefore, it is important to measure and record the temperature accurately. Always keep in mind that “room temperature” (ambient temperature) is a figure of speech and not an actual temperature. Ideal room temperature is different for everyone!

Also note, conventional laboratory glass and mercury thermometers can be highly inaccurate. Most have an error of +/- 2°C, but some can have an error of up to +/- 4°C! Digital thermometers (LED outputs) can be just as poor in accuracy. Some labs calibrate their conventional glass thermometers with a certified thermometer, but the best way to measure temperature accurately is to use a certified validated thermometer and re-certify validated equipment before the expiration date.

Figure 6.12 Calibrated digital thermometer can be used to accurately measure temperatures. In this figure, a (yellow) calibrated digital thermometer is being used to measure the temperature of a water bath. Image credit: Jack O'Grady, Austin Community College.

DEVIATIONS & CORRECTIVE ACTIONS

After completing validation activities, the collected data is analyzed as described in the validation protocol and summarized in a report. *Successful validation demonstrates that a process is efficient and reliable.* With careful validation design, planning and implementation problems are easily avoided. However, even in the most carefully

designed facilities, unplanned occurrences do happen. These unexpected occurrences are called ***deviations***, and every company must be prepared to deal with them. Typically, the validation plan will have a form for documenting the deviation. The supervisor and the quality department will review the deviation to determine the plan of action to correct the deviation. Along with the deviation, the ***corrective action*** is also carefully documented and implemented.

SUMMARY

In a typical biotechnology company bound by GMP or ISO9000 regulations, ***Equipment validation*** is part of routine Quality Control procedures, usually performed in a committed period (weekly, daily, or monthly). The technician follows a ***Standard Operating Procedure*** (SOP) and fills out the ***associated form*** documenting they have performed the task correctly and what the outcome of the task was. If the equipment fails the validation, the quality department must be notified through a ***deviation report***. This report outlines the parameters of the equipment that did not meet the outlined specifications on the form. The equipment may need to be recalibrated or repaired, reinstalled or revalidated, or even replaced.

7 | CELL STRUCTURE AND FUNCTION

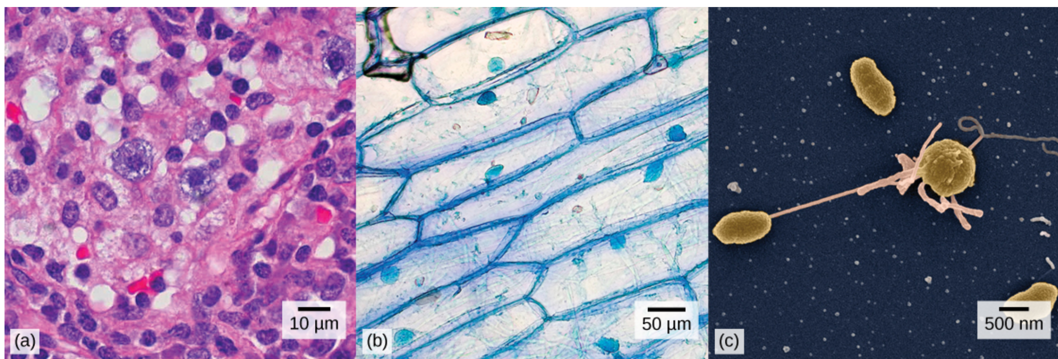


Figure 7.1 (a) Nasal sinus cells (viewed with a light microscope), (b) onion cells (viewed with a light microscope), and (c) *Vibrio tasmaniensis* bacterial cells (viewed using a scanning electron microscope) are from very different organisms, yet all share certain characteristics of basic cell structure. (credit a: modification of work by Ed Uthman, MD; credit b: modification of work by Umberto Salvagnin; credit c: modification of work by Anthony D'Onofrio; scale-bar data from Matt Russell)

Chapter Outline

7.1: Comparing Prokaryotic and Eukaryotic Cells
7.2: Eukaryotic Cells
7.3: The Cell Membrane
7.4: Passive Transport
7.5: Active Transport

Introduction

Close your eyes and picture a brick wall. What is the basic building block of that wall? It is a single brick, of course. Like a brick wall, your body is composed of basic building blocks, and the building blocks of your body are cells.

Your body has many kinds of cells, each specialized for a specific purpose. Just as a home is made from a variety of building materials, the human body is constructed from many cell types. For example, epithelial cells protect the surface of the body and cover the organs and body cavities within. Bone cells help to support and protect the body. Cells of the immune system fight invading bacteria. Additionally, red blood cells carry oxygen throughout the body. Each of these cell types plays a vital role during the growth, development, and day-to-day maintenance of the body. In spite of their enormous variety, however, all cells share certain fundamental characteristics.

7.1 | Comparing Prokaryotic and Eukaryotic Cells

By the end of this section, you will be able to:

- Name examples of prokaryotic and eukaryotic organisms
- Compare and contrast prokaryotic cells and eukaryotic cells
- Describe the relative sizes of different kinds of cells

Cells fall into one of two broad categories: prokaryotic and eukaryotic. The predominantly single-celled organisms of the domains Bacteria and Archaea are classified as prokaryotes (*pro-* = before; *-karyon-* = nucleus). Animal cells, plant cells, fungi, and protists are eukaryotes (*eu-* = true).

Components of Prokaryotic Cells

All cells share four common components: 1) a plasma membrane, an outer covering that separates the cell's interior from its surrounding environment; 2) cytoplasm, consisting of a jelly-like region within the cell in which other cellular components are found; 3) DNA, the genetic material of the cell; and 4) ribosomes, particles that synthesize proteins. However, prokaryotes differ from eukaryotic cells in several ways.

A **prokaryotic cell** is a simple, single-celled (unicellular) organism that lacks a nucleus, or any other membrane-bound organelle. We will shortly come to see that this is significantly different in eukaryotes. Prokaryotic DNA is found in the central part of the cell: a darkened region called the nucleoid (Figure 7.2).

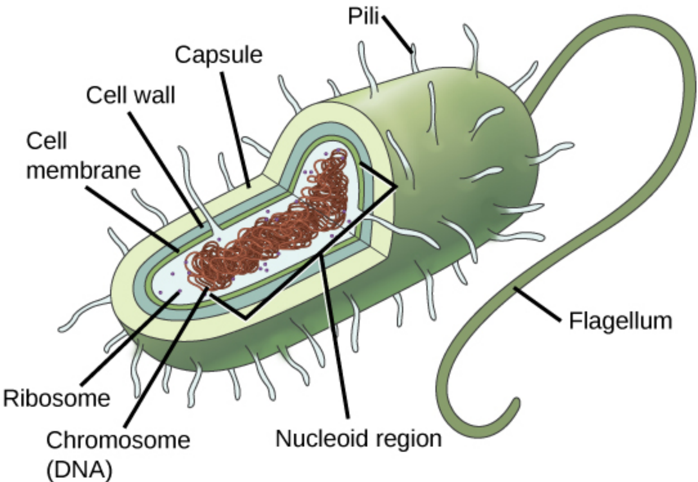


Figure 7.2 This figure shows the generalized structure of a prokaryotic cell.

Unlike Archaea and eukaryotes, bacteria have a cell wall made of peptidoglycan, comprised of sugars and amino acids, and many have a polysaccharide capsule (Figure 7.2). The cell wall acts as an extra layer of protection, helps the cell maintain its shape, and prevents dehydration. The capsule enables the cell to attach to surfaces in its environment. Some prokaryotes have flagella, pili, or fimbriae. Flagella are used for locomotion, while most pili are used to exchange genetic material during a type of reproduction called conjugation.

Eukaryotic Cells

In nature, the relationship between form and function is apparent at all levels, including the level of the cell, and this will become clear as we explore eukaryotic cells. The principle "form follows function" is found in many contexts. For example, birds and fish have streamlined bodies that allow them to move quickly through the medium in which they live, be it air or water. It means that, in general, one can deduce the function of a structure by looking at its form, because the two are matched.

A **eukaryotic cell** is a cell that has a membrane-bound nucleus and other membrane-bound compartments or sacs, called **organelles**, which have specialized functions. The word eukaryotic means "true kernel" or "true nucleus," alluding to the presence of the membrane-bound nucleus in these cells. The word "organelle" means

"little organ," and, as already mentioned, organelles have specialized cellular functions, just as the organs of your body have specialized functions.

Cell Size

At 0.1–5.0 µm in diameter, prokaryotic cells are significantly smaller than eukaryotic cells, which have diameters ranging from 10–100 µm (**Figure 7.3**). The small size of prokaryotes allows ions and organic molecules that enter them to quickly spread to other parts of the cell. Similarly, any wastes produced within a prokaryotic cell can quickly move out. However, larger eukaryotic cells have evolved different structural adaptations to enhance cellular transport. Indeed, the large size of these cells would not be possible without these adaptations. In general, cell size is limited because volume increases much more quickly than does cell surface area. As a cell becomes larger, it becomes more and more difficult for the cell to acquire sufficient materials to support the processes inside the cell, because the relative size of the surface area across which materials must be transported declines.

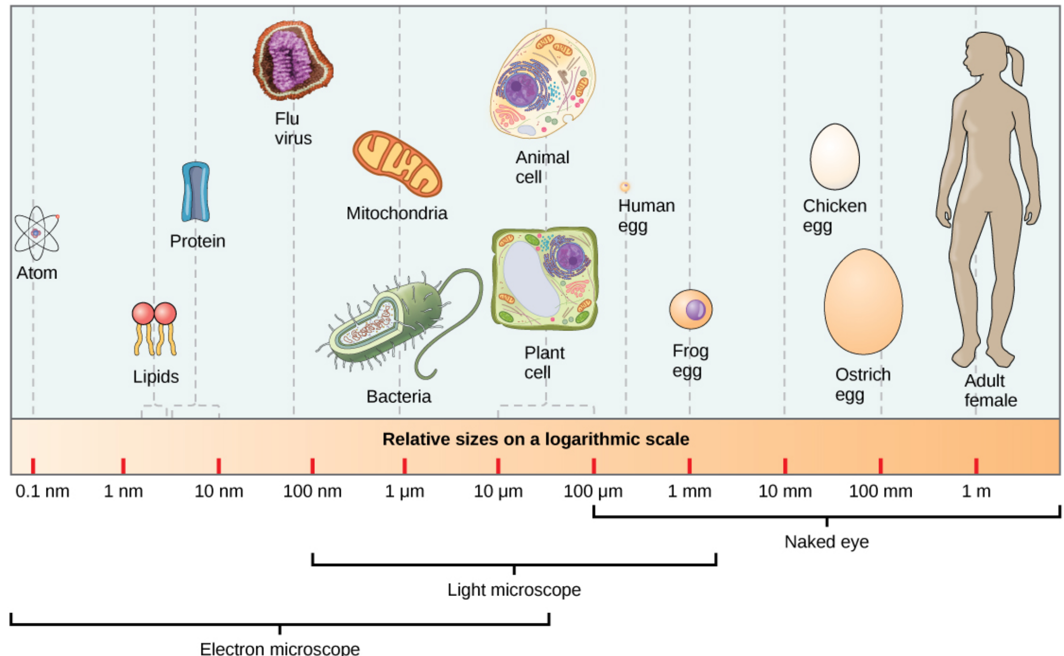


Figure 7.3 This figure shows the relative sizes of different kinds of cells and cellular components. An adult human is shown for comparison.

7.2 | Eukaryotic Cells

By the end of this section, you will be able to:

- Describe the structure of eukaryotic plant and animal cells
- State the role of the plasma membrane
- Summarize the functions of the major cell organelles
- Describe the cytoskeleton and extracellular matrix

At this point, it should be clear that eukaryotic cells have a more complex structure than do prokaryotic cells. Organelles allow for various functions to occur in the cell at the same time. Before discussing the functions of organelles within a eukaryotic cell, let us first examine two important components of the cell: the plasma membrane and the cytoplasm.

Visual Connection

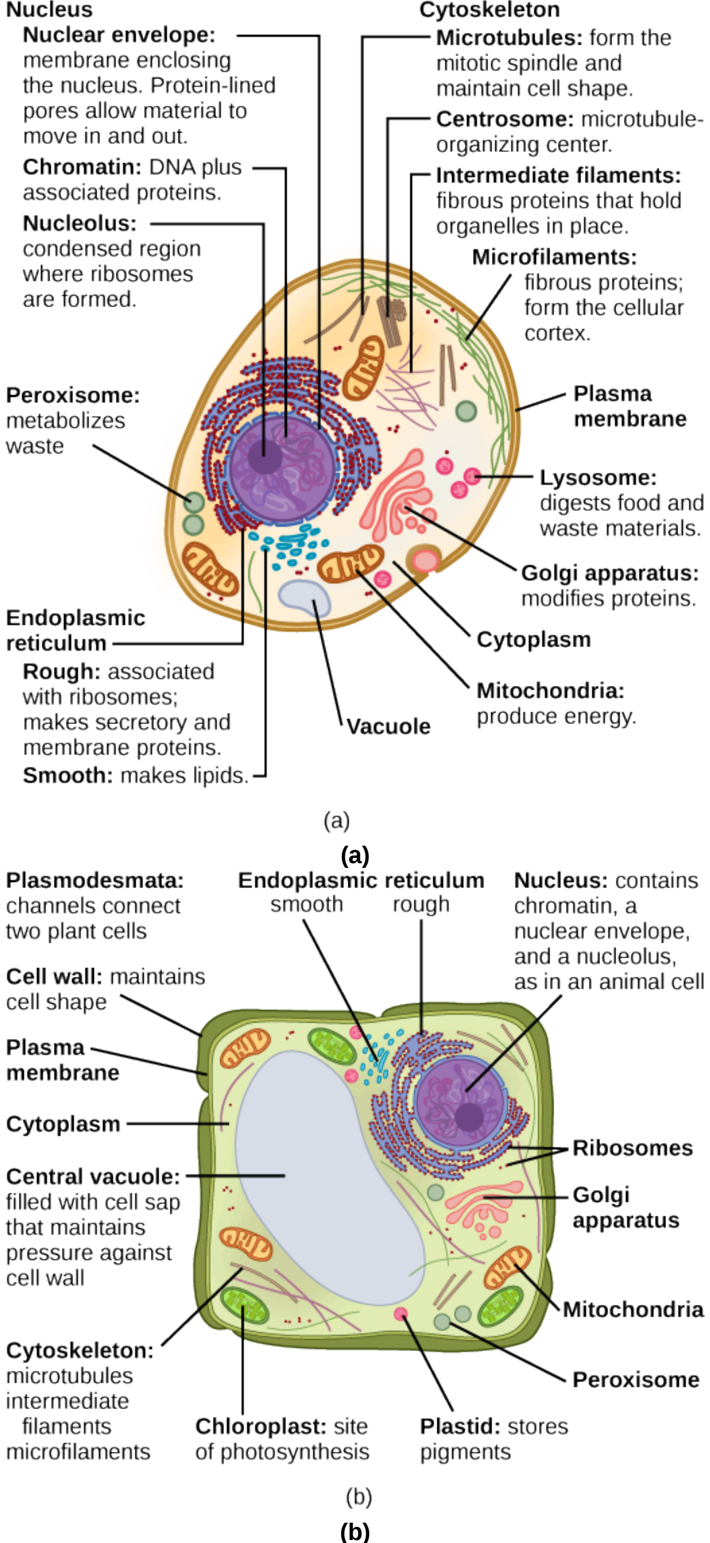


Figure 7.4 This figure shows (a) a typical animal cell and (b) a typical plant cell.

What structures does a plant cell have that an animal cell does not have? What structures does an animal cell have that a plant cell does not have?

The Plasma Membrane

Like prokaryotes, eukaryotic cells have a **plasma membrane** (Figure 7.5) made up of a phospholipid bilayer with embedded proteins that separates the internal contents of the cell from its surrounding environment. A phospholipid is a lipid molecule composed of two fatty acid chains, a glycerol backbone, and a phosphate group. The plasma membrane regulates the passage of some substances, such as organic molecules, ions, and water, preventing the passage of some to maintain internal conditions, while actively bringing in or removing others. Other compounds move passively across the membrane.

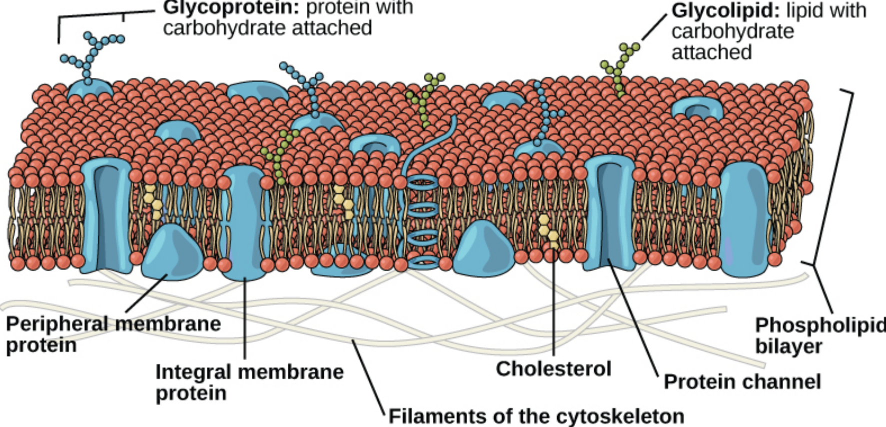


Figure 7.5 The plasma membrane is a phospholipid bilayer with embedded proteins. There are other components, such as cholesterol and carbohydrates, which can be found in the membrane in addition to phospholipids and protein.

The plasma membranes of cells that specialize in absorption are folded into fingerlike projections called microvilli (singular = microvillus). This folding increases the surface area of the plasma membrane. Such cells are typically found lining the small intestine, the organ that absorbs nutrients from digested food. This is an excellent example of form matching the function of a structure.

People with celiac disease have an immune response to gluten, which is a protein found in wheat, barley, and rye. The immune response damages microvilli, and thus, afflicted individuals cannot absorb nutrients. This leads to malnutrition, cramping, and diarrhea. Patients suffering from celiac disease must follow a gluten-free diet.

The Cytoplasm

The **cytoplasm** comprises the contents of a cell between the plasma membrane and the nuclear envelope (a structure to be discussed shortly). It is made up of organelles suspended in the gel-like **cytosol**, the cytoskeleton, and various chemicals (Figure 7.4). Even though the cytoplasm consists of 70 to 80 percent water, it has a semi-solid consistency, which comes from the proteins within it. However, proteins are not the only organic molecules found in the cytoplasm. Glucose and other simple sugars, polysaccharides, amino acids, nucleic acids, fatty acids, and derivatives of glycerol are found there too. Ions of sodium, potassium, calcium, and many other elements are also dissolved in the cytoplasm. Many metabolic reactions, including protein synthesis, take place in the cytoplasm.

The Cytoskeleton

If you were to remove all the organelles from a cell, would the plasma membrane and the cytoplasm be the only components left? No. Within the cytoplasm, there would still be ions and organic molecules, plus a network of protein fibers that helps to maintain the shape of the cell, secures certain organelles in specific positions, allows cytoplasm and vesicles to move within the cell, and enables unicellular organisms to move independently. Collectively, this network of protein fibers is known as the **cytoskeleton**. There are three types of fibers within the cytoskeleton: microfilaments, also known as actin filaments, intermediate filaments, and microtubules (Figure 7.6).

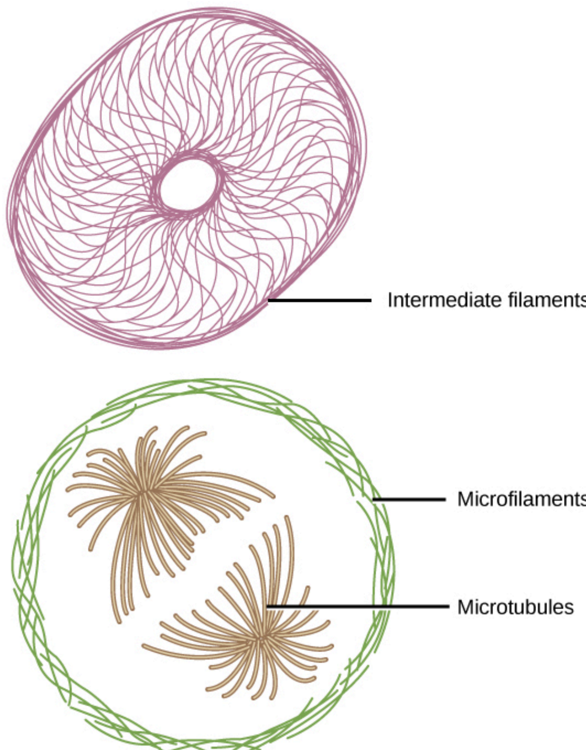


Figure 7.6 Microfilaments, intermediate filaments, and microtubules compose a cell's cytoskeleton.

Microfilaments are the thinnest of the cytoskeletal fibers and function in moving cellular components, for example, during cell division. They also maintain the structure of microvilli, the extensive folding of the plasma membrane found in cells dedicated to absorption. These components are also common in muscle cells and are responsible for muscle cell contraction. Intermediate filaments are of intermediate diameter and have structural functions, such as maintaining the shape of the cell and anchoring organelles. Keratin, the compound that strengthens hair and nails, forms one type of intermediate filament. Microtubules are the thickest of the cytoskeletal fibers. These are hollow tubes that can dissolve and reform quickly. Microtubules guide organelle movement and are the structures that pull chromosomes to their poles during cell division. They are also the structural components of flagella and cilia. In cilia and flagella, the microtubules are organized as a circle of nine double microtubules on the outside and two microtubules in the center.

The centrosome is a region near the nucleus of animal cells that functions as a microtubule-organizing center. It contains a pair of centrioles, two structures that lie perpendicular to each other. Each centriole is a cylinder of nine triplets of microtubules.

The centrosome replicates itself before a cell divides, and the centrioles play a role in pulling the duplicated chromosomes to opposite ends of the dividing cell. However, the exact function of the centrioles in cell division is not clear, since cells that have the centrioles removed can still divide, and plant cells, which lack centrioles, are capable of cell division.

Flagella and Cilia

Flagella (singular = flagellum) are long, hair-like structures that extend from the plasma membrane and are used to move an entire cell, (for example, sperm, *Euglena*). When present, the cell has just one flagellum or a few flagella. When **cilia** (singular = cilium) are present, however, they are many in number and extend along the entire surface of the plasma membrane. They are short, hair-like structures that are used to move entire cells (such as paramecium) or move substances along the outer surface of the cell (for example, the cilia of cells lining the fallopian tubes that move the ovum toward the uterus, or cilia lining the cells of the respiratory tract that move particulate matter toward the throat that mucus has trapped).

The Endomembrane System

The **endomembrane system** (*endo* = within) is a group of membranes and organelles (**Figure 7.10**) in eukaryotic cells that work together to modify, package, and transport lipids and proteins. It includes the nuclear

envelope, lysosomes, and vesicles, the endoplasmic reticulum and Golgi apparatus, which we will cover shortly. Although not technically *within* the cell, the plasma membrane is included in the endomembrane system because, as you will see, it interacts with the other endomembranous organelles.

The Nucleus

Typically, the nucleus is the most prominent organelle in a cell (**Figure 7.4**). The **nucleus** (plural = nuclei) houses the cell's DNA in the form of chromatin and directs the synthesis of ribosomes and proteins. Let us look at it in more detail (**Figure 7.7**).

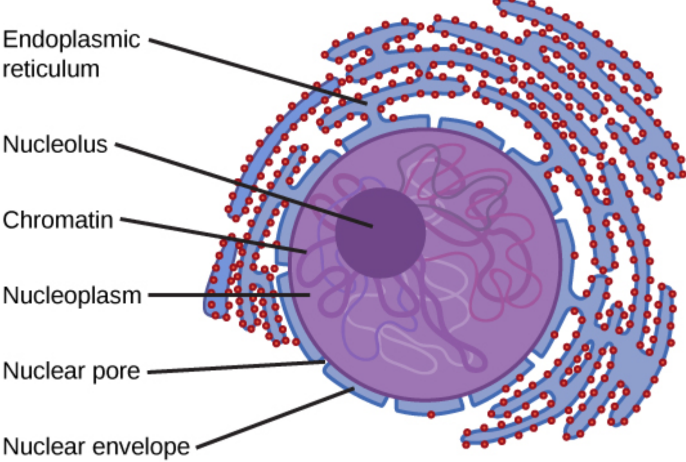


Figure 7.7 The outermost boundary of the nucleus is the nuclear envelope. Notice that the nuclear envelope consists of two phospholipid bilayers (membranes)—an outer membrane and an inner membrane—in contrast to the plasma membrane (**Figure 7.5**), which consists of only one phospholipid bilayer. (credit: modification of work by NIGMS, NIH)

The **nuclear envelope** is a double-membrane structure that constitutes the outermost portion of the nucleus (**Figure 7.7**). Both the inner and outer membranes of the nuclear envelope are phospholipid bilayers.

The nuclear envelope is punctuated with pores that control the passage of ions, molecules, and RNA between the nucleoplasm and the cytoplasm.

To understand chromatin, it is helpful to first consider chromosomes. Chromosomes are structures within the nucleus that are made up of DNA, the hereditary material, and proteins. This combination of DNA and proteins is called chromatin. In eukaryotes, chromosomes are linear structures. Every species has a specific number of chromosomes in the nucleus of its body cells. For example, in humans, the chromosome number is 46, whereas in fruit flies, the chromosome number is eight.

Chromosomes are only visible and distinguishable from one another when the cell is getting ready to divide. When the cell is in the growth and maintenance phases of its life cycle, the chromosomes resemble an unwound, jumbled bunch of threads.

We already know that the nucleus directs the synthesis of ribosomes, but how does it do this? Some chromosomes have sections of DNA that encode ribosomal RNA. A darkly staining area within the nucleus, called the **nucleolus** (plural = nucleoli), aggregates the ribosomal RNA with associated proteins to assemble the ribosomal subunits that are then transported through the nuclear pores into the cytoplasm.

The Endoplasmic Reticulum

The **endoplasmic reticulum (ER)** (**Figure 7.10**) is a series of interconnected membranous tubules that collectively modify proteins and synthesize lipids. However, these two functions are performed in separate areas of the endoplasmic reticulum: the rough endoplasmic reticulum and the smooth endoplasmic reticulum, respectively.

The hollow portion of the ER tubules is called the lumen or cisternal space. The membrane of the ER, which is a phospholipid bilayer embedded with proteins, is continuous with the nuclear envelope.

The **rough endoplasmic reticulum (RER)** is so named because the ribosomes attached to its cytoplasmic surface give it a studded appearance when viewed through an electron microscope.

The ribosomes synthesize proteins while attached to the ER, resulting in transfer of their newly synthesized proteins into the lumen of the RER where they undergo modifications such as folding or addition of sugars. The

RER also makes phospholipids for cell membranes.

If the phospholipids or modified proteins are not destined to stay in the RER, they will be packaged within vesicles and transported from the RER by budding from the membrane (**Figure 7.10**). Since the RER is engaged in modifying proteins that will be secreted from the cell, it is abundant in cells that secrete proteins, such as the liver.

The **smooth endoplasmic reticulum (SER)** is continuous with the RER but has few or no ribosomes on its cytoplasmic surface (see **Figure 7.4**). The SER's functions include synthesis of carbohydrates, lipids (including phospholipids), and steroid hormones; detoxification of medications and poisons; alcohol metabolism; and storage of calcium ions.

The Golgi Apparatus

We have already mentioned that vesicles can bud from the ER, but where do the vesicles go? Before reaching their final destination, the lipids or proteins within the transport vesicles need to be sorted, packaged, and tagged so that they wind up in the right place. The sorting, tagging, packaging, and distribution of lipids and proteins take place in the **Golgi apparatus** (also called the Golgi body), a series of flattened membranous sacs (**Figure 7.8**).

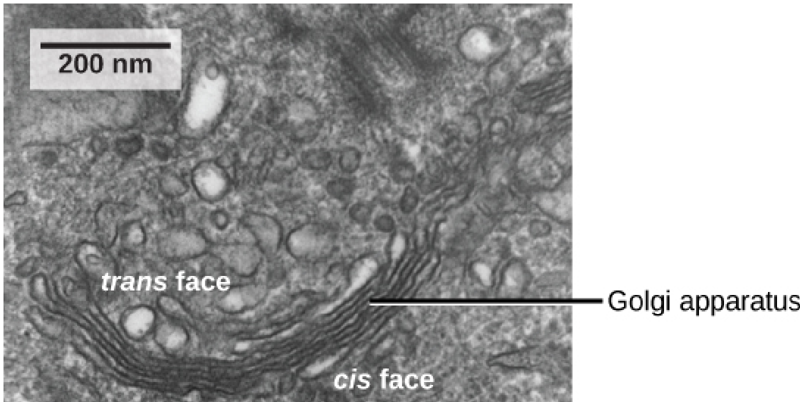


Figure 7.8 The Golgi apparatus in this transmission electron micrograph of a white blood cell is visible as a stack of semicircular flattened rings in the lower portion of this image. Several vesicles can be seen near the Golgi apparatus. (credit: modification of work by Louisa Howard; scale-bar data from Matt Russell)

The Golgi apparatus has a receiving face near the endoplasmic reticulum and a releasing face on the side away from the ER, toward the cell membrane. The transport vesicles that form from the ER travel to the receiving face, fuse with it, and empty their contents into the lumen of the Golgi apparatus. As the proteins and lipids travel through the Golgi, they undergo further modifications. The most frequent modification is the addition of short chains of sugar molecules. The newly modified proteins and lipids are then tagged with small molecular groups to enable them to be routed to their proper destinations.

Finally, the modified and tagged proteins are packaged into vesicles that bud from the opposite face of the Golgi. While some of these vesicles, transport vesicles, deposit their contents into other parts of the cell where they will be used, others, secretory vesicles, fuse with the plasma membrane and release their contents outside the cell.

The amount of Golgi in different cell types again illustrates that form follows function within cells. Cells that engage in a great deal of secretory activity (such as cells of the salivary glands that secrete digestive enzymes or cells of the immune system that secrete antibodies) have an abundant number of Golgi.

In plant cells, the Golgi has an additional role of synthesizing polysaccharides, some of which are incorporated into the cell wall and some of which are used in other parts of the cell.

Lysosomes

In animal cells, the **lysosomes** are the cell's "garbage disposal." Digestive enzymes within the lysosomes aid the breakdown of proteins, polysaccharides, lipids, nucleic acids, and even worn-out organelles. In single-celled eukaryotes, lysosomes are important for digestion of the food they ingest and the recycling of organelles. These enzymes are active at a much lower pH (more acidic) than those located in the cytoplasm. Many reactions that take place in the cytoplasm could not occur at a low pH, thus the advantage of compartmentalizing the eukaryotic cell into organelles is apparent.

Lysosomes also use their hydrolytic enzymes to destroy disease-causing organisms that might enter the cell. A

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good example of this occurs in a group of white blood cells called macrophages, which are part of your body's immune system. In a process known as phagocytosis, a section of the plasma membrane of the macrophage invaginates (folds in) and engulfs a pathogen. The invaginated section, with the pathogen inside, then pinches itself off from the plasma membrane and becomes a vesicle. The vesicle fuses with a lysosome. The lysosome's hydrolytic enzymes then destroy the pathogen (Figure 7.9).

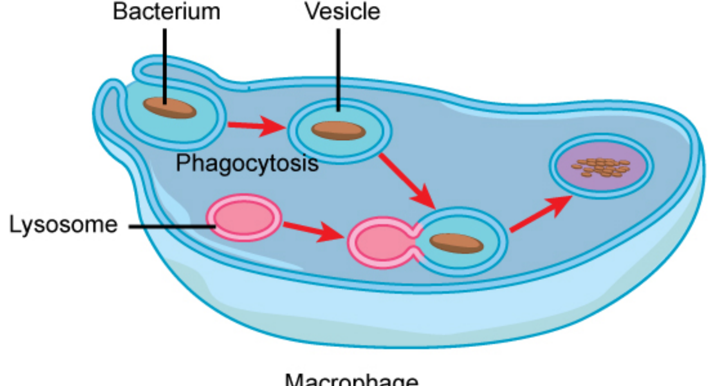

Figure 7.9 A macrophage has phagocytized a potentially pathogenic bacterium into a vesicle, which then fuses with a lysosome within the cell so that the pathogen can be destroyed. Other organelles are present in the cell, but for simplicity, are not shown.

Vesicles and Vacuoles

Vesicles and **vacuoles** are membrane-bound sacs that function in storage and transport. Vacuoles are somewhat larger than vesicles, and the membrane of a vacuole does not fuse with the membranes of other cellular components. Vesicles can fuse with other membranes within the cell system. Additionally, enzymes within plant vacuoles can break down macromolecules.

Visual Connection

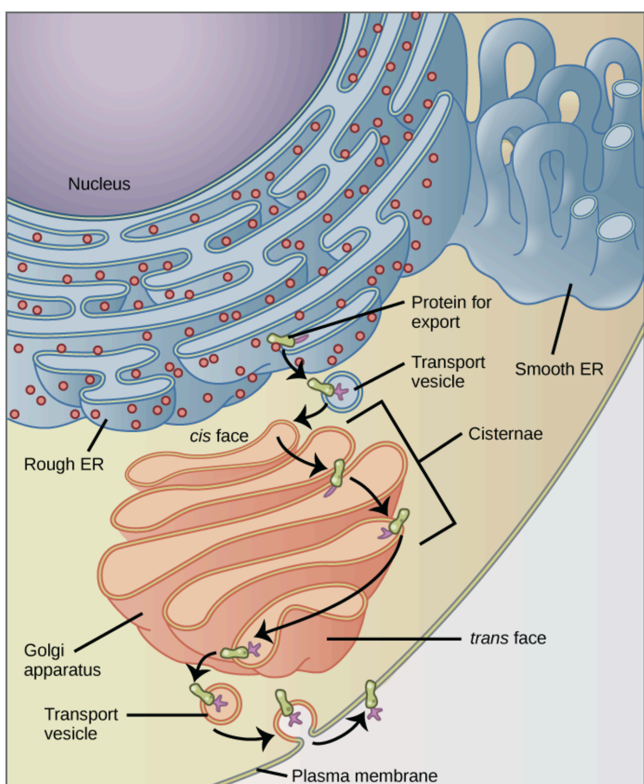

Figure 7.10 The endomembrane system works to modify, package, and transport lipids and proteins. (credit: modification of work by Magnus Manske)

Why does the *cis* face of the Golgi not face the plasma membrane?

Ribosomes

Ribosomes are the cellular structures responsible for protein synthesis. When viewed through an electron microscope, free ribosomes appear as either clusters or single tiny dots floating freely in the cytoplasm. Ribosomes may be attached to either the cytoplasmic side of the plasma membrane or the cytoplasmic side of the endoplasmic reticulum (Figure 7.4). Electron microscopy has shown that ribosomes consist of large and small subunits. Ribosomes are enzyme complexes that are responsible for protein synthesis.

Because protein synthesis is essential for all cells, ribosomes are found in practically every cell, although they are smaller in prokaryotic cells. They are particularly abundant in immature red blood cells for the synthesis of hemoglobin, which functions in the transport of oxygen throughout the body.

Mitochondria

Mitochondria (singular = mitochondrion) are often called the “powerhouses” or “energy factories” of a cell because they are responsible for making adenosine triphosphate (ATP), the cell’s main energy-carrying molecule. The formation of ATP from the breakdown of glucose is known as cellular respiration. Mitochondria are oval-shaped, double-membrane organelles (Figure 7.11) that have their own ribosomes and DNA. Each membrane is a phospholipid bilayer embedded with proteins. The inner layer has folds called cristae, which increase the surface area of the inner membrane. The area surrounded by the folds is called the mitochondrial matrix. The cristae and the matrix have different roles in cellular respiration.

In keeping with our theme of form following function, it is important to point out that muscle cells have a very high concentration of mitochondria because muscle cells need a lot of energy to contract.

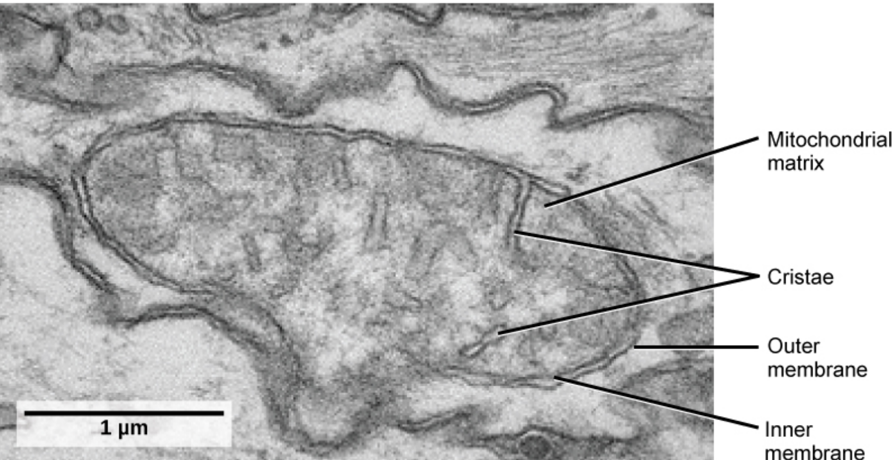

Figure 7.11 This transmission electron micrograph shows a mitochondrion as viewed with an electron microscope. Notice the inner and outer membranes, the cristae, and the mitochondrial matrix. (credit: modification of work by Matthew Britton; scale-bar data from Matt Russell)

Peroxisomes

Peroxisomes are small, round organelles enclosed by single membranes. They carry out oxidation reactions that break down fatty acids and amino acids. They also detoxify many poisons that may enter the body. Alcohol is detoxified by peroxisomes in liver cells. A byproduct of these oxidation reactions is hydrogen peroxide, H_2O_2, which is contained within the peroxisomes to prevent the chemical from causing damage to cellular components outside of the organelle. Hydrogen peroxide is safely broken down by peroxisomal enzymes into water and oxygen.

Animal Cells versus Plant Cells

Despite their fundamental similarities, there are some striking differences between animal and plant cells (see Table 7.1). Animal cells have centrioles, centrosomes (discussed under the cytoskeleton), and lysosomes, whereas plant cells do not. Plant cells have a cell wall, chloroplasts, plasmodesmata, and plastids used for

storage, and a large central vacuole, whereas animal cells do not.

The Cell Wall

In Figure 7.4**b**, the diagram of a plant cell, you see a structure external to the plasma membrane called the cell wall. The **cell wall** is a rigid covering that protects the cell, provides structural support, and gives shape to the cell. Fungal and protist cells also have cell walls.

While the chief component of prokaryotic cell walls is peptidoglycan, the major organic molecule in the plant cell wall is cellulose, a polysaccharide made up of long, straight chains of glucose units. When nutritional information refers to dietary fiber, it is referring to the cellulose content of food.

Chloroplasts

Like mitochondria, chloroplasts also have their own DNA and ribosomes. **Chloroplasts** function in photosynthesis and can be found in eukaryotic cells such as plants and algae. In photosynthesis, carbon dioxide, water, and light energy are used to make glucose and oxygen. This is the major difference between plants and animals: Plants (autotrophs) are able to make their own food, like glucose, whereas animals (heterotrophs) must rely on other organisms for their organic compounds or food source.

Like mitochondria, chloroplasts have outer and inner membranes, but within the space enclosed by a chloroplast’s inner membrane is a set of interconnected and stacked, fluid-filled membrane sacs called thylakoids (Figure 7.12). Each stack of thylakoids is called a granum (plural = grana). The fluid enclosed by the inner membrane and surrounding the grana is called the stroma.

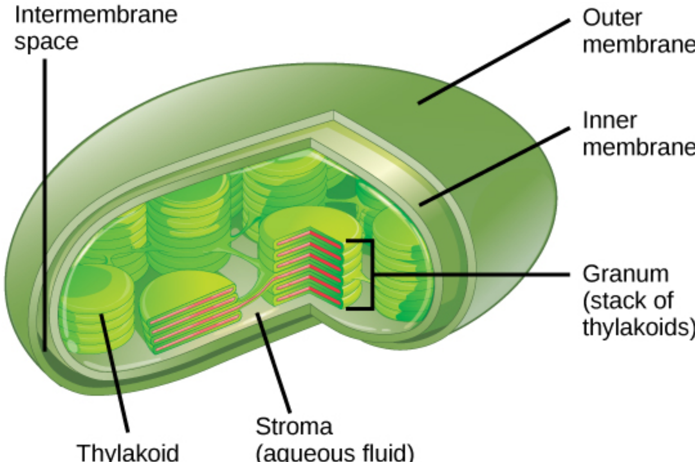

Figure 7.12 This simplified diagram of a chloroplast shows the outer membrane, inner membrane, thylakoids, grana, and stroma.

The chloroplasts contain a green pigment called chlorophyll, which captures the energy of sunlight for photosynthesis. Like plant cells, photosynthetic protists also have chloroplasts. Some bacteria also perform photosynthesis, but they do not have chloroplasts. Their photosynthetic pigments are located in the thylakoid membrane within the cell itself.

evolution IN ACTION

Endosymbiosis

We have mentioned that both mitochondria and chloroplasts contain DNA and ribosomes. Have you wondered why? Strong evidence points to endosymbiosis as the explanation.

Symbiosis is a relationship in which organisms from two separate species live in close association and typically exhibit specific adaptations to each other. Endosymbiosis (*endo*-= within) is a relationship in which one organism lives inside the other. Endosymbiotic relationships abound in nature. Microbes that produce vitamin K live inside the human gut. This relationship is beneficial for us because we are unable to synthesize vitamin K. It is also beneficial for the microbes because they are protected from other organisms and are provided a stable habitat and abundant food by living within the large intestine.

Scientists have long noticed that bacteria, mitochondria, and chloroplasts are similar in size. We also know that mitochondria and chloroplasts have DNA and ribosomes, just as bacteria do. Scientists believe that host cells and bacteria formed a mutually beneficial endosymbiotic relationship when the host cells ingested aerobic bacteria and cyanobacteria but did not destroy them. Through evolution, these ingested bacteria became more specialized in their functions, with the aerobic bacteria becoming mitochondria and the photosynthetic bacteria becoming chloroplasts.

The Central Vacuole

Previously, we mentioned vacuoles as essential components of plant cells. If you look at **Figure 7.4**, you will see that plant cells each have a large, central vacuole that occupies most of the cell. The **central vacuole** plays a key role in regulating the cell’s concentration of water in changing environmental conditions. In plant cells, the liquid inside the central vacuole provides turgor pressure, which is the outward pressure caused by the fluid inside the cell. Have you ever noticed that if you forget to water a plant for a few days, it wilts? That is because as the water concentration in the soil becomes lower than the water concentration in the plant, water moves out of the central vacuoles and cytoplasm and into the soil. As the central vacuole shrinks, it leaves the cell wall unsupported. This loss of support to the cell walls of a plant results in the wilted appearance. Additionally, this fluid has a very bitter taste, which discourages consumption by insects and animals. The central vacuole also functions to store proteins in developing seed cells.

Extracellular Matrix of Animal Cells

Most animal cells release materials into the extracellular space. The primary components of these materials are glycoproteins and the protein collagen. Collectively, these materials are called the **extracellular matrix** (**Figure 7.13**). Not only does the extracellular matrix hold the cells together to form a tissue, but it also allows the cells within the tissue to communicate with each other.

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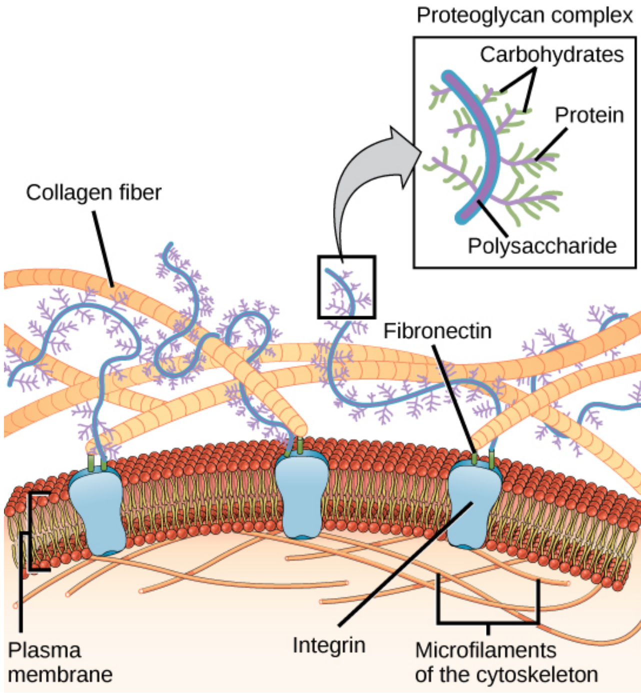


Figure 7.13 The extracellular matrix consists of a network of substances secreted by cells.

Blood clotting provides an example of the role of the extracellular matrix in cell communication. When the cells lining a blood vessel are damaged, they display a protein receptor called tissue factor. When tissue factor binds with another factor in the extracellular matrix, it causes platelets to adhere to the wall of the damaged blood vessel, stimulates adjacent smooth muscle cells in the blood vessel to contract (thus constricting the blood vessel), and initiates a series of steps that stimulate the platelets to produce clotting factors.

Intercellular Junctions

Cells can also communicate with each other by direct contact, referred to as intercellular junctions. There are some differences in the ways that plant and animal cells do this. **Plasmodesmata** (singular = plasmodesma) are junctions between plant cells, whereas animal cell contacts include tight and gap junctions, and desmosomes.

In general, long stretches of the plasma membranes of neighboring plant cells cannot touch one another because they are separated by the cell walls surrounding each cell. Plasmodesmata are numerous channels that pass between the cell walls of adjacent plant cells, connecting their cytoplasm and enabling signal molecules and nutrients to be transported from cell to cell (Figure 7.14**a**).

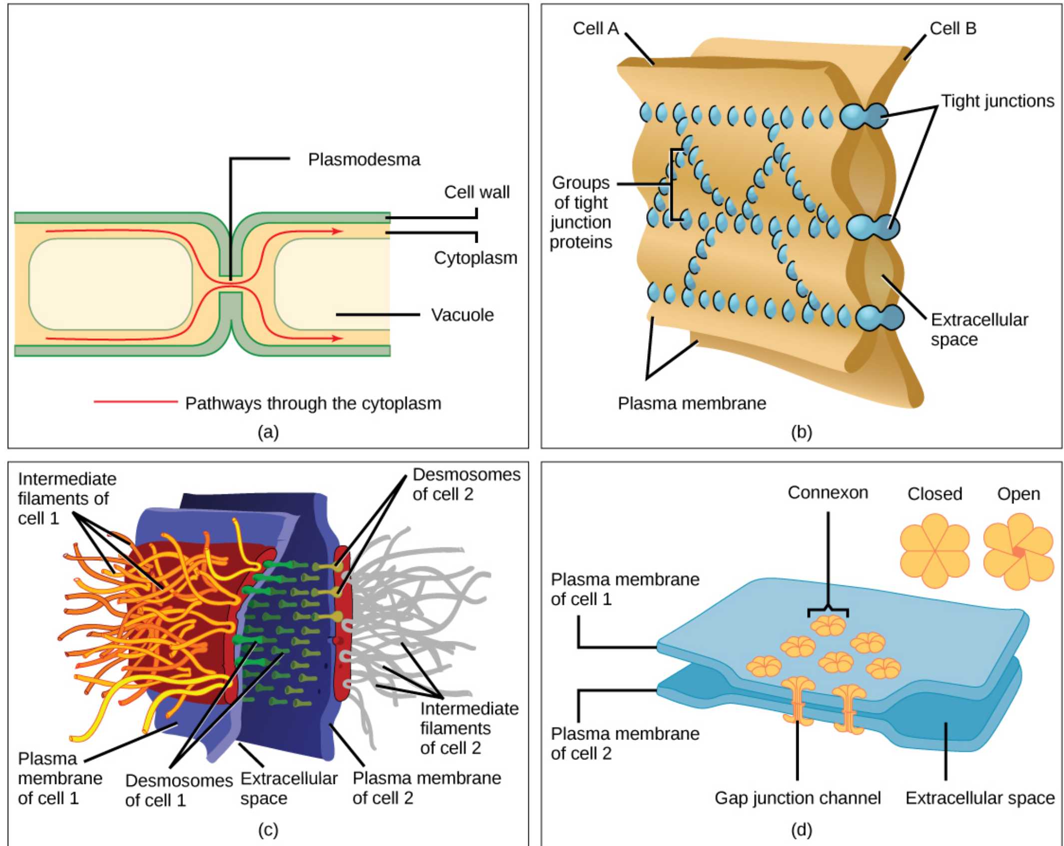


Figure 7.14 There are four kinds of connections between cells. (a) A plasmodesma is a channel between the cell walls of two adjacent plant cells. (b) Tight junctions join adjacent animal cells. (c) Desmosomes join two animal cells together. (d) Gap junctions act as channels between animal cells. (credit b, c, d: modification of work by Mariana Ruiz Villareal)

A **tight junction** is a watertight seal between two adjacent animal cells (Figure 7.14**b**). Proteins hold the cells tightly against each other. This tight adhesion prevents materials from leaking between the cells. Tight junctions are typically found in the epithelial tissue that lines internal organs and cavities, and composes most of the skin. For example, the tight junctions of the epithelial cells lining the urinary bladder prevent urine from leaking into the extracellular space.

Also found only in animal cells are **desmosomes**, which act like spot welds between adjacent epithelial cells (Figure 7.14**c**). They keep cells together in a sheet-like formation in organs and tissues that stretch, like the skin, heart, and muscles.

Gap junctions in animal cells are like plasmodesmata in plant cells in that they are channels between adjacent cells that allow for the transport of ions, nutrients, and other substances that enable cells to communicate (Figure 7.14**d**). Structurally, however, gap junctions and plasmodesmata differ.

Components of Prokaryotic and Eukaryotic Cells and Their Functions

Cell Component	Function	Present in Prokaryotes?	Present in Animal Cells?	Present in Plant Cells?
Plasma membrane	Separates cell from external environment; controls passage of organic molecules, ions, water, oxygen, and wastes into and out of the cell	Yes	Yes	Yes
Cytoplasm	Provides structure to cell; site of many metabolic reactions; medium in which organelles are found	Yes	Yes	Yes
Nucleoid	Location of DNA	Yes	No	No
Nucleus	Cell organelle that houses DNA and directs synthesis of ribosomes and proteins	No	Yes	Yes
Ribosomes	Protein synthesis	Yes	Yes	Yes
Mitochondria	ATP production/cellular respiration	No	Yes	Yes
Peroxisomes	Oxidizes and breaks down fatty acids and amino acids, and detoxifies poisons	No	Yes	Yes
Vesicles and vacuoles	Storage and transport; digestive function in plant cells	No	Yes	Yes
Centrosome	Unspecified role in cell division in animal cells; organizing center of microtubules in animal cells	No	Yes	No
Lysosomes	Digestion of macromolecules; recycling of worn-out organelles	No	Yes	No
Cell wall	Protection, structural support and maintenance of cell shape	Yes, primarily peptidoglycan in bacteria but not Archaea	No	Yes, primarily cellulose
Chloroplasts	Photosynthesis	No	No	Yes
Endoplasmic reticulum	Modifies proteins and synthesizes lipids	No	Yes	Yes
Golgi apparatus	Modifies, sorts, tags, packages, and distributes lipids and proteins	No	Yes	Yes
Cytoskeleton	Maintains cell's shape, secures organelles in specific positions, allows cytoplasm and vesicles to move within the cell, and enables unicellular organisms to move independently	Yes	Yes	Yes
Flagella	Cellular locomotion	Some	Some	No, except for some plant sperm.
Cilia	Cellular locomotion, movement of particles along extracellular surface of plasma membrane, and filtration	No	Some	No

Table 7.1

This table provides the components of prokaryotic and eukaryotic cells and their respective functions.

7.3 | The Cell Membrane

By the end of this section, you will be able to:

- Understand the fluid mosaic model of membranes
- Describe the functions of phospholipids, proteins, and carbohydrates in membranes

A cell's plasma membrane defines the boundary of the cell and determines the nature of its contact with the environment. Cells exclude some substances, take in others, and excrete still others, all in controlled quantities. Plasma membranes enclose the borders of cells, but rather than being a static bag, they are dynamic and constantly in flux. The plasma membrane must be sufficiently flexible to allow certain cells, such as red blood cells and white blood cells, to change shape as they pass through narrow capillaries. These are the more obvious functions of a plasma membrane. In addition, the surface of the plasma membrane carries markers that allow cells to recognize one another, which is vital as tissues and organs form during early development, and which later plays a role in the "self" versus "non-self" distinction of the immune response.

The plasma membrane also carries receptors, which are attachment sites for specific substances that interact with the cell. Each receptor is structured to bind with a specific substance. For example, surface receptors of the membrane create changes in the interior, such as changes in enzymes of metabolic pathways. These metabolic pathways might be vital for providing the cell with energy, making specific substances for the cell, or breaking down cellular waste or toxins for disposal. Receptors on the plasma membrane's exterior surface interact with hormones or neurotransmitters, and allow their messages to be transmitted into the cell. Some recognition sites are used by viruses as attachment points. Although they are highly specific, pathogens like viruses may evolve to exploit receptors to gain entry to a cell by mimicking the specific substance that the receptor is meant to bind. This specificity helps to explain why human immunodeficiency virus (HIV) or any of the five types of hepatitis viruses invade only specific cells.

Fluid Mosaic Model

In 1972, S. J. Singer and Garth L. Nicolson proposed a new model of the plasma membrane that, compared to earlier understanding, better explained both microscopic observations and the function of the plasma membrane. This was called the **fluid mosaic model**. The model has evolved somewhat over time, but still best accounts for the structure and functions of the plasma membrane as we now understand them. The fluid mosaic model describes the structure of the plasma membrane as a mosaic of components—including phospholipids, cholesterol, proteins, and carbohydrates—in which the components are able to flow and change position, while maintaining the basic integrity of the membrane. Both phospholipid molecules and embedded proteins are able to diffuse rapidly and laterally in the membrane. The fluidity of the plasma membrane is necessary for the activities of certain enzymes and transport molecules within the membrane. Plasma membranes range from 5–10 nm thick. As a comparison, human red blood cells, visible via light microscopy, are approximately 8 µm thick, or approximately 1,000 times thicker than a plasma membrane. (**Figure 7.15**)

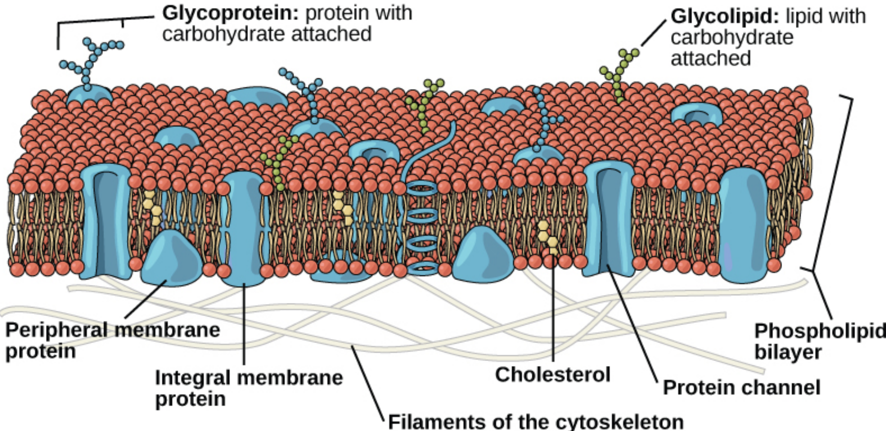


Figure 7.15 The fluid mosaic model of the plasma membrane structure describes the plasma membrane as a fluid combination of phospholipids, cholesterol, proteins, and carbohydrates.

The plasma membrane is made up primarily of a bilayer of phospholipids with embedded proteins, carbohydrates, glycolipids, and glycoproteins, and, in animal cells, cholesterol. The amount of cholesterol in animal plasma membranes regulates the fluidity of the membrane and changes based on the temperature of the cell's environment. In other words, cholesterol acts as antifreeze in the cell membrane and is more abundant in animals that live in cold climates.

The main fabric of the membrane is composed of two layers of phospholipid molecules, and the polar ends of these molecules (which look like a collection of balls in an artist's rendition of the model) (**Figure 7.15**) are in contact with aqueous fluid both inside and outside the cell. Thus, both surfaces of the plasma membrane are hydrophilic. In contrast, the interior of the membrane, between its two surfaces, is a hydrophobic or nonpolar region because of the fatty acid tails. This region has no attraction for water or other polar molecules.

Proteins make up the second major chemical component of plasma membranes. Integral proteins are embedded in the plasma membrane and may span all or part of the membrane. Integral proteins may serve as channels or pumps to move materials into or out of the cell. Peripheral proteins are found on the exterior or interior surfaces of membranes, attached either to integral proteins or to phospholipid molecules. Both integral and peripheral proteins may serve as enzymes, as structural attachments for the fibers of the cytoskeleton, or as part of the cell's recognition sites.

Carbohydrates are the third major component of plasma membranes. They are always found on the exterior surface of cells and are bound either to proteins (forming glycoproteins) or to lipids (forming glycolipids). These carbohydrate chains may consist of 2–60 monosaccharide units and may be either straight or branched. Along with peripheral proteins, carbohydrates form specialized sites on the cell surface that allow cells to recognize each other.

evolution IN ACTION

How Viruses Infect Specific Organs

Specific glycoprotein molecules exposed on the surface of the cell membranes of host cells are exploited by many viruses to infect specific organs. For example, HIV is able to penetrate the plasma membranes of specific kinds of white blood cells called T-helper cells and monocytes, as well as some cells of the central nervous system. The hepatitis virus attacks only liver cells.

These viruses are able to invade these cells, because the cells have binding sites on their surfaces that the viruses have exploited with equally specific glycoproteins in their coats. (Figure 7.16). The cell is tricked by the mimicry of the virus coat molecules, and the virus is able to enter the cell. Other recognition sites on the virus's surface interact with the human immune system, prompting the body to produce antibodies. Antibodies are made in response to the antigens (or proteins associated with invasive pathogens). These same sites serve as places for antibodies to attach, and either destroy or inhibit the activity of the virus. Unfortunately, these sites on HIV are encoded by genes that change quickly, making the production of an effective vaccine against the virus very difficult. The virus population within an infected individual quickly evolves through mutation into different populations, or variants, distinguished by differences in these recognition sites. This rapid change of viral surface markers decreases the effectiveness of the person's immune system in attacking the virus, because the antibodies will not recognize the new variations of the surface patterns.

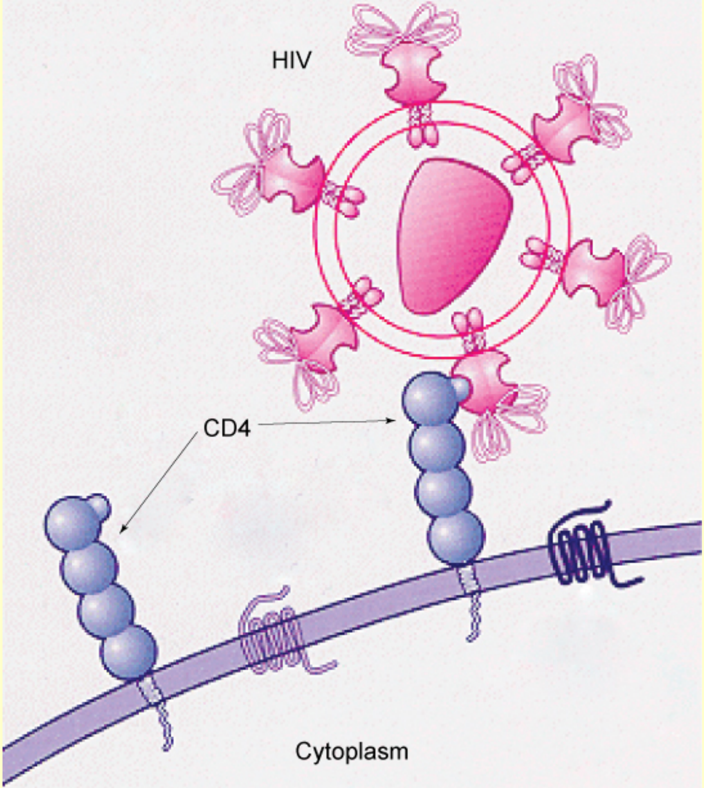


Figure 7.16 HIV docks at and binds to the CD4 receptor, a glycoprotein on the surface of T cells, before entering, or infecting, the cell. (credit: modification of work by US National Institutes of Health/National Institute of Allergy and Infectious Diseases)

7.4 | Passive Transport

By the end of this section, you will be able to:

- Explain why and how passive transport occurs
- Understand the processes of osmosis and diffusion
- Define tonicity and describe its relevance to passive transport

Plasma membranes must allow certain substances to enter and leave a cell, while preventing harmful material from entering and essential material from leaving. In other words, plasma membranes are **selectively permeable**—they allow some substances through but not others. If they were to lose this selectivity, the cell would no longer be able to sustain itself, and it would be destroyed. Some cells require larger amounts of specific substances than do other cells; they must have a way of obtaining these materials from the extracellular fluids. This may happen passively, as certain materials move back and forth, or the cell may have special mechanisms that ensure transport. Most cells expend most of their energy, in the form of adenosine triphosphate (ATP), to create and maintain an uneven distribution of ions on the opposite sides of their membranes. The structure of the plasma membrane contributes to these functions, but it also presents some problems.

The most direct forms of membrane transport are passive. **Passive transport** is a naturally occurring phenomenon and does not require the cell to expend energy to accomplish the movement. In passive transport, substances move from an area of higher concentration to an area of lower concentration in a process called diffusion. A physical space in which there is a different concentration of a single substance is said to have a **concentration gradient**.

Selective Permeability

Plasma membranes are asymmetric, meaning that despite the mirror image formed by the phospholipids, the interior of the membrane is not identical to the exterior of the membrane. Integral proteins that act as channels or pumps work in one direction. Carbohydrates, attached to lipids or proteins, are also found on the exterior surface of the plasma membrane. These carbohydrate complexes help the cell bind substances that the cell needs in the extracellular fluid. This adds considerably to the selective nature of plasma membranes.

Recall that plasma membranes have hydrophilic and hydrophobic regions. This characteristic helps the movement of certain materials through the membrane and hinders the movement of others. Lipid-soluble material can easily slip through the hydrophobic lipid core of the membrane. Substances such as the fat-soluble vitamins A, D, E, and K readily pass through the plasma membranes in the digestive tract and other tissues. Fat-soluble drugs also gain easy entry into cells and are readily transported into the body's tissues and organs. Molecules of oxygen and carbon dioxide have no charge and pass through by simple diffusion.

Polar substances present problems for the membrane. While some polar molecules connect easily with the outside of a cell, they cannot readily pass through the lipid core of the plasma membrane. Additionally, whereas small ions could easily slip through the spaces in the mosaic of the membrane, their charge prevents them from doing so. Ions such as sodium, potassium, calcium, and chloride must have a special means of penetrating plasma membranes. Simple sugars and amino acids also need help with transport across plasma membranes.

Diffusion

Diffusion is a passive process of transport. A single substance tends to move from an area of high concentration to an area of low concentration until the concentration is equal across the space. You are familiar with diffusion of substances through the air. For example, think about someone opening a bottle of perfume in a room filled with people. The perfume is at its highest concentration in the bottle and is at its lowest at the edges of the room. The perfume vapor will diffuse, or spread away, from the bottle, and gradually, more and more people will smell the perfume as it spreads. Materials move within the cell's cytosol by diffusion, and certain materials move through the plasma membrane by diffusion (Figure 7.17). Diffusion expends no energy. Rather the different concentrations of materials in different areas are a form of potential energy, and diffusion is the dissipation of that potential energy as materials move down their concentration gradients, from high to low.

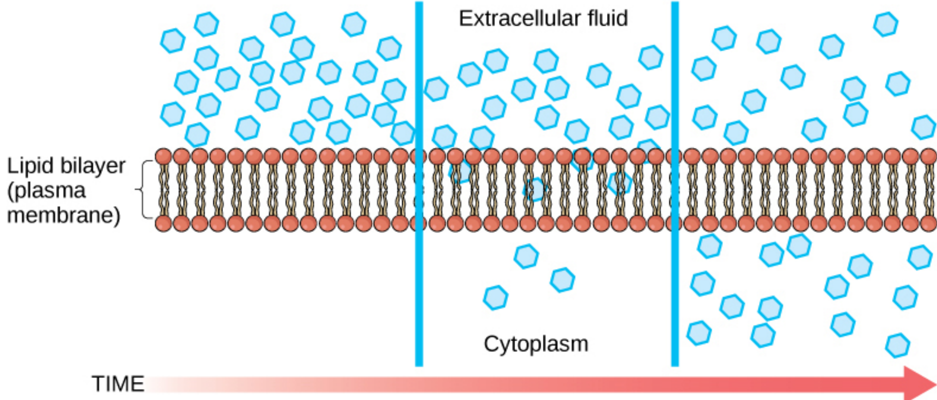


Figure 7.17 Diffusion through a permeable membrane follows the concentration gradient of a substance, moving the substance from an area of high concentration to one of low concentration. (credit: modification of work by Mariana Ruiz Villarreal)

Each separate substance in a medium, such as the extracellular fluid, has its own concentration gradient, independent of the concentration gradients of other materials. Additionally, each substance will diffuse according to that gradient.

Several factors affect the rate of diffusion.

- Extent of the concentration gradient: The greater the difference in concentration, the more rapid the diffusion. The closer the distribution of the material gets to equilibrium, the slower the rate of diffusion becomes.
- Mass of the molecules diffusing: More massive molecules move more slowly, because it is more difficult for them to move between the molecules of the substance they are moving through; therefore, they diffuse more slowly.
- Temperature: Higher temperatures increase the energy and therefore the movement of the molecules, increasing the rate of diffusion.
- Solvent density: As the density of the solvent increases, the rate of diffusion decreases. The molecules slow down because they have a more difficult time getting through the denser medium.

For an animation of the diffusion process in action, view **this short video (http://openstax.org/l/passive_trnsprt)** on cell membrane transport.

Facilitated transport

In **facilitated transport**, also called facilitated diffusion, material moves across the plasma membrane with the assistance of transmembrane proteins down a concentration gradient (from high to low concentration) without the expenditure of cellular energy. However, the substances that undergo facilitated transport would otherwise not diffuse easily or quickly across the plasma membrane. The solution to moving polar substances and other substances across the plasma membrane rests in the proteins that span its surface. The material being transported is first attached to protein or glycoprotein receptors on the exterior surface of the plasma membrane. This allows the material that is needed by the cell to be removed from the extracellular fluid. The substances are then passed to specific integral proteins that facilitate their passage, because they form channels or pores that allow certain substances to pass through the membrane. The integral proteins involved in facilitated transport are collectively referred to as transport proteins, and they function as either channels for the material or carriers.

Osmosis

Osmosis is the movement of free water molecules through a semipermeable membrane according to the water's concentration gradient across the membrane, which is inversely proportional to the solutes' concentration. Whereas diffusion transports material across membranes and within cells, osmosis transports *only water* across a membrane and the membrane limits the diffusion of solutes in the water. Osmosis is a special case of diffusion. Water, like other substances, moves from an area of high concentration of free water molecules to one of low free water molecule concentration. Imagine a beaker with a semipermeable membrane, separating the two sides or halves (**Figure 7.18**). On both sides of the membrane, the water level is the same, but there are different concentrations on each side of a dissolved substance, or **solute**, that cannot cross the membrane. If the volume of the water is the same, but the concentrations of solute are different, then there are also different concentrations of water, the solvent, on either side of the membrane.

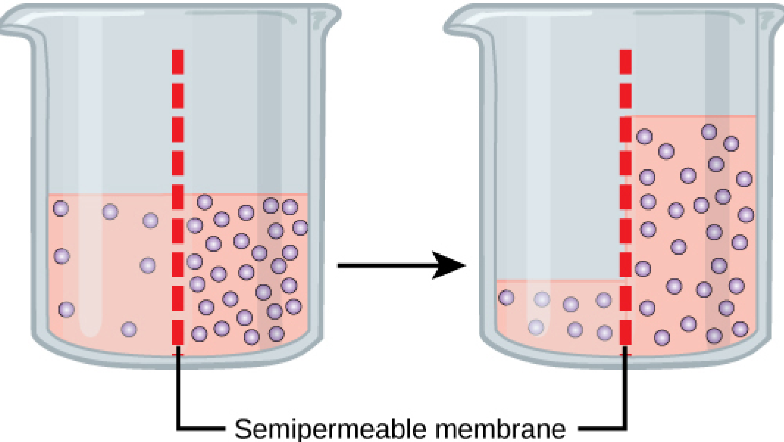


Figure 7.18 In osmosis, water always moves from an area of higher concentration (of water) to one of lower concentration (of water). In this system, the solute cannot pass through the selectively permeable membrane.

A principle of diffusion is that the molecules move around and will spread evenly throughout the medium if they can. However, only the material capable of getting through the membrane will diffuse through it. In this example, the solute cannot diffuse through the membrane, but the water can. Water has a concentration gradient in this system. Therefore, water will diffuse down its concentration gradient, crossing the membrane to the side where it is less concentrated. This diffusion of water through the membrane—osmosis—will continue until the concentration gradient of water goes to zero. Osmosis proceeds constantly in living systems.

Watch this **video (http://openstax.org/l/passive_trnsprt)** that illustrates diffusion in hot versus cold solutions.

Tonicity

Tonicity describes the amount of solute in a solution. The measure of the tonicity of a solution, or the total amount of solutes dissolved in a specific amount of solution, is called its **osmolarity**. Three terms—hypotonic, isotonic, and hypertonic—are used to relate the osmolarity of a cell to the osmolarity of the extracellular fluid that contains the cells. In a **hypotonic** solution, such as tap water, the extracellular fluid has a lower concentration of solutes than the fluid inside the cell, and water enters the cell. (In living systems, the point of reference is always the cytoplasm, so the prefix *hypo-* means that the extracellular fluid has a lower concentration of solutes, or a lower osmolarity, than the cell cytoplasm.) It also means that the extracellular fluid has a higher concentration of water than does the cell. In this situation, water will follow its concentration gradient and enter the cell. This may cause an animal cell to burst, or lyse.

In a **hypertonic** solution (the prefix *hyper-* refers to the extracellular fluid having a higher concentration of solutes

than the cell’s cytoplasm), the fluid contains less water than the cell does, such as seawater. Because the cell has a lower concentration of solutes, the water will leave the cell. In effect, the solute is drawing the water out of the cell. This may cause an animal cell to shrivel, or crenate.

In an **isotonic** solution, the extracellular fluid has the same osmolarity as the cell. If the concentration of solutes of the cell matches that of the extracellular fluid, there will be no net movement of water into or out of the cell. Blood cells in hypertonic, isotonic, and hypotonic solutions take on characteristic appearances (Figure 7.19).

Visual Connection

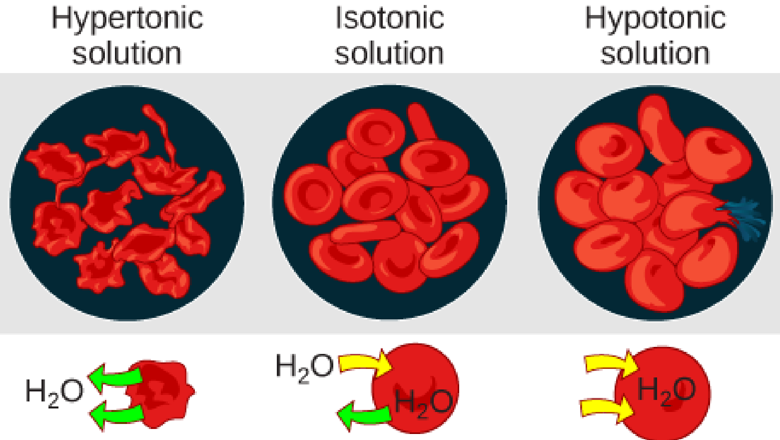


Figure 7.19 Osmotic pressure changes the shape of red blood cells in hypertonic, isotonic, and hypotonic solutions. (credit: modification of work by Mariana Ruiz Villarreal)

A doctor injects a patient with what the doctor thinks is isotonic saline solution. The patient dies, and autopsy reveals that many red blood cells have been destroyed. Do you think the solution the doctor injected was really isotonic?

Some organisms, such as plants, fungi, bacteria, and some protists, have cell walls that surround the plasma membrane and prevent cell lysis. The plasma membrane can only expand to the limit of the cell wall, so the cell will not lyse. In fact, the cytoplasm in plants is always slightly hypertonic compared to the cellular environment, and water will always enter a cell if water is available. This influx of water produces turgor pressure, which stiffens the cell walls of the plant (Figure 7.20). In nonwoody plants, turgor pressure supports the plant. If the plant cells become hypertonic, as occurs in drought or if a plant is not watered adequately, water will leave the cell. Plants lose turgor pressure in this condition and wilt.

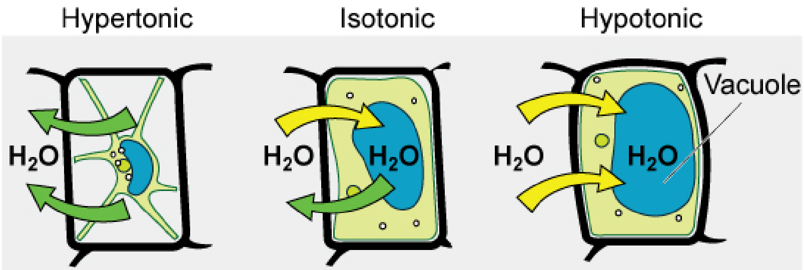


Figure 7.20 The turgor pressure within a plant cell depends on the tonicity of the solution that it is bathed in. (credit: modification of work by Mariana Ruiz Villarreal)

7.5 | Active Transport

By the end of this section, you will be able to:

- Understand how electrochemical gradients affect ions
- Describe endocytosis, including phagocytosis, pinocytosis, and receptor-mediated endocytosis
- Understand the process of exocytosis

Active transport mechanisms require the use of the cell’s energy, usually in the form of adenosine triphosphate (ATP). If a substance must move into the cell against its concentration gradient, that is, if the concentration of the substance inside the cell must be greater than its concentration in the extracellular fluid, the cell must use energy to move the substance. Some active transport mechanisms move small-molecular weight material, such as ions, through the membrane.

In addition to moving small ions and molecules through the membrane, cells also need to remove and take in larger molecules and particles. Some cells are even capable of engulfing entire unicellular microorganisms. You might have correctly hypothesized that the uptake and release of large particles by the cell requires energy. A large particle, however, cannot pass through the membrane, even with energy supplied by the cell.

Electrochemical Gradient

We have discussed simple concentration gradients—differential concentrations of a substance across a space or a membrane—but in living systems, gradients are more complex. Because cells contain proteins, most of which are negatively charged, and because ions move into and out of cells, there is an electrical gradient, a difference of charge, across the plasma membrane. The interior of living cells is electrically negative with respect to the extracellular fluid in which they are bathed; at the same time, cells have higher concentrations of potassium (K^+) and lower concentrations of sodium (Na^+) than does the extracellular fluid. Thus, in a living cell, the concentration gradient and electrical gradient of Na^+ promotes diffusion of the ion into the cell, and the electrical gradient of Na^+ (a positive ion) tends to drive it inward to the negatively charged interior. The situation is more complex, however, for other elements such as potassium. The electrical gradient of K^+ promotes diffusion of the ion *into* the cell, but the concentration gradient of K^+ promotes diffusion *out* of the cell (**Figure 7.21**). The combined gradient that affects an ion is called its **electrochemical gradient**, and it is especially important to muscle and nerve cells.

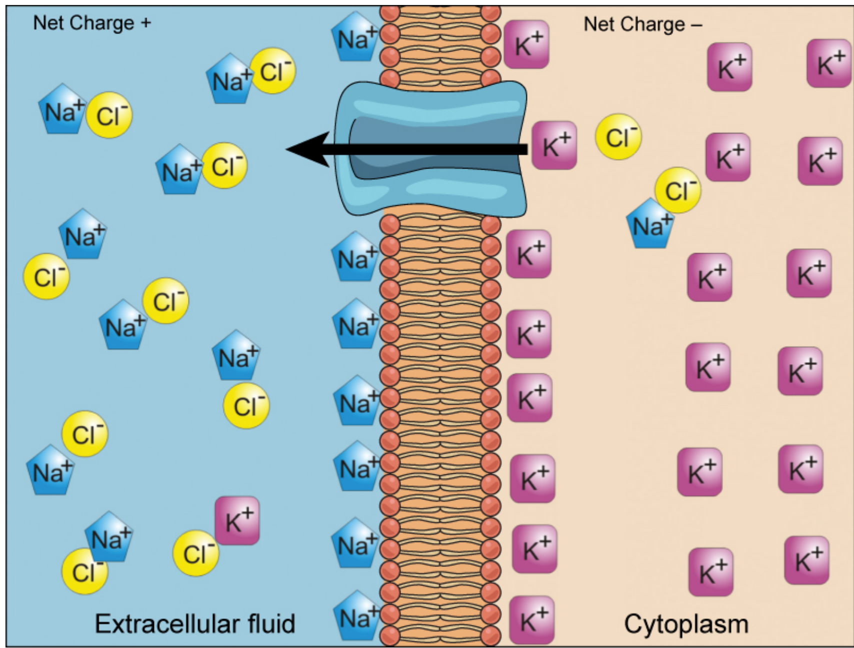


Figure 7.21 Electrochemical gradients arise from the combined effects of concentration gradients and electrical gradients. (credit: modification of work by “Synaptitude”/Wikimedia Commons)

Moving Against a Gradient

To move substances against a concentration or an electrochemical gradient, the cell must use energy. This energy is harvested from ATP that is generated through cellular metabolism. Active transport mechanisms, collectively called pumps or carrier proteins, work against electrochemical gradients. With the exception of ions, small substances constantly pass through plasma membranes. Active transport maintains concentrations of ions and other substances needed by living cells in the face of these passive changes. Much of a cell’s supply of metabolic energy may be spent maintaining these processes. Because active transport mechanisms depend on cellular metabolism for energy, they are sensitive to many metabolic poisons that interfere with the supply of ATP.

Two mechanisms exist for the transport of small-molecular weight material and macromolecules. Primary active transport moves ions across a membrane and creates a difference in charge across that membrane. The primary active transport system uses ATP to move a substance, such as an ion, into the cell, and often at the same time, a second substance is moved out of the cell. The sodium-potassium pump, an important pump in animal cells, expends energy to move potassium ions into the cell and a different number of sodium ions out of the cell (Figure 7.22). The action of this pump results in a concentration and charge difference across the membrane.

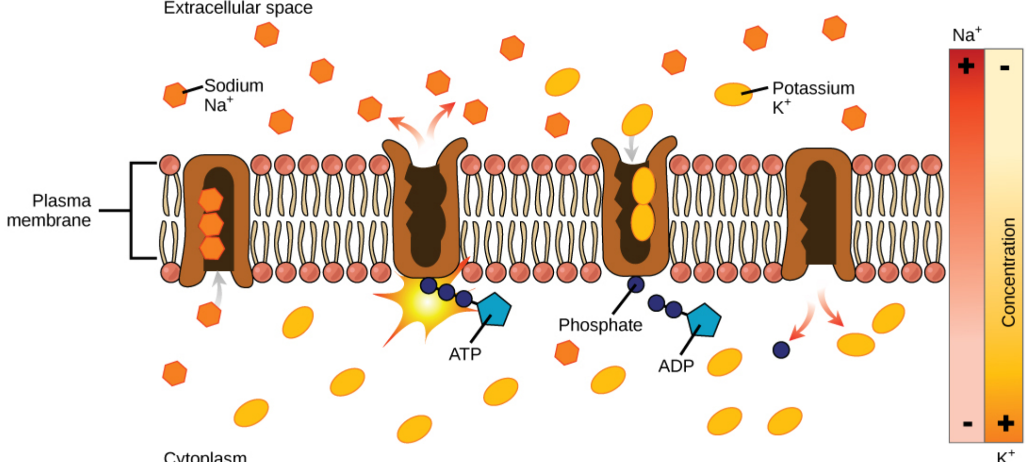


Figure 7.22 The sodium-potassium pump move potassium and sodium ions across the plasma membrane. (credit: modification of work by Mariana Ruiz Villarreal)

Secondary active transport describes the movement of material using the energy of the electrochemical gradient established by primary active transport. Using the energy of the electrochemical gradient created by the primary active transport system, other substances such as amino acids and glucose can be brought into the cell through membrane channels. ATP itself is formed through secondary active transport using a hydrogen ion gradient in the mitochondrion.

Endocytosis

Endocytosis is a type of active transport that moves particles, such as large molecules, parts of cells, and even whole cells, into a cell. There are different variations of endocytosis, but all share a common characteristic: The plasma membrane of the cell invaginates, forming a pocket around the target particle. The pocket pinches off, resulting in the particle being contained in a newly created vacuole that is formed from the plasma membrane.

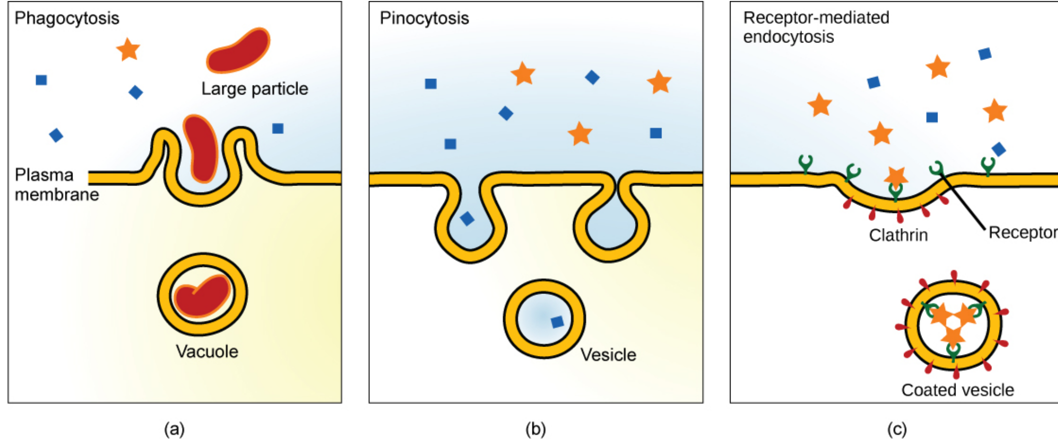


Figure 7.23 Three variations of endocytosis are shown. (a) In one form of endocytosis, phagocytosis, the cell membrane surrounds the particle and pinches off to form an intracellular vacuole. (b) In another type of endocytosis, pinocytosis, the cell membrane surrounds a small volume of fluid and pinches off, forming a vesicle. (c) In receptor-mediated endocytosis, uptake of substances by the cell is targeted to a single type of substance that binds at the receptor on the external cell membrane. (credit: modification of work by Mariana Ruiz Villarreal)

Phagocytosis is the process by which large particles, such as cells, are taken in by a cell. For example, when microorganisms invade the human body, a type of white blood cell called a neutrophil removes the invader through this process, surrounding and engulfing the microorganism, which is then destroyed by the neutrophil (Figure 7.23).

A variation of endocytosis is called **pinocytosis**. This literally means "cell drinking" and was named at a time when the assumption was that the cell was purposefully taking in extracellular fluid. In reality, this process takes in solutes that the cell needs from the extracellular fluid (Figure 7.23).

A targeted variation of endocytosis employs binding proteins in the plasma membrane that are specific for certain substances (Figure 7.23). The particles bind to the proteins and the plasma membrane invaginates, bringing the substance and the proteins into the cell. If passage across the membrane of the target of **receptor-mediated endocytosis** is ineffective, it will not be removed from the tissue fluids or blood. Instead, it will stay in those fluids and increase in concentration. Some human diseases are caused by a failure of receptor-mediated endocytosis. For example, the form of cholesterol termed low-density lipoprotein or LDL (also referred to as "bad" cholesterol) is removed from the blood by receptor-mediated endocytosis. In the human genetic disease familial hypercholesterolemia, the LDL receptors are defective or missing entirely. People with this condition have life-threatening levels of cholesterol in their blood, because their cells cannot clear the chemical from their blood.

See receptor-mediated endocytosis animation (https://www.youtube.com/watch?v=hLbjLWNA5c0) in action.

Exocytosis

In contrast to these methods of moving material into a cell is the process of exocytosis. **Exocytosis** is the opposite of the processes discussed above in that its purpose is to expel material from the cell into the extracellular fluid. A particle enveloped in membrane fuses with the interior of the plasma membrane. This fusion opens the membranous envelope to the exterior of the cell, and the particle is expelled into the extracellular space (Figure 7.24).

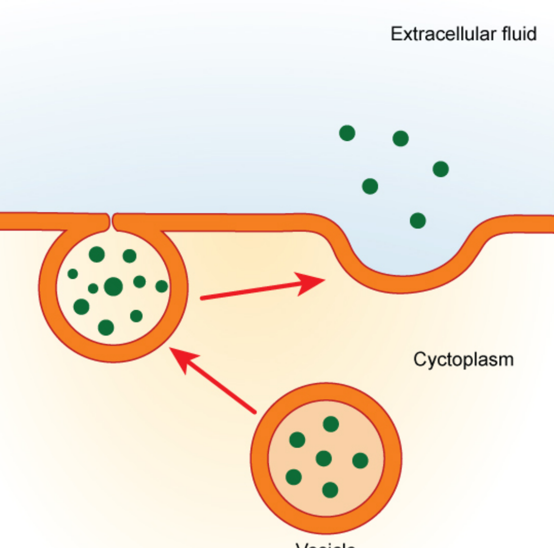


Figure 7.24 In exocytosis, a vesicle migrates to the plasma membrane, binds, and releases its contents to the outside of the cell. (credit: modification of work by Mariana Ruiz Villarreal)

KEY TERMS

active transport the method of transporting material that requires energy

cell wall a rigid cell covering made of cellulose in plants, peptidoglycan in bacteria, non-peptidoglycan compounds in Archaea, and chitin in fungi that protects the cell, provides structural support, and gives shape to the cell

central vacuole a large plant cell organelle that acts as a storage compartment, water reservoir, and site of macromolecule degradation

chloroplast a plant cell organelle that carries out photosynthesis

cilium (plural: cilia) a short, hair-like structure that extends from the plasma membrane in large numbers and is used to move an entire cell or move substances along the outer surface of the cell

concentration gradient an area of high concentration across from an area of low concentration

cytoplasm the entire region between the plasma membrane and the nuclear envelope, consisting of organelles suspended in the gel-like cytosol, the cytoskeleton, and various chemicals

cytoskeleton the network of protein fibers that collectively maintains the shape of the cell, secures some organelles in specific positions, allows cytoplasm and vesicles to move within the cell, and enables unicellular organisms to move

cytosol the gel-like material of the cytoplasm in which cell structures are suspended

desmosome a linkage between adjacent epithelial cells that forms when cadherins in the plasma membrane attach to intermediate filaments

diffusion a passive process of transport of low-molecular weight material down its concentration gradient

electrochemical gradient a gradient produced by the combined forces of the electrical gradient and the chemical gradient

endocytosis a type of active transport that moves substances, including fluids and particles, into a cell

endomembrane system the group of organelles and membranes in eukaryotic cells that work together to modify, package, and transport lipids and proteins

endoplasmic reticulum (ER) a series of interconnected membranous structures within eukaryotic cells that collectively modify proteins and synthesize lipids

eukaryotic cell a cell that has a membrane-bound nucleus and several other membrane-bound compartments or sacs

exocytosis a process of passing material out of a cell

extracellular matrix the material, primarily collagen, glycoproteins, and proteoglycans, secreted from animal cells that holds cells together as a tissue, allows cells to communicate with each other, and provides mechanical protection and anchoring for cells in the tissue

facilitated transport a process by which material moves down a concentration gradient (from high to low concentration) using integral membrane proteins

flagellum (plural: flagella) the long, hair-like structure that extends from the plasma membrane and is used to move the cell

fluid mosaic model a model of the structure of the plasma membrane as a mosaic of components, including phospholipids, cholesterol, proteins, and glycolipids, resulting in a fluid rather than static character

gap junction a channel between two adjacent animal cells that allows ions, nutrients, and other low-molecular

weight substances to pass between the cells, enabling the cells to communicate

Golgi apparatus a eukaryotic organelle made up of a series of stacked membranes that sorts, tags, and packages lipids and proteins for distribution

hypertonic describes a solution in which extracellular fluid has higher osmolarity than the fluid inside the cell

hypotonic describes a solution in which extracellular fluid has lower osmolarity than the fluid inside the cell

isotonic describes a solution in which the extracellular fluid has the same osmolarity as the fluid inside the cell

lysosome an organelle in an animal cell that functions as the cell’s digestive component; it breaks down proteins, polysaccharides, lipids, nucleic acids, and even worn-out organelles

mitochondria (singular: mitochondrion) the cellular organelles responsible for carrying out cellular respiration, resulting in the production of ATP, the cell’s main energy-carrying molecule

nuclear envelope the double-membrane structure that constitutes the outermost portion of the nucleus

nucleolus the darkly staining body within the nucleus that is responsible for assembling ribosomal subunits

nucleus the cell organelle that houses the cell’s DNA and directs the synthesis of ribosomes and proteins

organelle a membrane-bound compartment or sac within a cell

osmolarity the total amount of substances dissolved in a specific amount of solution

osmosis the transport of water through a semipermeable membrane from an area of high water concentration to an area of low water concentration across a membrane

passive transport a method of transporting material that does not require energy

peroxisome a small, round organelle that contains hydrogen peroxide, oxidizes fatty acids and amino acids, and detoxifies many poisons

phagocytosis a process that takes particulate matter like macromolecules, cells, or cell fragments that the cell needs from the extracellular fluid; a variation of endocytosis

pinocytosis a process that takes solutes that the cell needs from the extracellular fluid; a variation of endocytosis

plasma membrane a phospholipid bilayer with embedded (integral) or attached (peripheral) proteins that separates the internal contents of the cell from its surrounding environment

plasmodesma (plural: plasmodesmata) a channel that passes between the cell walls of adjacent plant cells, connects their cytoplasm, and allows materials to be transported from cell to cell

prokaryotic cell a unicellular organism that lacks a nucleus or any other membrane-bound organelle

receptor-mediated endocytosis a variant of endocytosis that involves the use of specific binding proteins in the plasma membrane for specific molecules or particles

ribosome a cellular structure that carries out protein synthesis

rough endoplasmic reticulum (RER) the region of the endoplasmic reticulum that is studded with ribosomes and engages in protein modification

selectively permeable the characteristic of a membrane that allows some substances through but not others

smooth endoplasmic reticulum (SER) the region of the endoplasmic reticulum that has few or no ribosomes on its cytoplasmic surface and synthesizes carbohydrates, lipids, and steroid hormones; detoxifies chemicals like pesticides, preservatives, medications, and environmental pollutants, and stores calcium ions

solute a substance dissolved in another to form a solution

tight junction a firm seal between two adjacent animal cells created by protein adherence

tonicity the amount of solute in a solution.

vacuole a membrane-bound sac, somewhat larger than a vesicle, that functions in cellular storage and transport

vesicle a small, membrane-bound sac that functions in cellular storage and transport; its membrane is capable of fusing with the plasma membrane and the membranes of the endoplasmic reticulum and Golgi apparatus

CHAPTER SUMMARY

7.1 Comparing Prokaryotic and Eukaryotic Cells

Prokaryotes are predominantly single-celled organisms of the domains Bacteria and Archaea. All prokaryotes have plasma membranes, cytoplasm, ribosomes, a cell wall, DNA, and lack membrane-bound organelles. Many also have polysaccharide capsules. Prokaryotic cells range in diameter from 0.1–5.0 µm.

Like a prokaryotic cell, a eukaryotic cell has a plasma membrane, cytoplasm, and ribosomes, but a eukaryotic cell is typically larger than a prokaryotic cell, has a true nucleus (meaning its DNA is surrounded by a membrane), and has other membrane-bound organelles that allow for compartmentalization of functions. Eukaryotic cells tend to be 10 to 100 times the size of prokaryotic cells.

7.2 Eukaryotic Cells

Like a prokaryotic cell, a eukaryotic cell has a plasma membrane, cytoplasm, and ribosomes, but a eukaryotic cell is typically larger than a prokaryotic cell, has a true nucleus (meaning its DNA is surrounded by a membrane), and has other membrane-bound organelles that allow for compartmentalization of functions. The plasma membrane is a phospholipid bilayer embedded with proteins. The nucleolus within the nucleus is the site for ribosome assembly. Ribosomes are found in the cytoplasm or are attached to the cytoplasmic side of the plasma membrane or endoplasmic reticulum. They perform protein synthesis. Mitochondria perform cellular respiration and produce ATP. Peroxisomes break down fatty acids, amino acids, and some toxins. Vesicles and vacuoles are storage and transport compartments. In plant cells, vacuoles also help break down macromolecules.

Animal cells also have a centrosome and lysosomes. The centrosome has two bodies, the centrioles, with an unknown role in cell division. Lysosomes are the digestive organelles of animal cells.

Plant cells have a cell wall, chloroplasts, and a central vacuole. The plant cell wall, whose primary component is cellulose, protects the cell, provides structural support, and gives shape to the cell. Photosynthesis takes place in chloroplasts. The central vacuole expands, enlarging the cell without the need to produce more cytoplasm.

The endomembrane system includes the nuclear envelope, the endoplasmic reticulum, Golgi apparatus, lysosomes, vesicles, as well as the plasma membrane. These cellular components work together to modify, package, tag, and transport membrane lipids and proteins.

The cytoskeleton has three different types of protein elements. Microfilaments provide rigidity and shape to the cell, and facilitate cellular movements. Intermediate filaments bear tension and anchor the nucleus and other organelles in place. Microtubules help the cell resist compression, serve as tracks for motor proteins that move vesicles through the cell, and pull replicated chromosomes to opposite ends of a dividing cell. They are also the structural elements of centrioles, flagella, and cilia.

Animal cells communicate through their extracellular matrices and are connected to each other by tight junctions, desmosomes, and gap junctions. Plant cells are connected and communicate with each other by plasmodesmata.

7.3 The Cell Membrane

The modern understanding of the plasma membrane is referred to as the fluid mosaic model. The plasma membrane is composed of a bilayer of phospholipids, with their hydrophobic, fatty acid tails in contact with each other. The landscape of the membrane is studded with proteins, some of which span the membrane.

Some of these proteins serve to transport materials into or out of the cell. Carbohydrates are attached to some of the proteins and lipids on the outward-facing surface of the membrane. These form complexes that function to identify the cell to other cells. The fluid nature of the membrane owes itself to the configuration of the fatty acid tails, the presence of cholesterol embedded in the membrane (in animal cells), and the mosaic nature of the proteins and protein-carbohydrate complexes, which are not firmly fixed in place. Plasma membranes enclose the borders of cells, but rather than being a static bag, they are dynamic and constantly in flux.

7.4 Passive Transport

The passive forms of transport, diffusion and osmosis, move material of small molecular weight. Substances diffuse from areas of high concentration to areas of low concentration, and this process continues until the substance is evenly distributed in a system. In solutions of more than one substance, each type of molecule diffuses according to its own concentration gradient. Many factors can affect the rate of diffusion, including concentration gradient, the sizes of the particles that are diffusing, and the temperature of the system.

In living systems, diffusion of substances into and out of cells is mediated by the plasma membrane. Some materials diffuse readily through the membrane, but others are hindered, and their passage is only made possible by protein channels and carriers. The chemistry of living things occurs in aqueous solutions, and balancing the concentrations of those solutions is an ongoing problem. In living systems, diffusion of some substances would be slow or difficult without membrane proteins.

7.5 Active Transport

The combined gradient that affects an ion includes its concentration gradient and its electrical gradient. Living cells need certain substances in concentrations greater than they exist in the extracellular space. Moving substances up their electrochemical gradients requires energy from the cell. Active transport uses energy stored in ATP to fuel the transport. Active transport of small molecular-size material uses integral proteins in the cell membrane to move the material—these proteins are analogous to pumps. Some pumps, which carry out primary active transport, couple directly with ATP to drive their action. In secondary transport, energy from primary transport can be used to move another substance into the cell and up its concentration gradient.

Endocytosis methods require the direct use of ATP to fuel the transport of large particles such as macromolecules; parts of cells or whole cells can be engulfed by other cells in a process called phagocytosis. In phagocytosis, a portion of the membrane invaginates and flows around the particle, eventually pinching off and leaving the particle wholly enclosed by an envelope of plasma membrane. Vacuoles are broken down by the cell, with the particles used as food or dispatched in some other way. Pinocytosis is a similar process on a smaller scale. The cell expels waste and other particles through the reverse process, exocytosis. Wastes are moved outside the cell, pushing a membranous vesicle to the plasma membrane, allowing the vesicle to fuse with the membrane and incorporating itself into the membrane structure, releasing its contents to the exterior of the cell.

VISUAL CONNECTION QUESTIONS

7.2 Eukaryotic Cells

1. Figure 7.4 What structures does a plant cell have that an animal cell does not have? What structures does an animal cell have that a plant cell does not have?

2. Figure 7.10 Why does the *cis* face of the Golgi not face the plasma membrane?

7.4 Passive Transport

3. Figure 7.19 A doctor injects a patient with what he thinks is isotonic saline solution. The patient dies, and autopsy reveals that many red blood cells have been destroyed. Do you think the solution the doctor injected was really isotonic?

REVIEW QUESTIONS

4. Which of these do all prokaryotes and eukaryotes share?

a. nuclear envelope
b. cell walls
c. organelles
d. plasma membrane

5. A typical prokaryotic cell __________________

compared to a eukaryotic cell.

a. is smaller in size by a factor of 100
b. is similar in size
c. is smaller in size by a factor of one million
d. is larger in size by a factor of 10

6. Which of the following is found both in eukaryotic and prokaryotic cells?

a. nucleus
b. mitochondrion
c. vacuole
d. ribosome

7. Which of the following is not a component of the endomembrane system?

a. mitochondrion
b. Golgi apparatus
c. endoplasmic reticulum
d. lysosome

8. Which plasma membrane component can be either found on its surface or embedded in the membrane structure?

a. protein
b. cholesterol
c. carbohydrate
d. phospholipid

9. The tails of the phospholipids of the plasma membrane are composed of _____ and are _______?

a. phosphate groups; hydrophobic
b. fatty acid groups; hydrophilic
c. phosphate groups; hydrophilic
d. fatty acid groups; hydrophobic

10. Water moves via osmosis _________.

a. throughout the cytoplasm
b. from an area with a high concentration of other solutes to a lower one
c. from an area with a low concentration of solutes to an area with a higher one
d. from an area with a low concentration of water to one of higher concentration

11. The principal force driving movement in diffusion is __________.

a. temperature
b. particle size
c. concentration gradient
d. membrane surface area

12. Active transport must function continuously because __________.

a. plasma membranes wear out
b. cells must be in constant motion
c. facilitated transport opposes active transport
d. diffusion is constantly moving the solutes in the other direction

CRITICAL THINKING QUESTIONS

13. Describe the structures that are characteristic of a prokaryote cell.

14. In the context of cell biology, what do we mean by form follows function? What are at least two examples of this concept?

15. Why is it advantageous for the cell membrane to be fluid in nature?

16. Why does osmosis occur?

17. Where does the cell get energy for active transport processes?

8 | MICROBES

Figure 8.1 A veterinarian gets ready to clean a sea turtle covered in oil following the Deepwater Horizon oil spill in the Gulf of Mexico in 2010. After the spill, the population of a naturally occurring oil-eating marine bacterium called *Alcanivorax borkumensis* skyrocketed, helping to get rid of the oil. Scientists are working on ways to genetically engineer this bacterium to be more efficient in cleaning up future spills. (credit: modification of work by NOAA's National Ocean Service)

Chapter Outline

8.1: What Our Ancestors Knew
8.2: A Systematic Approach
8.3: Types of Microorganisms
8.4: Beneficial Prokaryotes

Introduction

From boiling thermal hot springs to deep beneath the Antarctic ice, microorganisms can be found almost everywhere on earth in great quantities. Microorganisms (or microbes, as they are also called) are small organisms. Most are so small that they cannot be seen without a microscope.

Most microorganisms are harmless to humans and, in fact, many are helpful. They play fundamental roles in ecosystems everywhere on earth, forming the backbone of many food webs. People use them to make biofuels, medicines, and even foods. Without microbes, there would be no bread, cheese, or beer. Our bodies are filled with microbes, and our skin alone is home to trillions of them.[1] Some of them we can't live without; others cause diseases that can make us sick or even kill us.

Although much more is known today about microbial life than ever before, the vast majority of this invisible world remains unexplored. Microbiologists continue to identify new ways that microbes benefit and threaten humans.

8.1 | What Our Ancestors Knew

1. J. Hulcr et al. "A Jungle in There: Bacteria in Belly Buttons are Highly Diverse, but Predictable." *PLoS ONE* 7 no. 11 (2012): e47712. doi:10.1371/journal.pone.0047712.

Learning Objectives

By the end of this section, you will be able to:

- Describe how our ancestors improved food with the use of invisible microbes
- Describe how the causes of sickness and disease were explained in ancient times, prior to the invention of the microscope
- Describe key historical events associated with the birth of microbiology

Part 1

Cora, a 41-year-old lawyer and mother of two, has recently been experiencing severe headaches, a high fever, and a stiff neck. Her husband, who has accompanied Cora to see a doctor, reports that Cora also seems confused at times and unusually drowsy. Based on these symptoms, the doctor suspects that Cora may have meningitis, a potentially life-threatening infection of the tissue that surrounds the brain and spinal cord.

Meningitis has several potential causes. It can be brought on by bacteria, fungi, viruses, or even a reaction to medication or exposure to heavy metals. Although people with viral meningitis usually heal on their own, bacterial and fungal meningitis are quite serious and require treatment.

Cora's doctor orders a lumbar puncture (spinal tap) to take three samples of cerebrospinal fluid (CSF) from around the spinal cord (**Figure 8.2**). The samples will be sent to laboratories in three different departments for testing: clinical chemistry, microbiology, and hematology. The samples will first be visually examined to determine whether the CSF is abnormally colored or cloudy; then the CSF will be examined under a microscope to see if it contains a normal number of red and white blood cells and to check for any abnormal cell types. In the microbiology lab, the specimen will be centrifuged to concentrate any cells in a sediment; this sediment will be smeared on a slide and stained with a Gram stain. Gram staining is a procedure used to differentiate between two different types of bacteria (gram-positive and gram-negative).

About 80% of patients with bacterial meningitis will show bacteria in their CSF with a Gram stain.[2] Cora's Gram stain did not show any bacteria, but her doctor decides to prescribe her antibiotics just in case. Part of the CSF sample will be cultured—put in special dishes to see if bacteria or fungi will grow. It takes some time for most microorganisms to reproduce in sufficient quantities to be detected and analyzed.

- What types of microorganisms would be killed by antibiotic treatment?

2. Rebecca Buxton. "Examination of Gram Stains of Spinal Fluid—Bacterial Meningitis." *American Society for Microbiology*. 2007. http://www.microbelibrary.org/library/gram-stain/3065-examination-of-gram-stains-of-spinal-fluid-bacterial-meningitis

This OpenStax book is available for free at https://legacy.cnx.org/content/col26095/1.5

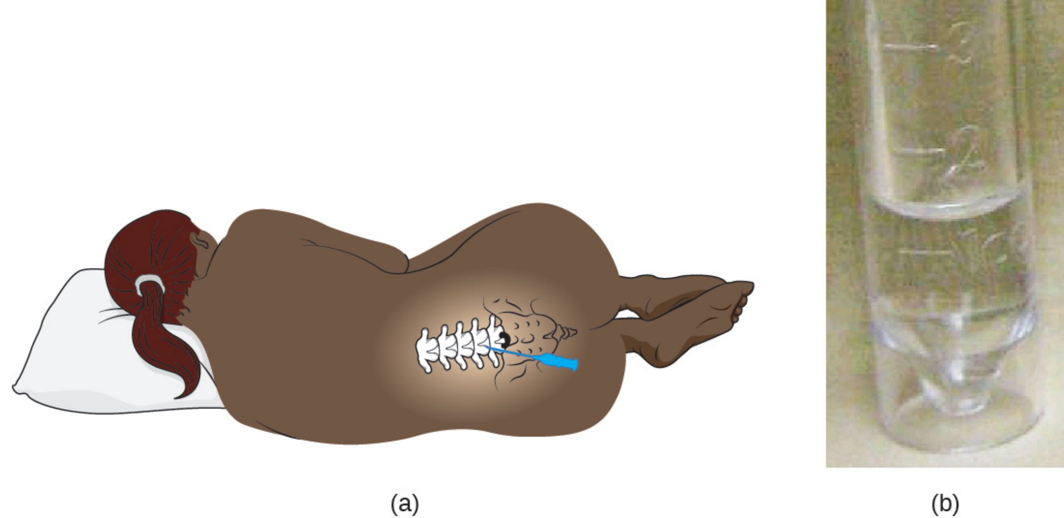


Figure 8.2 (a) A lumbar puncture is used to take a sample of a patient's cerebrospinal fluid (CSF) for testing. A needle is inserted between two vertebrae of the lower back, called the lumbar region. (b) CSF should be clear, as in this sample. Abnormally cloudy CSF may indicate an infection but must be tested further to confirm the presence of microorganisms. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by James Heilman)

*Jump to the **next** Clinical Focus box.*

Most people today, even those who know very little about microbiology, are familiar with the concept of microbes, or "germs," and their role in human health. Schoolchildren learn about bacteria, viruses, and other microorganisms, and many even view specimens under a microscope. But a few hundred years ago, before the invention of the microscope, the existence of many types of microbes was impossible to prove. By definition, **microorganisms**, or **microbes**, are very small organisms; many types of microbes are too small to see without a microscope, although some parasites and fungi are visible to the naked eye.

Humans have been living with—and using—microorganisms for much longer than they have been able to see them. Historical evidence suggests that humans have had some notion of microbial life since prehistoric times and have used that knowledge to develop foods as well as prevent and treat disease. In this section, we will explore some of the historical applications of microbiology as well as the early beginnings of microbiology as a science.

Fermented Foods and Beverages

People across the world have enjoyed fermented foods and beverages like beer, wine, bread, yogurt, cheese, and pickled vegetables for all of recorded history. Discoveries from several archeological sites suggest that even prehistoric people took advantage of fermentation to preserve and enhance the taste of food. Archaeologists studying pottery jars from a Neolithic village in China found that people were making a fermented beverage from rice, honey, and fruit as early as 7000 BC.[3]

Production of these foods and beverages requires microbial fermentation, a process that uses bacteria, mold, or yeast to convert sugars (carbohydrates) to alcohol, gases, and organic acids (Figure 8.3). While it is likely that people first learned about fermentation by accident—perhaps by drinking old milk that had curdled or old grape juice that had fermented—they later learned to harness the power of fermentation to make products like bread, cheese, and wine.

3. P.E. McGovern et al. "Fermented Beverages of Pre- and Proto-Historic China." *Proceedings of the National Academy of Sciences of the United States of America* 1 no. 51 (2004):17593–17598. doi:10.1073/pnas.0407921102.

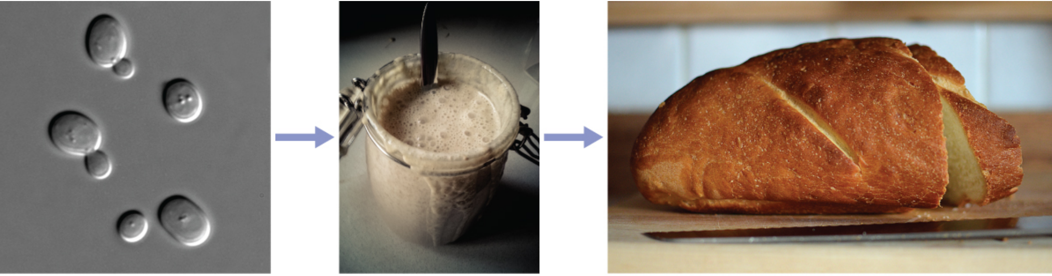


Figure 8.3 A microscopic view of *Saccharomyces cerevisiae*, the yeast responsible for making bread rise (left). Yeast is a microorganism. Its cells metabolize the carbohydrates in flour (middle) and produce carbon dioxide, which causes the bread to rise (right). (credit middle: modification of work by Janus Sandsgaard; credit right: modification of work by "MDreibelbis"/Flickr)

The Iceman Treateth

Prehistoric humans had a very limited understanding of the causes of disease, and various cultures developed different beliefs and explanations. While many believed that illness was punishment for angering the gods or was simply the result of fate, archaeological evidence suggests that prehistoric people attempted to treat illnesses and infections. One example of this is Ötzi the Iceman, a 5300-year-old mummy found frozen in the ice of the Ötzal Alps on the Austrian-Italian border in 1991. Because Ötzi was so well preserved by the ice, researchers discovered that he was infected with the eggs of the parasite *Trichuris trichiura*, which may have caused him to have abdominal pain and anemia. Researchers also found evidence of *Borrelia burgdorferi*, a bacterium that causes Lyme disease.[4] Some researchers think Ötzi may have been trying to treat his infections with the woody fruit of the *Fomitopsis betulinus* fungus, which was discovered tied to his belongings.[5] This fungus has both laxative and antibiotic properties. Ötzi was also covered in tattoos that were made by cutting incisions into his skin, filling them with herbs, and then burning the herbs.[6] There is speculation that this may have been another attempt to treat his health ailments.

Early Notions of Disease, Contagion, and Containment

Several ancient civilizations appear to have had some understanding that disease could be transmitted by things they could not see. This is especially evident in historical attempts to contain the spread of disease. For example, the Bible refers to the practice of quarantining people with leprosy and other diseases, suggesting that people understood that diseases could be communicable. Ironically, while leprosy is communicable, it is also a disease that progresses slowly. This means that people were likely quarantined after they had already spread the disease to others.

The ancient Greeks attributed disease to bad air, *mal'aria*, which they called "miasmatic odors." They developed hygiene practices that built on this idea. The Romans also believed in the miasma hypothesis and created a complex sanitation infrastructure to deal with sewage. In Rome, they built aqueducts, which brought fresh water into the city, and a giant sewer, the *Cloaca Maxima*, which carried waste away and into the river Tiber (**Figure 8.4**). Some researchers believe that this infrastructure helped protect the Romans from epidemics of waterborne illnesses.

4. A. Keller et al. "New Insights into the Tyrolean Iceman's Origin and Phenotype as Inferred by Whole-Genome Sequencing." *Nature Communications*, 3 (2012): 698. doi:10.1038/ncomms1701.
5. L. Capasso. "5300 Years Ago, the Ice Man Used Natural Laxatives and Antibiotics." *The Lancet*, 352 (1998) 9143: 1864. doi: 10.1016/s0140-6736(05)79939-6.
6. L. Capasso, L. "5300 Years Ago, the Ice Man Used Natural Laxatives and Antibiotics." *The Lancet*, 352 no. 9143 (1998): 1864. doi: 10.1016/s0140-6736(05)79939-6.

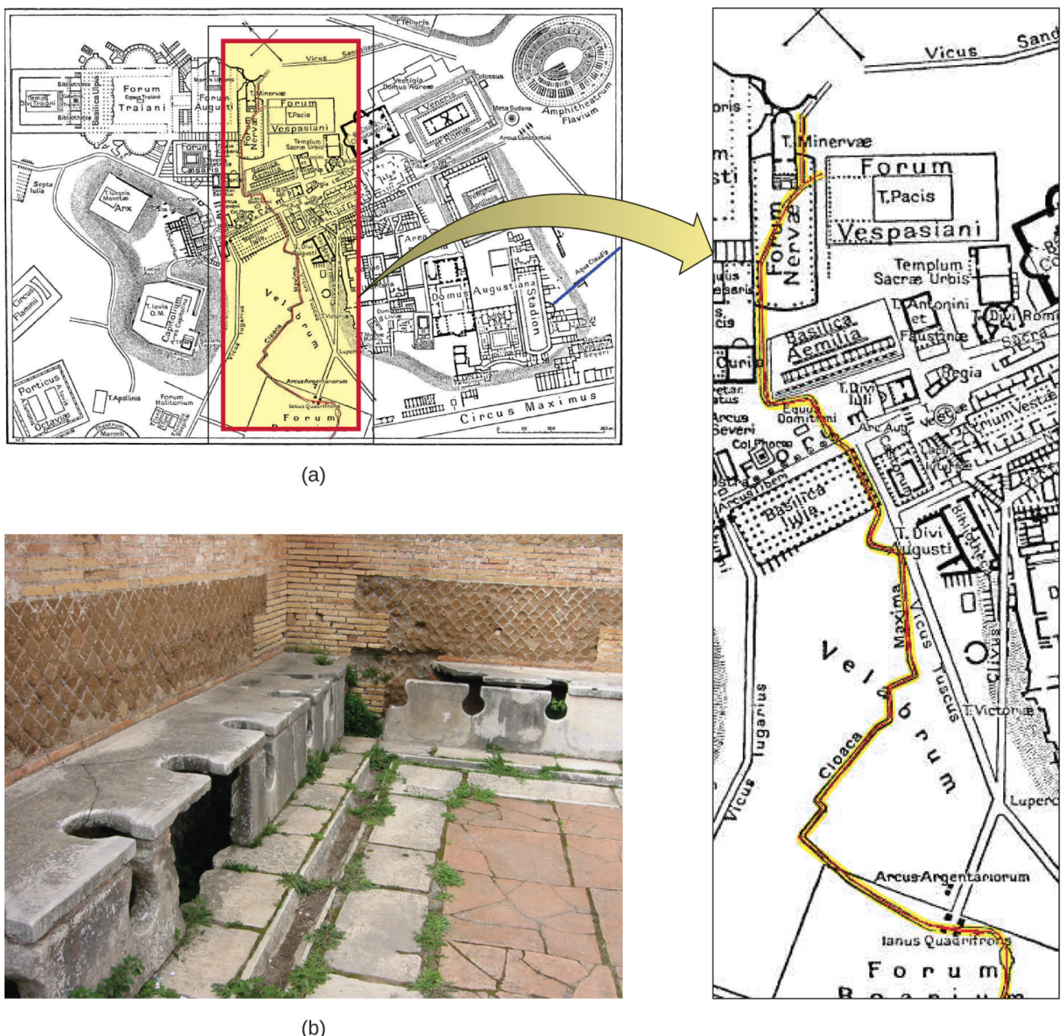


Figure 8.4 (a) The *Cloaca Maxima*, or "Greatest Sewer" (shown in red), ran through ancient Rome. It was an engineering marvel that carried waste away from the city and into the river Tiber. (b) These ancient latrines emptied into the *Cloaca Maxima*.

Even before the invention of the microscope, some doctors, philosophers, and scientists made great strides in understanding the invisible forces—what we now know as microbes—that can cause infection, disease, and death.

The Greek physician Hippocrates (460–370 BC) is considered the "father of Western medicine" (**Figure 8.5**). Unlike many of his ancestors and contemporaries, he dismissed the idea that disease was caused by supernatural forces. Instead, he posited that diseases had natural causes from within patients or their environments. Hippocrates and his heirs are believed to have written the *Hippocratic Corpus*, a collection of texts that make up some of the oldest surviving medical books.[7] Hippocrates is also often credited as the author of the Hippocratic Oath, taken by new physicians to pledge their dedication to diagnosing and treating patients without causing harm.

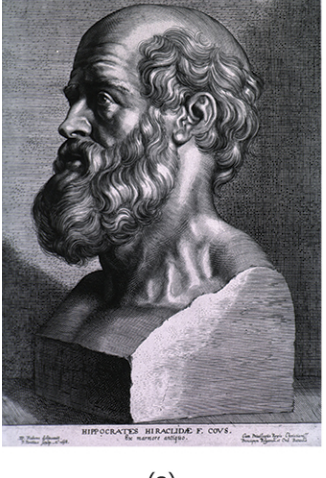
While Hippocrates is considered the father of Western medicine, the Greek philosopher and historian Thucydides (460–395 BC) is considered the father of scientific history because he advocated for evidence-based analysis of cause-and-effect reasoning (**Figure 8.5**). Among his most important contributions are his observations regarding the Athenian plague that killed one-third of the population of Athens between 430 and 410 BC. Having survived the epidemic himself, Thucydides made the important observation that survivors did not get re-infected with the disease, even when taking care of actively sick people.[8] This observation shows an

7. G. Pappas et al. "Insights Into Infectious Disease in the Era of Hippocrates." *International Journal of Infectious Diseases* 12 (2008) 4:347–350. doi: http://dx.doi.org/10.1016/j.ijid.2007.11.003.

8. Thucydides. *The History of the Peloponnesian War. The Second Book*. 431 BC. Translated by Richard Crawley. http://classics.mit.edu/Thucydides/pelopwar.2.second.html.

early understanding of the concept of immunity.

Marcus Terentius Varro (116–27 BC) was a prolific Roman writer who was one of the first people to propose the concept that things we cannot see (what we now call microorganisms) can cause disease (**Figure 8.5**). In *Res Rusticae* (*On Farming*), published in 36 BC, he said that “precautions must also be taken in neighborhood swamps
. . . because certain minute creatures [*animalia minuta*] grow there which cannot be seen by the eye, which float in the air and enter the body through the mouth and nose and there cause serious diseases.”[9]



(a) (b) (c)

Figure 8.5 (a) Hippocrates, the “father of Western medicine,” believed that diseases had natural, not supernatural, causes. (b) The historian Thucydides observed that survivors of the Athenian plague were subsequently immune to the infection. (c) Marcus Terentius Varro proposed that disease could be caused by “certain minute creatures . . . which cannot be seen by the eye.” (credit c: modification of work by Alessandro Antonelli)

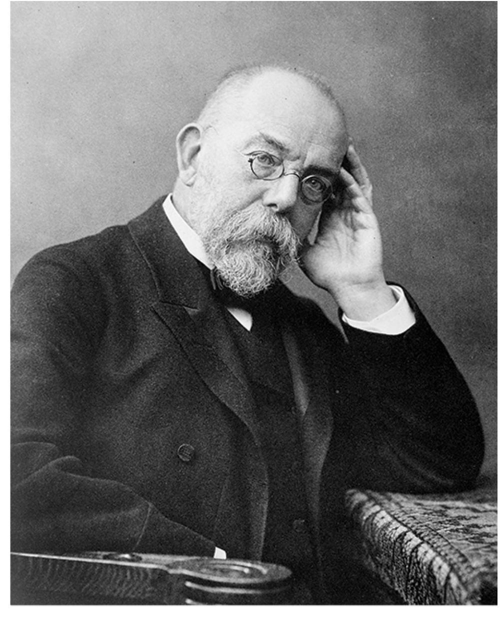
- Give two examples of foods that have historically been produced by humans with the aid of microbes.
- Explain how historical understandings of disease contributed to attempts to treat and contain disease.

The Birth of Microbiology

While the ancients may have suspected the existence of invisible “minute creatures,” it wasn’t until the invention of the microscope that their existence was definitively confirmed. While it is unclear who exactly invented the microscope, a Dutch cloth merchant named Antonie van Leeuwenhoek (1632–1723) was the first to develop a lens powerful enough to view microbes. In 1675, using a simple but powerful microscope, Leeuwenhoek was able to observe single-celled organisms, which he described as “animalcules” or “wee little beasties,” swimming in a drop of rain water. From his drawings of these little organisms, we now know he was looking at bacteria and protists. (We will explore Leeuwenhoek’s contributions to microscopy further in **How We See the Invisible World (https://legacy.cnx.org/content/m58784/latest/)** .)

Nearly 200 years after van Leeuwenhoek got his first glimpse of microbes, the “ Golden Age of Microbiology” spawned a host of new discoveries between 1857 and 1914. Two famous microbiologists, Louis Pasteur and Robert Koch, were especially active in advancing our understanding of the unseen world of microbes (**Figure 8.6**). Pasteur, a French chemist, showed that individual microbial strains had unique properties and demonstrated that fermentation is caused by microorganisms. He also invented pasteurization, a process used to kill microorganisms responsible for spoilage, and developed vaccines for the treatment of diseases, including rabies, in animals and humans. Koch, a German physician, was the first to demonstrate the connection between a single, isolated microbe and a known human disease. For example, he discovered the bacteria that cause anthrax (*Bacillus anthracis*), cholera (*Vibrio cholera*), and tuberculosis (*Mycobacterium tuberculosis*).[10] We will discuss these famous microbiologists, and others, in later chapters.

9. Plinio Prioreschi. *A History of Medicine: Roman Medicine*. Lewiston, NY: Edwin Mellen Press, 1998: p. 215.
10. S.M. Blevins and M.S. Bronze. “Robert Koch and the ‘Golden Age’ of Bacteriology.” *International Journal of Infectious Diseases*. 14 no. 9 (2010): e744-e751. doi:10.1016/j.ijid.2009.12.003.



(a) (b)

Figure 8.6 (a) Louis Pasteur (1822–1895) is credited with numerous innovations that advanced the fields of microbiology and immunology. (b) Robert Koch (1843–1910) identified the specific microbes that cause anthrax, cholera, and tuberculosis.

As microbiology has developed, it has allowed the broader discipline of biology to grow and flourish in previously unimagined ways. Much of what we know about human cells comes from our understanding of microbes, and many of the tools we use today to study cells and their genetics derive from work with microbes.

- How did the discovery of microbes change human understanding of disease?

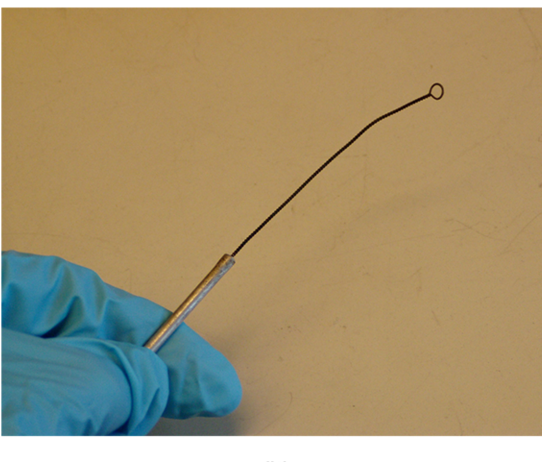
Microbiology Toolbox

Because individual microbes are generally too small to be seen with the naked eye, the science of microbiology is dependent on technology that can artificially enhance the capacity of our natural senses of perception. Early microbiologists like Pasteur and Koch had fewer tools at their disposal than are found in modern laboratories, making their discoveries and innovations that much more impressive. Later chapters of this text will explore many applications of technology in depth, but for now, here is a brief overview of some of the fundamental tools of the microbiology lab.

- **Microscopes** produce magnified images of microorganisms, human cells and tissues, and many other types of specimens too small to be observed with the naked eye.
- **Stains and dyes** are used to add color to microbes so they can be better observed under a microscope. Some dyes can be used on living microbes, whereas others require that the specimens be fixed with chemicals or heat before staining. Some stains only work on certain types of microbes because of differences in their cellular chemical composition.
- **Growth media** are used to grow microorganisms in a lab setting. Some media are liquids; others are more solid or gel-like. A growth medium provides nutrients, including water, various salts, a source of carbon (like glucose), and a source of nitrogen and amino acids (like yeast extract) so microorganisms can grow and reproduce. Ingredients in a growth medium can be modified to grow unique types of microorganisms.
- **A Petri dish** is a flat-lidded dish that is typically 10–11 centimeters (cm) in diameter and 1–1.5 cm high. Petri dishes made out of either plastic or glass are used to hold growth media (Figure 8.7).
- **Test tubes** are cylindrical plastic or glass tubes with rounded bottoms and open tops. They can be used to grow microbes in broth, or semisolid or solid growth media.
- A **Bunsen burner** is a metal apparatus that creates a flame that can be used to sterilize pieces of

equipment. A rubber tube carries gas (fuel) to the burner. In many labs, Bunsen burners are being phased out in favor of infrared **microincinerators**, which serve a similar purpose without the safety risks of an open flame.

- An **inoculation loop** is a handheld tool that ends in a small wire loop (Figure 8.7). The loop can be used to streak microorganisms on agar in a Petri dish or to transfer them from one test tube to another. Before each use, the inoculation loop must be sterilized so cultures do not become contaminated.



(a) (b)

Figure 8.7 (a) This Petri dish filled with agar has been streaked with *Legionella*, the bacterium responsible for causing Legionnaire's disease. (b) An inoculation loop like this one can be used to streak bacteria on agar in a Petri dish. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by Jeffrey M. Vinocur)

8.2 | A Systematic Approach

Learning Objectives

By the end of this section, you will be able to:

- Describe how microorganisms are classified and distinguished as unique species
- Compare historical and current systems of taxonomy used to classify microorganisms

Once microbes became visible to humans with the help of microscopes, scientists began to realize their enormous diversity. Microorganisms vary in all sorts of ways, including their size, their appearance, and their rates of reproduction. To study this incredibly diverse new array of organisms, researchers needed a way to systematically organize them.

The Science of Taxonomy

Taxonomy is the classification, description, identification, and naming of living organisms. Classification is the practice of organizing organisms into different groups based on their shared characteristics. The most famous early taxonomist was a Swedish botanist, zoologist, and physician named Carolus Linnaeus (1701–1778). In 1735, Linnaeus published *Systema Naturae*, an 11-page booklet in which he proposed the Linnaean taxonomy, a system of categorizing and naming organisms using a standard format so scientists could discuss organisms using consistent terminology. He continued to revise and add to the book, which grew into multiple volumes (Figure 8.8).

Figure 8.8 Swedish botanist, zoologist, and physician Carolus Linnaeus developed a new system for categorizing plants and animals. In this 1853 portrait by Hendrik Hollander, Linnaeus is holding a twinflower, named *Linnaea borealis* in his honor.

In his taxonomy, Linnaeus divided the natural world into three kingdoms: animal, plant, and mineral (the mineral kingdom was later abandoned). Within the animal and plant kingdoms, he grouped organisms using a hierarchy of increasingly specific levels and sublevels based on their similarities. The names of the levels in Linnaeus's original taxonomy were kingdom, class, order, family, genus (plural: genera), and species. Species was, and continues to be, the most specific and basic taxonomic unit.

Evolving Trees of Life (Phylogenies)

With advances in technology, other scientists gradually made refinements to the Linnaean system and eventually created new systems for classifying organisms. In the 1800s, there was a growing interest in developing

taxonomies that took into account the evolutionary relationships, or **phylogenies**, of all different species of organisms on earth. One way to depict these relationships is via a diagram called a phylogenetic tree (or tree of life). In these diagrams, groups of organisms are arranged by how closely related they are thought to be. In early phylogenetic trees, the relatedness of organisms was inferred by their visible similarities, such as the presence or absence of hair or the number of limbs. Now, the analysis is more complicated. Today, phylogenic analyses include genetic, biochemical, and embryological comparisons, as will be discussed later in this chapter.

Linnaeus's tree of life contained just two main branches for all living things: the animal and plant kingdoms. In 1866, Ernst Haeckel, a German biologist, philosopher, and physician, proposed another kingdom, Protista, for unicellular organisms (Figure 8.9). He later proposed a fourth kingdom, Monera, for unicellular organisms whose cells lack nuclei, like bacteria.

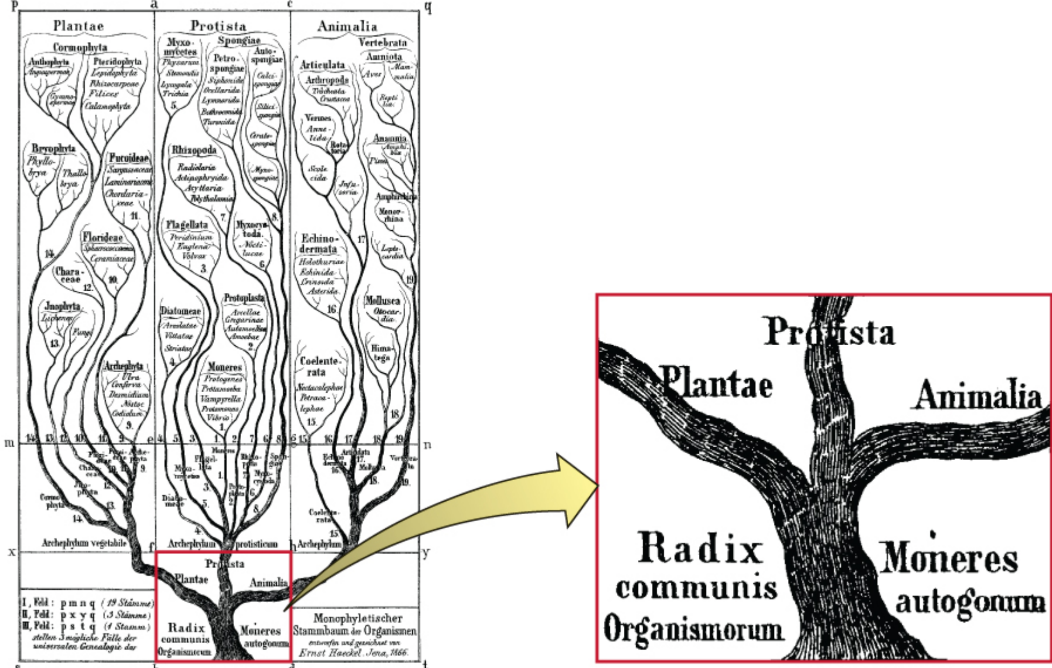


Figure 8.9 Ernst Haeckel's rendering of the tree of life, from his 1866 book *General Morphology of Organisms*, contained three kingdoms: Plantae, Protista, and Animalia. He later added a fourth kingdom, Monera, for unicellular organisms lacking a nucleus.

Nearly 100 years later, in 1969, American ecologist Robert Whittaker (1920–1980) proposed adding another kingdom—Fungi—in his tree of life. Whittaker's tree also contained a level of categorization above the kingdom level—the empire or superkingdom level—to distinguish between organisms that have membrane-bound nuclei in their cells (**eukaryotes**) and those that do not (**prokaryotes**). Empire Prokaryota contained just the Kingdom Monera. The Empire Eukaryota contained the other four kingdoms: Fungi, Protista, Plantae, and Animalia. Whittaker's five-kingdom tree was considered the standard phylogeny for many years.

Figure 8.10 shows how the tree of life has changed over time. Note that viruses are not found in any of these trees. That is because they are not made up of cells and thus it is difficult to determine where they would fit into a tree of life.

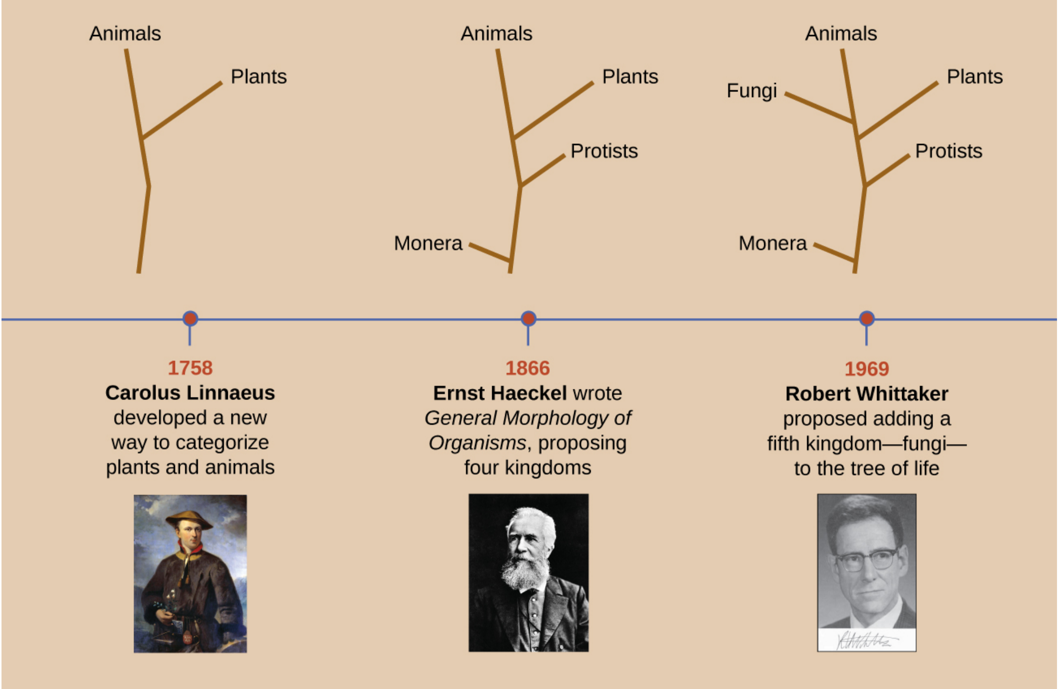


Figure 8.10 This timeline shows how the shape of the tree of life has changed over the centuries. Even today, the taxonomy of living organisms is continually being reevaluated and refined with advances in technology.

- Briefly summarize how our evolving understanding of microorganisms has contributed to changes in the way that organisms are classified.

Part 2

Antibiotic drugs are specifically designed to kill or inhibit the growth of bacteria. But after a couple of days on antibiotics, Cora shows no signs of improvement. Also, her CSF cultures came back from the lab negative. Since bacteria or fungi were not isolated from Cora's CSF sample, her doctor rules out bacterial and fungal meningitis. Viral meningitis is still a possibility.

However, Cora now reports some troubling new symptoms. She is starting to have difficulty walking. Her muscle stiffness has spread from her neck to the rest of her body, and her limbs sometimes jerk involuntarily. In addition, Cora's cognitive symptoms are worsening. At this point, Cora's doctor becomes very concerned and orders more tests on the CSF samples.

- What types of microorganisms could be causing Cora's symptoms?

*Jump to the **next** Clinical Focus box. Go back to the **previous** Clinical Focus box.*

The Role of Genetics in Modern Taxonomy

Haeckel's and Whittaker's trees presented hypotheses about the phylogeny of different organisms based on readily observable characteristics. But the advent of molecular genetics in the late 20th century revealed other ways to organize phylogenetic trees. Genetic methods allow for a standardized way to compare all living organisms without relying on observable characteristics that can often be subjective. Modern taxonomy relies heavily on comparing the nucleic acids (deoxyribonucleic acid [DNA] or ribonucleic acid [RNA]) or proteins from different organisms. The more similar the nucleic acids and proteins are between two organisms, the more closely related they are considered to be.

In the 1970s, American microbiologist Carl Woese discovered what appeared to be a "living record" of the evolution of organisms. He and his collaborator George Fox created a genetics-based tree of life based on similarities and differences they observed in the gene sequences coding for small subunit ribosomal RNA (rRNA) of different organisms. In the process, they discovered that a certain type of bacteria, called archaebacteria (now known simply as archaea), were significantly different from other bacteria and eukaryotes in terms of their small subunit rRNA gene sequences. To accommodate this difference, they created a tree with three Domains

above the level of Kingdom: Archaea, Bacteria, and Eukarya (Figure 8.11). Analysis of small subunit rRNA gene sequences suggests archaea, bacteria, and eukaryotes all evolved from a common ancestral cell type. The tree is skewed to show a closer evolutionary relationship between Archaea and Eukarya than they have to Bacteria.

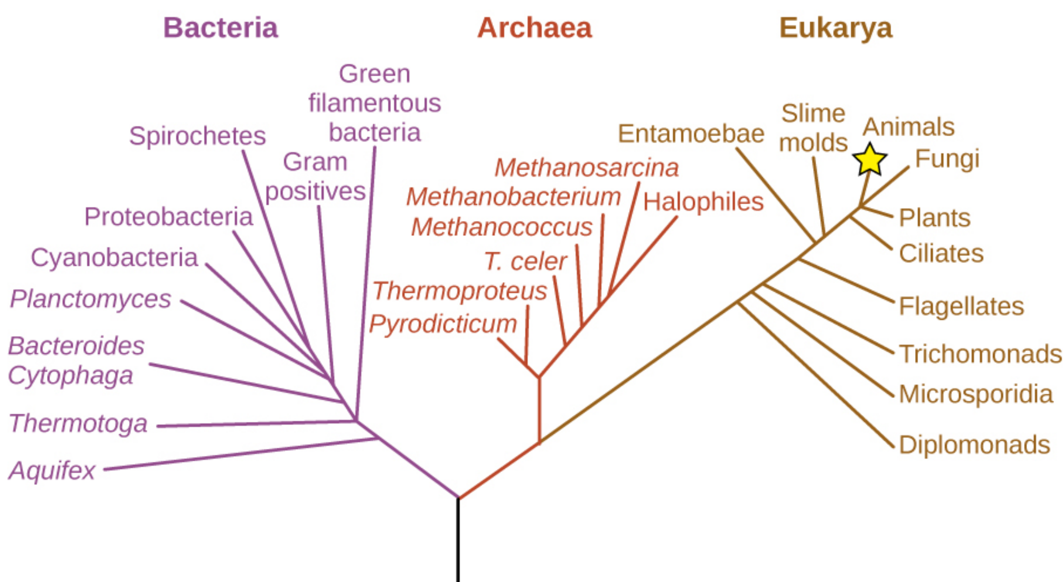


Figure 8.11 Woese and Fox's phylogenetic tree contains three domains: Bacteria, Archaea, and Eukarya. Domains Archaea and Bacteria contain all prokaryotic organisms, and Eukarya contains all eukaryotic organisms. (credit: modification of work by Eric Gaba)

Scientists continue to use analysis of RNA, DNA, and proteins to determine how organisms are related. One interesting, and complicating, discovery is that of horizontal gene transfer—when a gene of one species is absorbed into another organism's genome. Horizontal gene transfer is especially common in microorganisms and can make it difficult to determine how organisms are evolutionarily related. Consequently, some scientists now think in terms of "webs of life" rather than "trees of life."

- In modern taxonomy, how do scientists determine how closely two organisms are related?
- Explain why the branches on the "tree of life" all originate from a single "trunk."

Naming Microbes

In developing his taxonomy, Linnaeus used a system of **binomial nomenclature**, a two-word naming system for identifying organisms by genus and specific epithet. For example, modern humans are in the genus *Homo* and have the specific epithet name *sapiens*, so their scientific name in binomial nomenclature is *Homo sapiens*. In binomial nomenclature, the genus part of the name is always capitalized; it is followed by the specific epithet name, which is not capitalized. Both names are italicized. When referring to the species of humans, the binomial nomenclature would be *Homo sapiens*.

Taxonomic names in the 18th through 20th centuries were typically derived from Latin, since that was the common language used by scientists when taxonomic systems were first created. Today, newly discovered organisms can be given names derived from Latin, Greek, or English. Sometimes these names reflect some distinctive trait of the organism; in other cases, microorganisms are named after the scientists who discovered them. The archaeon *Haloquadratum walsbyi* is an example of both of these naming schemes. The genus, *Haloquadratum*, describes the microorganism's saltwater habitat (*halo* is derived from the Greek word for "salt") as well as the arrangement of its square cells, which are arranged in square clusters of four cells (*quadratum* is Latin for "foursquare"). The species, *walsbyi*, is named after Anthony Edward Walsby, the microbiologist who discovered *Haloquadratum walsbyi* in in 1980. While it might seem easier to give an organism a common descriptive name—like a red-headed woodpecker—we can imagine how that could become problematic. What happens when another species of woodpecker with red head coloring is discovered? The

systematic nomenclature scientists use eliminates this potential problem by assigning each organism a single, unique two-word name that is recognized by scientists all over the world.

In this text, we will typically abbreviate an organism's genus and species after its first mention. The abbreviated form is simply the first initial of the genus, followed by a period and the full name of the species. For example, the bacterium *Escherichia coli* is shortened to *E. coli* in its abbreviated form. You will encounter this same convention in other scientific texts as well.

Bergey's Manuals

Whether in a tree or a web, microbes can be difficult to identify and classify. Without easily observable macroscopic features like feathers, feet, or fur, scientists must capture, grow, and devise ways to study their biochemical properties to differentiate and classify microbes. Despite these hurdles, a group of microbiologists created and updated a set of manuals for identifying and classifying microorganisms. First published in 1923 and since updated many times, *Bergey's Manual of Determinative Bacteriology* and *Bergey's Manual of Systematic Bacteriology* are the standard references for identifying and classifying different prokaryotes. (**Appendix D (https://legacy.cnx.org/content/m58949/latest/)** of this textbook is partly based on Bergey's manuals; it shows how the organisms that appear in this textbook are classified.) Because so many bacteria look identical, methods based on nonvisual characteristics must be used to identify them. For example, biochemical tests can be used to identify chemicals unique to certain species. Likewise, serological tests can be used to identify specific antibodies that will react against the proteins found in certain species. Ultimately, DNA and rRNA sequencing can be used both for identifying a particular bacterial species and for classifying newly discovered species.

- What is binomial nomenclature and why is it a useful tool for naming organisms?
- Explain why a resource like one of Bergey's manuals would be helpful in identifying a microorganism in a sample.

Same Name, Different Strain

Within one species of microorganism, there can be several subtypes called strains. While different strains may be nearly identical genetically, they can have very different attributes. The bacterium *Escherichia coli* is infamous for causing food poisoning and traveler's diarrhea. However, there are actually many different strains of *E. coli*, and they vary in their ability to cause disease.

One pathogenic (disease-causing) *E. coli* strain that you may have heard of is *E. coli* O157:H7. In humans, infection from *E. coli* O157:H7 can cause abdominal cramps and diarrhea. Infection usually originates from contaminated water or food, particularly raw vegetables and undercooked meat. In the 1990s, there were several large outbreaks of *E. coli* O157:H7 thought to have originated in undercooked hamburgers.

While *E. coli* O157:H7 and some other strains have given *E. coli* a bad name, most *E. coli* strains do not cause disease. In fact, some can be helpful. Different strains of *E. coli* found naturally in our gut help us digest our food, provide us with some needed chemicals, and fight against pathogenic microbes.

Learn more about phylogenetic trees by exploring the Wellcome Trust's interactive Tree of Life. The **website (https://www.openstax.org/l/22wellcome)** contains information, photos, and animations about many different organisms. Select two organisms to see how they are evolutionarily related.

8.3 | Types of Microorganisms

Learning Objectives

By the end of this section, you will be able to:

- List the various types of microorganisms and describe their defining characteristics
- Give examples of different types of cellular and viral microorganisms and infectious agents

- Describe the similarities and differences between archaea and bacteria
- Provide an overview of the field of microbiology

Most microbes are unicellular and small enough that they require artificial magnification to be seen. However, there are some unicellular microbes that are visible to the naked eye, and some multicellular organisms that are microscopic. An object must measure about 100 micrometers (µm) to be visible without a microscope, but most microorganisms are many times smaller than that. For some perspective, consider that a typical animal cell measures roughly 10 µm across but is still microscopic. Bacterial cells are typically about 1 µm, and viruses can be 10 times smaller than bacteria (Figure 8.12). See Table 8.1 for units of length used in microbiology.

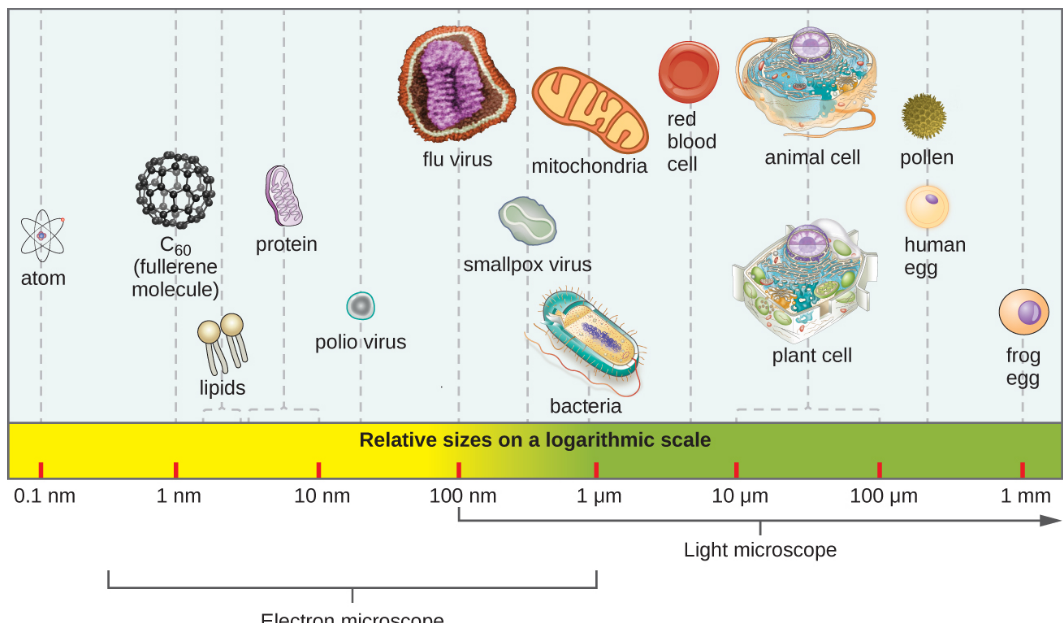


Figure 8.12 The relative sizes of various microscopic and nonmicroscopic objects. Note that a typical virus measures about 100 nm, 10 times smaller than a typical bacterium (~1 µm), which is at least 10 times smaller than a typical plant or animal cell (~10–100 µm). An object must measure about 100 µm to be visible without a microscope.

Units of Length Commonly Used in Microbiology

Metric Unit	Meaning of Prefix	Metric Equivalent
meter (m)	—	1 m = 10^{0} m
decimeter (dm)	1/10	1 dm = 0.1 m = 10^{-1} m
centimeter (cm)	1/100	1 cm = 0.01 m = 10^{-2} m
millimeter (mm)	1/1000	1 mm = 0.001 m = 10^{-3} m
micrometer (µm)	1/1,000,000	1 µm = 0.000001 m = 10^{-6} m
nanometer (nm)	1/1,000,000,000	1 nm = 0.000000001 m = 10^{-9} m

Table 8.1

Microorganisms differ from each other not only in size, but also in structure, habitat, metabolism, and many other characteristics. While we typically think of microorganisms as being unicellular, there are also many multicellular organisms that are too small to be seen without a microscope. Some microbes, such as viruses, are even **acellular** (not composed of cells).

Microorganisms are found in each of the three domains of life: Archaea, Bacteria, and Eukarya. Microbes within the domains Bacteria and Archaea are all prokaryotes (their cells lack a nucleus), whereas microbes in the

domain Eukarya are eukaryotes (their cells have a nucleus). Some microorganisms, such as viruses, do not fall within any of the three domains of life. In this section, we will briefly introduce each of the broad groups of microbes. Later chapters will go into greater depth about the diverse species within each group.

How big is a bacterium or a virus compared to other objects? Check out this **interactive website (https://www.openstax.org/l/22relsizes)** to get a feel for the scale of different microorganisms.

Prokaryotic Microorganisms

Bacteria are found in nearly every habitat on earth, including within and on humans. Most bacteria are harmless or helpful, but some are **pathogens**, causing disease in humans and other animals. Bacteria are prokaryotic because their genetic material (DNA) is not housed within a true nucleus. Most bacteria have cell walls that contain peptidoglycan.

Bacteria are often described in terms of their general shape. Common shapes include spherical (coccus), rod-shaped (bacillus), or curved (spirillum, spirochete, or vibrio). **Figure 8.13** shows examples of these shapes.

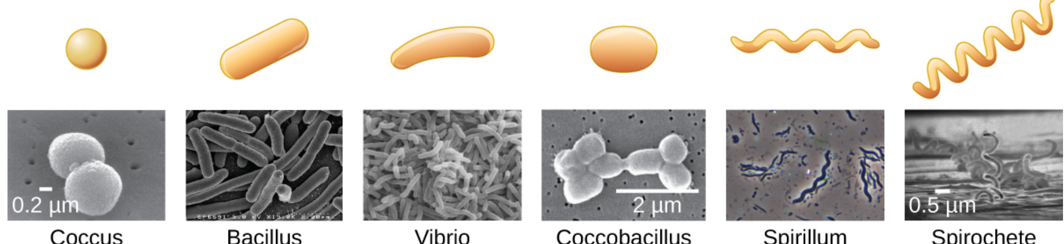


Figure 8.13 Common bacterial shapes. Note how coccobacillus is a combination of spherical (coccus) and rod-shaped (bacillus). (credit “Coccus”: modification of work by Janice Haney Carr, Centers for Disease Control and Prevention; credit “Coccobacillus”: modification of work by Janice Carr, Centers for Disease Control and Prevention; credit “Spirochete”: Centers for Disease Control and Prevention)

They have a wide range of metabolic capabilities and can grow in a variety of environments, using different combinations of nutrients. Some bacteria are photosynthetic, such as oxygenic cyanobacteria and anoxygenic green sulfur and green nonsulfur bacteria; these bacteria use energy derived from sunlight, and fix carbon dioxide for growth. Other types of bacteria are nonphotosynthetic, obtaining their energy from organic or inorganic compounds in their environment.

Archaea are also unicellular prokaryotic organisms. Archaea and bacteria have different evolutionary histories, as well as significant differences in genetics, metabolic pathways, and the composition of their cell walls and membranes. Unlike most bacteria, archaeal cell walls do not contain peptidoglycan, but their cell walls are often composed of a similar substance called pseudopeptidoglycan. Like bacteria, archaea are found in nearly every habitat on earth, even extreme environments that are very cold, very hot, very basic, or very acidic (**Figure 8.14**). Some archaea live in the human body, but none have been shown to be human pathogens.

Figure 8.14 Some archaea live in extreme environments, such as the Morning Glory pool, a hot spring in Yellowstone National Park. The color differences in the pool result from the different communities of microbes that are able to thrive at various water temperatures.

- What are the two main types of prokaryotic organisms?
- Name some of the defining characteristics of each type.

Eukaryotic Microorganisms

The domain Eukarya contains all eukaryotes, including uni- or multicellular eukaryotes such as protists, fungi, plants, and animals. The major defining characteristic of eukaryotes is that their cells contain a nucleus.

Protists

Protists are an informal grouping of eukaryotes that are not plants, animals, or fungi. Algae and protozoa are examples of protists.

Algae (singular: alga) are protists that can be either unicellular or multicellular and vary widely in size, appearance, and habitat (**Figure 8.15**). Their cells are surrounded by cell walls made of cellulose, a type of carbohydrate. Algae are photosynthetic organisms that extract energy from the sun and release oxygen and carbohydrates into their environment. Because other organisms can use their waste products for energy, algae are important parts of many ecosystems. Many consumer products contain ingredients derived from algae, such as carrageenan or alginic acid, which are found in some brands of ice cream, salad dressing, beverages, lipstick, and toothpaste. A derivative of algae also plays a prominent role in the microbiology laboratory. Agar, a gel derived from algae, can be mixed with various nutrients and used to grow microorganisms in a Petri dish. Algae are also being developed as a possible source for biofuels.

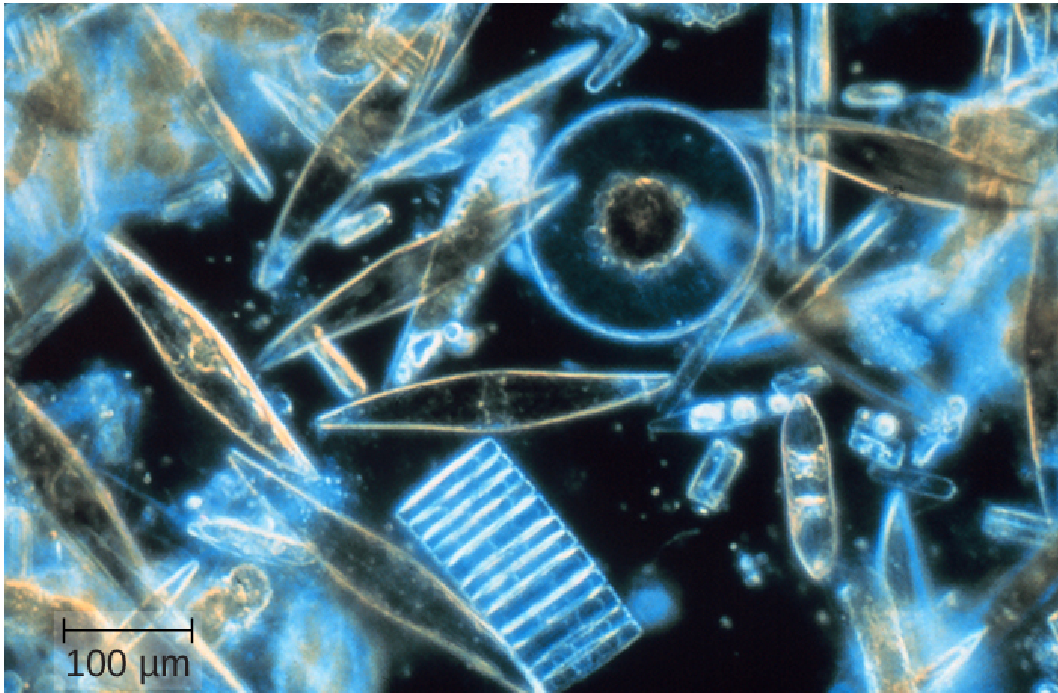


Figure 8.15 Assorted diatoms, a kind of algae, live in annual sea ice in McMurdo Sound, Antarctica. Diatoms range in size from 2 µm to 200 µm and are visualized here using light microscopy. (credit: modification of work by National Oceanic and Atmospheric Administration)

Protozoa (singular: protozoan) are protists that make up the backbone of many food webs by providing nutrients for other organisms. Protozoa are very diverse. Some protozoa move with help from hair-like structures called cilia or whip-like structures called flagella. Others extend part of their cell membrane and cytoplasm to propel themselves forward. These cytoplasmic extensions are called pseudopods ("false feet"). Some protozoa are photosynthetic; others feed on organic material. Some are free-living, whereas others are parasitic, only able to survive by extracting nutrients from a host organism. Most protozoa are harmless, but some are pathogens that can cause disease in animals or humans (**Figure 8.16**).

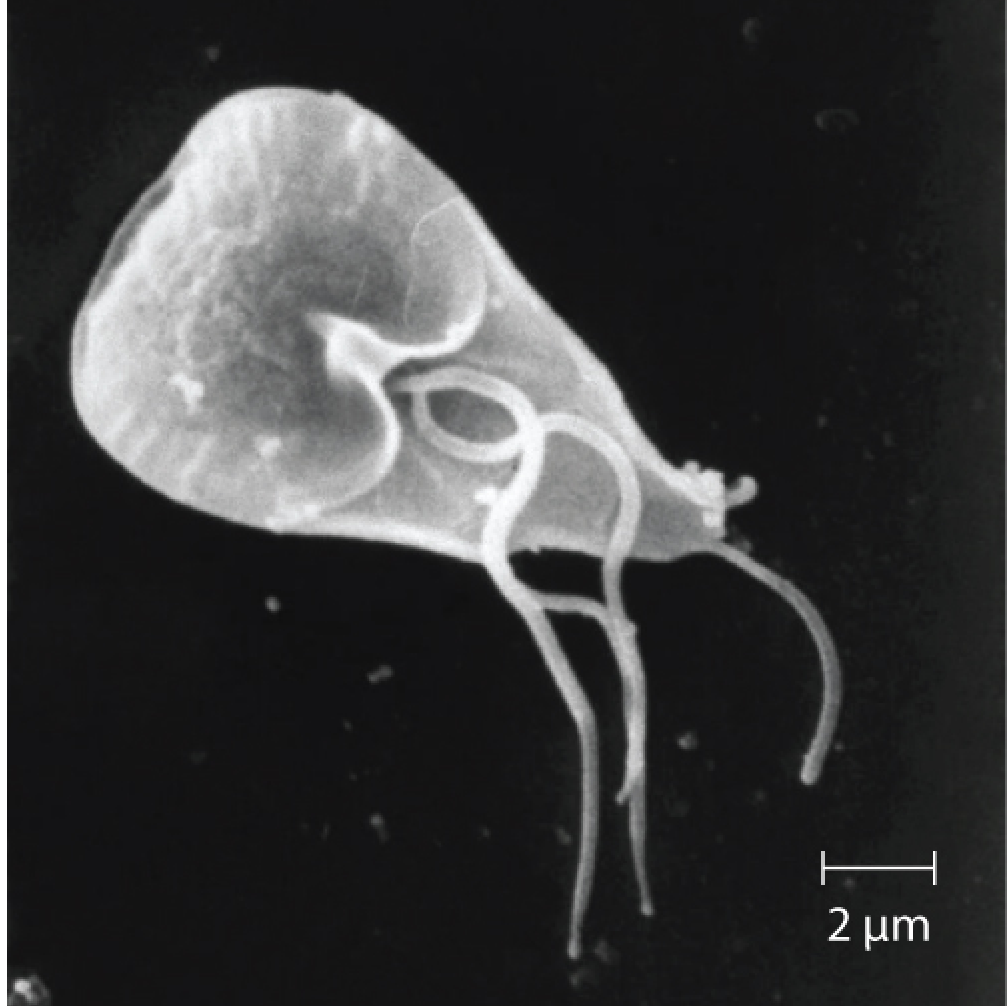


Figure 8.16 *Giardia lamblia*, an intestinal protozoan parasite that infects humans and other mammals, causing severe diarrhea. (credit: modification of work by Centers for Disease Control and Prevention)

Fungi

Fungi (singular: fungus) are also eukaryotes. Some multicellular fungi, such as mushrooms, resemble plants, but they are actually quite different. Fungi are not photosynthetic, and their cell walls are usually made out of chitin rather than cellulose.

Unicellular fungi—yeasts—are included within the study of microbiology. There are more than 1000 known species. Yeasts are found in many different environments, from the deep sea to the human navel. Some yeasts have beneficial uses, such as causing bread to rise and beverages to ferment; but yeasts can also cause food to spoil. Some even cause diseases, such as vaginal yeast infections and oral thrush (**Figure 8.17**).

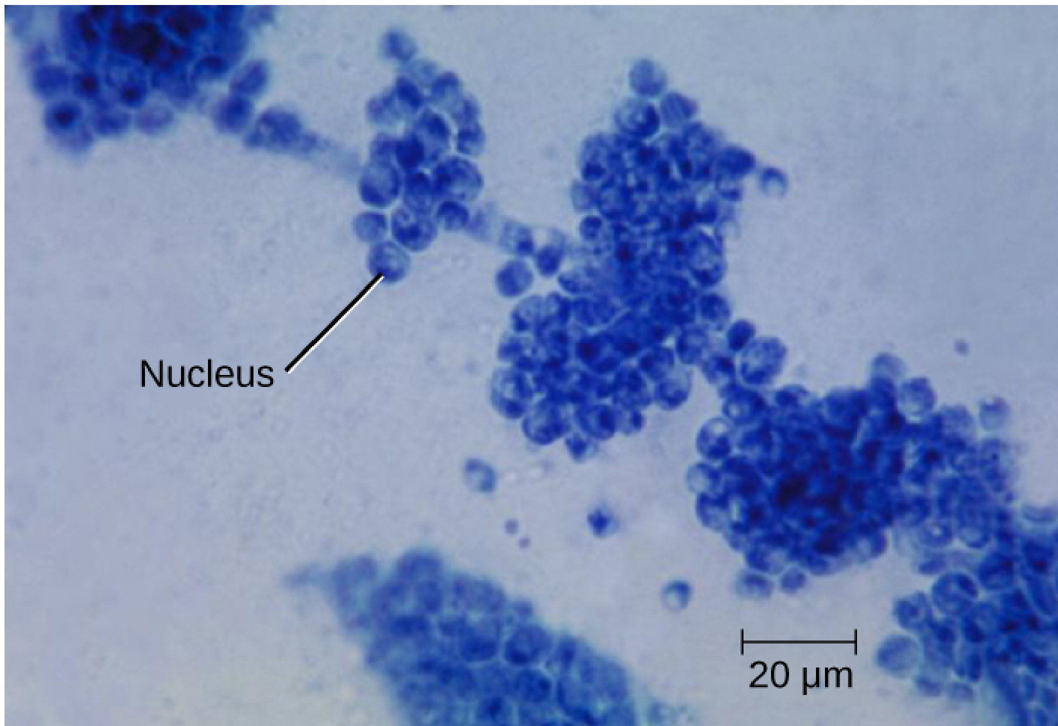


Figure 8.17 *Candida albicans* is a unicellular fungus, or yeast. It is the causative agent of vaginal yeast infections as well as oral thrush, a yeast infection of the mouth that commonly afflicts infants. *C. albicans* has a morphology similar to that of coccus bacteria; however, yeast is a eukaryotic organism (note the nuclei) and is much larger. (credit: modification of work by Centers for Disease Control and Prevention)

Other fungi of interest to microbiologists are multicellular organisms called **molds**. Molds are made up of long filaments that form visible colonies (**Figure 8.18**). Molds are found in many different environments, from soil to rotting food to dank bathroom corners. Molds play a critical role in the decomposition of dead plants and animals. Some molds can cause allergies, and others produce disease-causing metabolites called mycotoxins. Molds have been used to make pharmaceuticals, including penicillin, which is one of the most commonly prescribed antibiotics, and cyclosporine, used to prevent organ rejection following a transplant.

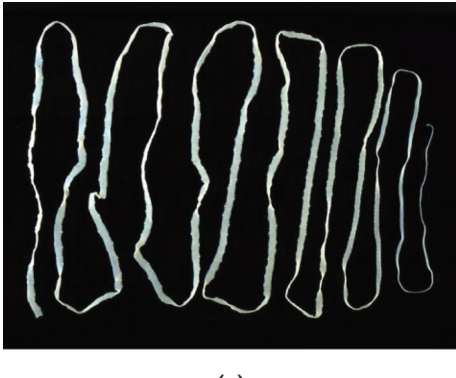
Figure 8.18 Large colonies of microscopic fungi can often be observed with the naked eye, as seen on the surface of these moldy oranges.

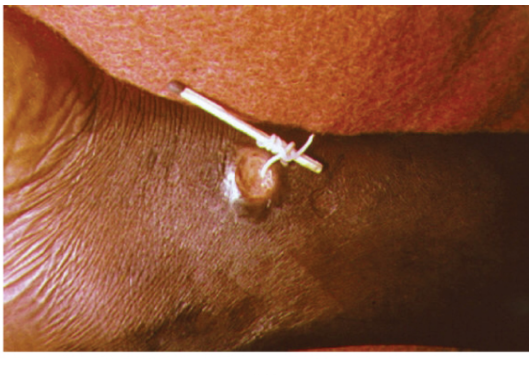
- Name two types of protists and two types of fungi.
- Name some of the defining characteristics of each type.

Helminths

Multicellular parasitic worms called **helminths** are not technically microorganisms, as most are large enough to see without a microscope. However, these worms fall within the field of microbiology because diseases caused by helminths involve microscopic eggs and larvae. One example of a helminth is the guinea worm, or *Dracunculus medinensis*, which causes dizziness, vomiting, diarrhea, and painful ulcers on the legs and feet when the worm works its way out of the skin (**Figure 8.19**). Infection typically occurs after a person drinks water containing water fleas infected by guinea-worm larvae. In the mid-1980s, there were an estimated 3.5 million cases of guinea-worm disease, but the disease has been largely eradicated. In 2014, there were only 126 cases reported, thanks to the coordinated efforts of the World Health Organization (WHO) and other groups committed to improvements in drinking water sanitation.[11][12]

11. C. Greenaway "Dracunculiasis (Guinea Worm Disease)." *Canadian Medical Association Journal* 170 no. 4 (2004):495–500.
12. World Health Organization. "Dracunculiasis (Guinea-Worm Disease)." *WHO*. 2015. http://www.who.int/mediacentre/factsheets/fs359/en/. Accessed October 2, 2015.





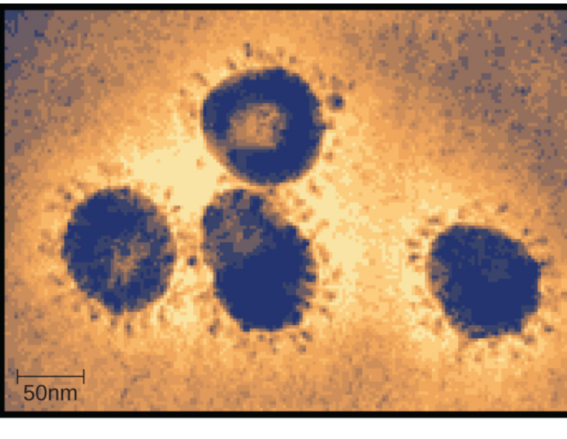
(a) (b)

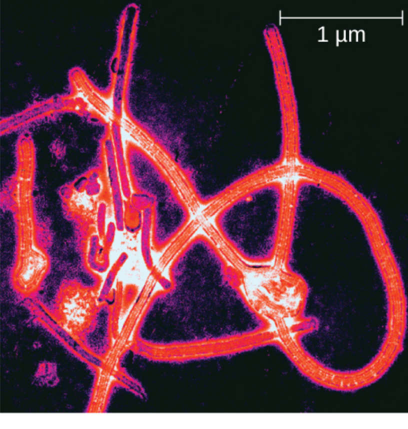
Figure 8.19 (a) The beef tapeworm, *Taenia saginata*, infects both cattle and humans. *T. saginata* eggs are microscopic (around 50 μm), but adult worms like the one shown here can reach 4–10 m, taking up residence in the digestive system. (b) An adult guinea worm, *Dracunculus medinensis*, is removed through a lesion in the patient's skin by winding it around a matchstick. (credit a, b: modification of work by Centers for Disease Control and Prevention)

Viruses

Viruses are **acellular** microorganisms, which means they are not composed of cells. Essentially, a virus consists of proteins and genetic material—either DNA or RNA, but never both—that are inert outside of a host organism. However, by incorporating themselves into a host cell, viruses are able to co-opt the host's cellular mechanisms to multiply and infect other hosts.

Viruses can infect all types of cells, from human cells to the cells of other microorganisms. In humans, viruses are responsible for numerous diseases, from the common cold to deadly Ebola (Figure 8.20). However, many viruses do not cause disease.





(a) (b)

Figure 8.20 (a) Members of the Coronavirus family can cause respiratory infections like the common cold, severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS). Here they are viewed under a transmission electron microscope (TEM). (b) Ebolavirus, a member of the Filovirus family, as visualized using a TEM. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by Thomas W. Geisbert)

- Are helminths microorganisms? Explain why or why not.
- How are viruses different from other microorganisms?

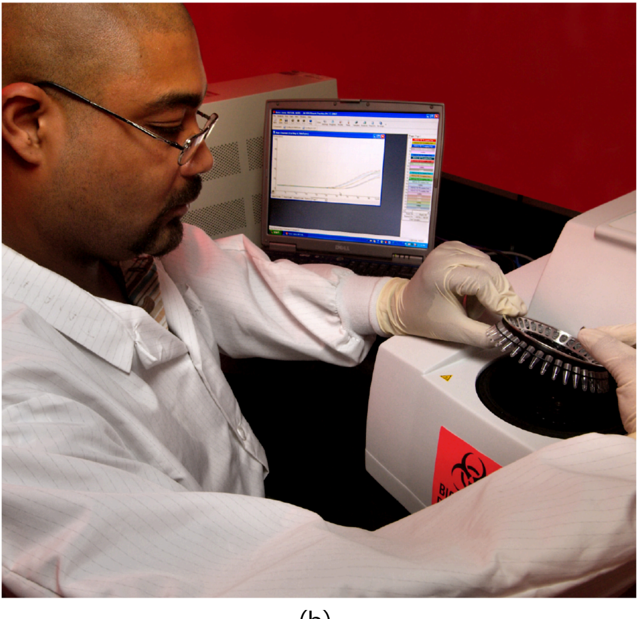
Microbiology as a Field of Study

Microbiology is a broad term that encompasses the study of all different types of microorganisms. But in practice, microbiologists tend to specialize in one of several subfields. For example, **bacteriology** is the study of bacteria; **mycology** is the study of fungi; **protozoology** is the study of protozoa; **parasitology** is the study of helminths and other parasites; and **virology** is the study of viruses (Figure 8.21). **Immunology**, the study

of the immune system, is often included in the study of microbiology because host–pathogen interactions are central to our understanding of infectious disease processes. Microbiologists can also specialize in certain areas of microbiology, such as clinical microbiology, environmental microbiology, applied microbiology, or food microbiology.

In this textbook, we are primarily concerned with clinical applications of microbiology, but since the various subfields of microbiology are highly interrelated, we will often discuss applications that are not strictly clinical.

(a)



(b)

Figure 8.21 (a) A virologist samples eggs from this nest to be tested for the influenza A virus, which causes avian flu in birds. (b) A biologist performs a procedure to identify an organism that causes ulcerations in humans (credit a: U.S. Fish and Wildlife Service; credit b: James Gathany / CDC; Public Domain)

Bioethics in Microbiology

In the 1940s, the U.S. government was looking for a solution to a medical problem: the prevalence of sexually transmitted diseases (STDs) among soldiers. Several now-infamous government-funded studies used human subjects to research common STDs and treatments. In one such study, American researchers intentionally exposed more than 1300 human subjects in Guatemala to syphilis, gonorrhea, and chancroid to determine the ability of penicillin and other antibiotics to combat these diseases. Subjects of the study included Guatemalan soldiers, prisoners, prostitutes, and psychiatric patients—none of whom were informed that they were taking part in the study. Researchers exposed subjects to STDs by various methods, from facilitating intercourse with infected prostitutes to inoculating subjects with the bacteria known to cause the diseases. This latter method involved making a small wound on the subject's genitals or elsewhere on the body, and then putting bacteria directly into the wound.[13] In 2011, a U.S. government commission tasked with investigating the experiment revealed that only some of the subjects were treated with penicillin, and 83 subjects died by 1953, likely as a result of the study.[14]

Unfortunately, this is one of many horrific examples of microbiology experiments that have violated basic ethical standards. Even if this study had led to a life-saving medical breakthrough (it did not), few would argue that its methods were ethically sound or morally justifiable. But not every case is so clear cut. Professionals working in clinical settings are frequently confronted with ethical dilemmas, such as working with patients who decline a vaccine or life-saving blood transfusion. These are just two examples of life-and-death decisions that may intersect with the religious and philosophical beliefs of both the patient and the health-care professional.

No matter how noble the goal, microbiology studies and clinical practice must be guided by a certain set

13. Kara Rogers. "Guatemala Syphilis Experiment: American Medical Research Project". *Encylopaedia Britannica*. http://www.britannica.com/event/Guatemala-syphilis-experiment. Accessed June 24, 2015.
14. Susan Donaldson James. "Syphilis Experiments Shock, But So Do Third-World Drug Trials." *ABC World News*. August 30, 2011. http://abcnews.go.com/Health/guatemala-syphilis-experiments-shock-us-drug-trials-exploit/story?id=14414902. Accessed June 24, 2015.

of ethical principles. Studies must be done with integrity. Patients and research subjects provide informed consent (not only agreeing to be treated or studied but demonstrating an understanding of the purpose of the study and any risks involved). Patients' rights must be respected. Procedures must be approved by an institutional review board. When working with patients, accurate record-keeping, honest communication, and confidentiality are paramount. Animals used for research must be treated humanely, and all protocols must be approved by an institutional animal care and use committee. These are just a few of the ethical principles explored in the *Eye on Ethics* boxes throughout this book.

Resolution

Cora's CSF samples show no signs of inflammation or infection, as would be expected with a viral infection. However, there is a high concentration of a particular protein, 14-3-3 protein, in her CSF. An electroencephalogram (EEG) of her brain function is also abnormal. The EEG resembles that of a patient with a neurodegenerative disease like Alzheimer's or Huntington's, but Cora's rapid cognitive decline is not consistent with either of these. Instead, her doctor concludes that Cora has Creutzfeldt-Jakob disease (CJD), a type of transmissible spongiform encephalopathy (TSE).

CJD is an extremely rare disease, with only about 300 cases in the United States each year. It is not caused by a bacterium, fungus, or virus, but rather by prions—which do not fit neatly into any particular category of microbe. Like viruses, prions are not found on the tree of life because they are acellular. Prions are extremely small, about one-tenth the size of a typical virus. They contain no genetic material and are composed solely of a type of abnormal protein.

CJD can have several different causes. It can be acquired through exposure to the brain or nervous-system tissue of an infected person or animal. Consuming meat from an infected animal is one way such exposure can occur. There have also been rare cases of exposure to CJD through contact with contaminated surgical equipment[15] and from cornea and growth-hormone donors who unknowingly had CJD.[16][17] In rare cases, the disease results from a specific genetic mutation that can sometimes be hereditary. However, in approximately 85% of patients with CJD, the cause of the disease is spontaneous (or sporadic) and has no identifiable cause.[18] Based on her symptoms and their rapid progression, Cora is diagnosed with sporadic CJD.

Unfortunately for Cora, CJD is a fatal disease for which there is no approved treatment. Approximately 90% of patients die within 1 year of diagnosis.[19] Her doctors focus on limiting her pain and cognitive symptoms as her disease progresses. Eight months later, Cora dies. Her CJD diagnosis is confirmed with a brain autopsy.

*Go back to the **previous** Clinical Focus box.*

8.4 | Beneficial Prokaryotes

By the end of this section, you will be able to do the following:

- Explain the need for nitrogen fixation and how it is accomplished
- Describe the beneficial effects of bacteria that colonize our skin and digestive tracts
- Identify prokaryotes used during the processing of food
- Describe the use of prokaryotes in bioremediation

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15. Greg Botelho. "Case of Creutzfeldt-Jakob Disease Confirmed in New Hampshire." *CNN*. 2013. http://www.cnn.com/2013/09/20/health/creutzfeldt-jakob-brain-disease/.
16. P. Rudge et al. "Iatrogenic CJD Due to Pituitary-Derived Growth Hormone With Genetically Determined Incubation Times of Up to 40 Years." *Brain* 138 no. 11 (2015): 3386–3399.
17. J.G. Heckmann et al. "Transmission of Creutzfeldt-Jakob Disease via a Corneal Transplant." *Journal of Neurology, Neurosurgery & Psychiatry* 63 no. 3 (1997): 388–390.
18. National Institute of Neurological Disorders and Stroke. "Creutzfeldt-Jakob Disease Fact Sheet." *NIH*. 2015. http://www.ninds.nih.gov/disorders/cjd/detail_cjd.htm#288133058.
19. National Institute of Neurological Disorders and Stroke. "Creutzfeldt-Jakob Disease Fact Sheet." *NIH*. 2015. http://www.ninds.nih.gov/disorders/cjd/detail_cjd.htm#288133058. Accessed June 22, 2015.

Fortunately, only a few species of prokaryotes are pathogenic! Prokaryotes also interact with humans and other organisms in a number of ways that are beneficial. For example, prokaryotes are major participants in the carbon and nitrogen cycles. They produce or process nutrients in the digestive tracts of humans and other animals. Prokaryotes are used in the production of some human foods, and also have been recruited for the degradation of hazardous materials. In fact, our life would not be possible without prokaryotes!

Cooperation between Bacteria and Eukaryotes: Nitrogen Fixation

Nitrogen is a very important element to living things, because it is part of nucleotides and amino acids that are the building blocks of nucleic acids and proteins, respectively. Nitrogen is usually the most limiting element in terrestrial ecosystems, with atmospheric nitrogen, N_2, providing the largest pool of available nitrogen. However, eukaryotes cannot use atmospheric, gaseous nitrogen to synthesize macromolecules. Fortunately, nitrogen can be “fixed,” meaning it is converted into a more accessible form—ammonia (NH_3)—either biologically or abiotically.

Abiotic nitrogen fixation occurs as a result of physical processes such as lightning or by industrial processes. **Biological nitrogen fixation** (BNF) is exclusively carried out by prokaryotes: soil bacteria, cyanobacteria, and *Frankia* spp. (filamentous bacteria interacting with actinorhizal plants such as alder, bayberry, and sweet fern). After photosynthesis, BNF is the most important biological process on Earth. The overall nitrogen fixation equation below represents a series of *redox reactions* (Pi stands for inorganic phosphate).

$$\mathrm{N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + 16ADP + 16Pi + H_2}$$

The total fixed nitrogen through BNF is about 100 to 180 million metric tons per year, which contributes about 65 percent of the nitrogen used in agriculture.

Cyanobacteria are the most important nitrogen fixers in aquatic environments. In soil, members of the genera *Clostridium* and *Azotobacter* are examples of free-living, nitrogen-fixing bacteria. Other bacteria live symbiotically with legume plants, providing the most important source of fixed nitrogen. Symbionts may fix more nitrogen in soils than free-living organisms by a factor of 10. Soil bacteria, collectively called rhizobia, are able to symbiotically interact with legumes to form **nodules**, specialized structures where nitrogen fixation occurs (Figure 8.22). *Nitrogenase*, the enzyme that fixes nitrogen, is inactivated by oxygen, so the nodule provides an oxygen-free area for nitrogen fixation to take place. The oxygen is sequestered by a form of plant hemoglobin called *leghemoglobin*, which protects the *nitrogenase*, but releases enough oxygen to support respiratory activity.

Symbiotic nitrogen fixation provides a natural and inexpensive plant fertilizer: It reduces atmospheric nitrogen to ammonia, which is easily usable by plants. The use of legumes is an excellent alternative to chemical fertilization and is of special interest to *sustainable agriculture*, which seeks to minimize the use of chemicals and conserve natural resources. Through symbiotic nitrogen fixation, the plant benefits from using an endless source of nitrogen: the atmosphere. The bacteria benefit from using photosynthates (carbohydrates produced during photosynthesis) from the plant and having a protected niche. In addition, the soil benefits from being naturally fertilized. Therefore, the use of rhizobia as biofertilizers is a sustainable practice.

Why are legumes so important? Some, like soybeans, are key sources of agricultural protein. Some of the most important legumes consumed by humans are soybeans, peanuts, peas, chickpeas, and beans. Other legumes, such as alfalfa, are used to feed cattle.

Figure 8.22 Nitrogen-fixation nodules on soybean roots. Soybean (*Glycine max*) is a legume that interacts symbiotically with the soil bacterium *Bradyrhizobium japonicum* to form specialized structures on the roots called *nodules* where nitrogen fixation occurs. (credit: USDA)

everyday CONNECTION

Microbes on the Human Body

The commensal bacteria that inhabit our skin and gastrointestinal tract do a host of good things for us. They protect us from pathogens, help us digest our food, and produce some of our vitamins and other nutrients. These activities have been known for a long time. More recently, scientists have gathered evidence that these bacteria may also help regulate our moods, influence our activity levels, and even help control weight by affecting our food choices and absorption patterns. The Human Microbiome Project has begun the process of cataloging our normal bacteria (and archaea) so we can better understand these functions.

A particularly fascinating example of our normal flora relates to our digestive systems. People who take high doses of antibiotics tend to lose many of their normal gut bacteria, allowing a naturally antibiotic-resistant species called *Clostridium difficile* to overgrow and cause severe gastric problems, especially chronic diarrhea (**Figure 8.23**). Obviously, trying to treat this problem with antibiotics only makes it worse. However, it has been successfully treated by giving the patients fecal transplants from healthy donors to reestablish the normal intestinal microbial community. Clinical trials are underway to ensure the safety and effectiveness of this technique.

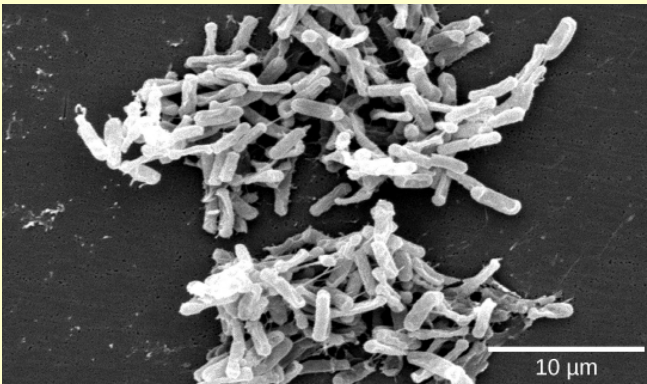


Figure 8.23 *Clostridium difficile*. This scanning electron micrograph shows *Clostridium difficile*, a Gram-positive, rod-shaped bacterium that causes severe diarrhea. Infection commonly occurs after the normal gut fauna are eradicated by antibiotics, and in the hospital can be deadly to seriously ill patients. (credit: modification of work by CDC, HHS; scale-bar data from Matt Russell)

Scientists are also discovering that the absence of certain key microbes from our intestinal tract may set us up for a variety of problems. This seems to be particularly true regarding the appropriate functioning of the immune system. There are intriguing findings that suggest that the absence of these microbes is an important contributor to the development of allergies and some autoimmune disorders. Research is currently underway to test whether adding certain microbes to our internal ecosystem may help in the treatment of these problems, as well as in treating some forms of autism.

Early Biotechnology: Cheese, Bread, Wine, Beer, and Yogurt

According to the United Nations Convention on Biological Diversity, **biotechnology** is “any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use."[20] The concept of “specific use” involves some sort of commercial application. Genetic engineering, artificial selection, antibiotic production, and cell culture are current topics of study in biotechnology and will be described in later chapters. However, humans were using prokaryotes before the term biotechnology was even coined. Some of the products of this early biotechnology are as familiar as cheese, bread, wine, beer, and yogurt, which employ both bacteria and other microbes, such as yeast, a fungus (**Figure 8.24**).

20. http://www.cbd.int/convention/articles/?a=cbd-02 (http://openstax.org/l/UN_convention) , United Nations Convention on Biological Diversity: Article 2: Use of Terms.

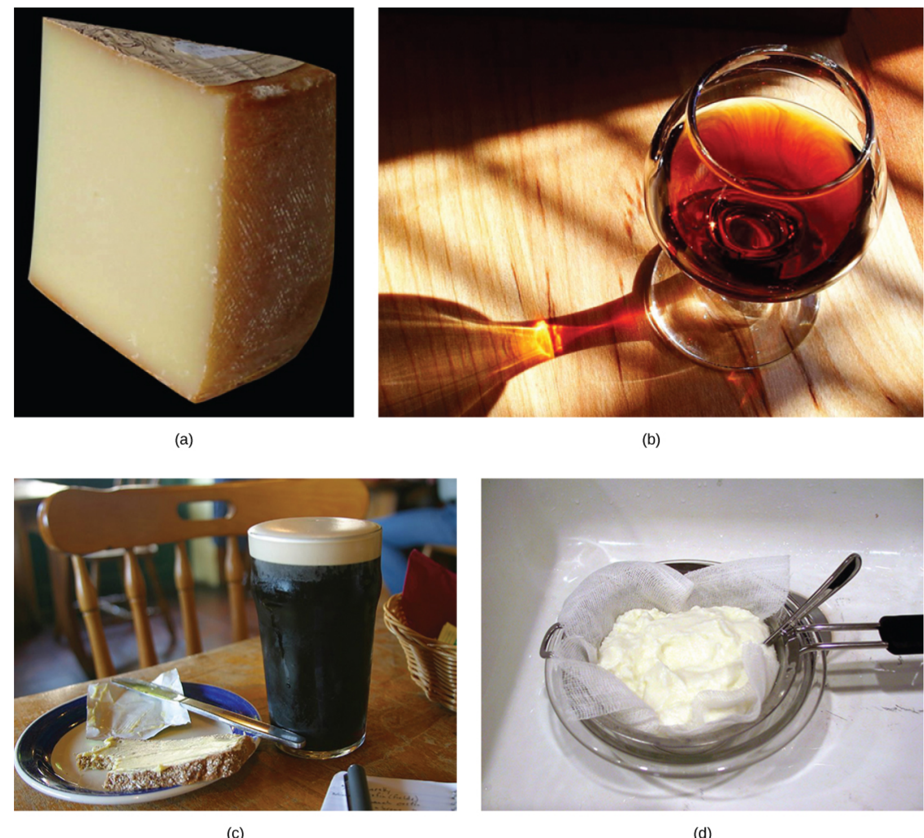


Figure 8.24 Some foods produced by microorganisms. Some of the products derived from the use of prokaryotes in early biotechnology include (a) cheese, (b) wine, (c) beer and bread, and (d) yogurt. (credit bread: modification of work by F. Rodrigo/Wikimedia Commons; credit wine: modification of work by Jon Sullivan; credit beer and bread: modification of work by Kris Miller; credit yogurt: modification of work by Jon Sullivan)

Cheese production began around 4,000 to 7,000 years ago when humans began to breed animals and process their milk. Fermentation in this case preserves nutrients: Milk will spoil relatively quickly, but when processed as cheese, it is more stable. As for beer, the oldest records of brewing are about 6,000 years old and were an integral part of the Sumerian culture. Evidence indicates that the Sumerians discovered fermentation by chance. Wine has been produced for about 4,500 years, and evidence suggests that cultured milk products, like yogurt, have existed for at least 4,000 years.

Using Prokaryotes to Clean up Our Planet: Bioremediation

Microbial **bioremediation** is the use of prokaryotes (or microbial metabolism) to remove pollutants. Bioremediation has been used to remove agricultural chemicals (e.g., pesticides, fertilizers) that leach from soil into groundwater and the subsurface. Certain toxic metals and oxides, such as selenium and arsenic compounds, can also be removed from water by bioremediation. The reduction of SeO_4^{-2} to SeO_3^{-2} and to Se^0 (metallic selenium) is a method used to remove selenium ions from water. Mercury (Hg) is an example of a toxic metal that can be removed from an environment by bioremediation. As an active ingredient of some pesticides, mercury is used in industry and is also a by-product of certain processes, such as battery production. Methyl mercury is usually present in very low concentrations in natural environments, but it is highly toxic because it accumulates in living tissues. Several species of bacteria can carry out the biotransformation of toxic mercury into nontoxic forms. These bacteria, such as *Pseudomonas aeruginosa*, can convert Hg^{+2} into Hg^0, which is nontoxic to humans.

One of the most useful and interesting examples of the use of prokaryotes for bioremediation purposes is the cleanup of oil spills. The significance of prokaryotes to petroleum bioremediation has been demonstrated in several oil spills in recent years, such as the Exxon Valdez spill in Alaska (1989) (**Figure 8.25**), the Prestige oil spill in Spain (2002), the spill into the Mediterranean from a Lebanon power plant (2006), and more recently, the BP oil spill in the Gulf of Mexico (2010). In the case of oil spills in the ocean, ongoing natural bioremediation tends to occur, since there are oil-consuming bacteria in the ocean prior to the spill. In addition to these naturally

occurring oil-degrading bacteria, humans select and engineer bacteria that possess the same capability with increased efficacy and spectrum of hydrocarbon compounds that can be processed. Bioremediation is enhanced by the addition of inorganic nutrients that help bacteria to grow.

Some hydrocarbon-degrading bacteria feed on hydrocarbons in the oil droplet, breaking down the hydrocarbons into smaller subunits. Some species, such as *Alcanivorax borkumensis*, produce surfactants that *solubilize* the oil (making it soluble in water), whereas other bacteria degrade the oil into carbon dioxide. Under ideal conditions, it has been reported that up to 80 percent of the nonvolatile components in oil can be degraded within one year of the spill. Other oil fractions containing aromatic and highly branched hydrocarbon chains are more difficult to remove and remain in the environment for longer periods of time.

(a)

(b)

Figure 8.25 Prokaryotes and bioremediation. (a) Cleaning up oil after the Exxon Valdez spill in Alaska, workers hosed oil from beaches and then used a floating boom to corral the oil, which was finally skimmed from the water surface. Some species of bacteria are able to solubilize and degrade the oil. (b) One of the most catastrophic consequences of oil spills is the damage to fauna. (credit a: modification of work by NOAA; credit b: modification of work by GOLUBENKOV, NGO: Saving Taman)

KEY TERMS

biological nitrogen fixation conversion of atmospheric nitrogen into ammonia exclusively carried out by prokaryotes

bioremediation use of microbial metabolism to remove pollutants

biotechnology any technological application that uses living organisms, biological systems, or their derivatives to produce or modify other products

nodule novel structure on the roots of certain plants (legumes) that results from the symbiotic interaction between the plant and soil bacteria, and is the site of nitrogen fixation

CHAPTER SUMMARY

8.1 What Our Ancestors Knew

- **Microorganisms** (or **microbes**) are living organisms that are generally too small to be seen without a microscope.
- Throughout history, humans have used microbes to make fermented foods such as beer, bread, cheese, and wine.
- Long before the invention of the microscope, some people theorized that infection and disease were spread by living things that were too small to be seen. They also correctly intuited certain principles regarding the spread of disease and immunity.
- Antonie van Leeuwenhoek, using a microscope, was the first to actually describe observations of bacteria, in 1675.
- During the Golden Age of Microbiology (1857–1914), microbiologists, including Louis Pasteur and Robert Koch, discovered many new connections between the fields of microbiology and medicine.

8.2 A Systematic Approach

- Carolus Linnaeus developed a taxonomic system for categorizing organisms into related groups.
- **Binomial nomenclature** assigns organisms Latinized scientific names with a genus and species designation.
- A **phylogenetic tree** is a way of showing how different organisms are thought to be related to one another from an evolutionary standpoint.
- The first phylogenetic tree contained kingdoms for plants and animals; Ernst Haeckel proposed adding kingdom for protists.
- Robert Whittaker’s tree contained five kingdoms: Animalia, Plantae, Protista, Fungi, and Monera.
- Carl Woese used small subunit ribosomal RNA to create a phylogenetic tree that groups organisms into three domains based on their genetic similarity.
- Bergey’s manuals of determinative and systemic bacteriology are the standard references for identifying and classifying bacteria, respectively.
- Bacteria can be identified through biochemical tests, DNA/RNA analysis, and serological testing methods.

8.3 Types of Microorganisms

- Microorganisms are very diverse and are found in all three domains of life: Archaea, Bacteria, and Eukarya.
- **Archaea** and **bacteria** are classified as prokaryotes because they lack a cellular nucleus. Archaea differ

from bacteria in evolutionary history, genetics, metabolic pathways, and cell wall and membrane composition.

- Archaea inhabit nearly every environment on earth, but no archaea have been identified as human pathogens.
- **Eukaryotes** studied in microbiology include algae, protozoa, fungi, and helminths.
- **Algae** are plant-like organisms that can be either unicellular or multicellular, and derive energy via photosynthesis.
- **Protozoa** are unicellular organisms with complex cell structures; most are motile.
- Microscopic **fungi** include **molds** and **yeasts**.
- **Helminths** are multicellular parasitic worms. They are included in the field of microbiology because their eggs and larvae are often microscopic.
- **Viruses** are acellular microorganisms that require a host to reproduce.
- The field of microbiology is extremely broad. Microbiologists typically specialize in one of many subfields, but all health professionals need a solid foundation in clinical microbiology.

8.4 Beneficial Prokaryotes

Pathogens are only a small percentage of all prokaryotes. In fact, prokaryotes provide essential services to humans and other organisms. Nitrogen, which is not usable by eukaryotes in its plentiful atmospheric form, can be “fixed,” or converted into ammonia (NH_3) either biologically or abiotically. Biological nitrogen fixation (BNF) is exclusively carried out by prokaryotes, and constitutes the second most important biological process on Earth. Although some terrestrial nitrogen is fixed by free-living bacteria, most BNF comes from the symbiotic interaction between soil rhizobia and the roots of legume plants.

Human life is only possible due to the action of microbes, both those in the environment and those species that call us home. Internally, they help us digest our food, produce vital nutrients for us, protect us from pathogenic microbes, and help train our immune systems to function properly.

Microbial bioremediation is the use of microbial metabolism to remove pollutants. Bioremediation has been used to remove agricultural chemicals that leach from soil into groundwater and the subsurface. Toxic metals and oxides, such as selenium and arsenic compounds, can also be removed by bioremediation. Probably one of the most useful and interesting examples of the use of prokaryotes for bioremediation purposes is the cleanup of oil spills.

REVIEW QUESTIONS

1. Which of the following foods is NOT made by fermentation?

A. beer
B. bread
C. cheese
D. orange juice

2. Who is considered the “father of Western medicine”?

A. Marcus Terentius Varro
B. Thucydides
C. Antonie van Leeuwenhoek
D. Hippocrates

3. Who was the first to observe “animalcules” under the microscope?

A. Antonie van Leeuwenhoek
B. Ötzi the Iceman
C. Marcus Terentius Varro
D. Robert Koch

4. Who proposed that swamps might harbor tiny, disease-causing animals too small to see?

A. Thucydides
B. Marcus Terentius Varro
C. Hippocrates
D. Louis Pasteur

5. Which of the following was NOT a kingdom in Linnaeus’s taxonomy?

A. animal
B. mineral
C. protist
D. plant

6. Which of the following is a correct usage of binomial nomenclature?
   A. Homo Sapiens
   B. *homo sapiens*
   C. *Homo sapiens*
   D. *Homo Sapiens*

7. Which scientist proposed adding a kingdom for protists?
   A. Carolus Linnaeus
   B. Carl Woese
   C. Robert Whittaker
   D. Ernst Haeckel

8. Which of the following is NOT a domain in Woese and Fox's phylogenetic tree?
   A. Plantae
   B. Bacteria
   C. Archaea
   D. Eukarya

9. Which of the following is the standard resource for identifying bacteria?
   A. *Systema Naturae*
   B. Bergey's *Manual of Determinative Bacteriology*
   C. Woese and Fox's phylogenetic tree
   D. Haeckel's *General Morphology of Organisms*

10. Which of the following types of microorganisms is photosynthetic?
   A. yeast
   B. virus
   C. helminth
   D. alga

11. Which of the following is a prokaryotic microorganism?
   A. helminth
   B. protozoan
   C. cyanobacterium
   D. mold

12. Which of the following is acellular?
   A. virus
   B. bacterium
   C. fungus
   D. protozoan

13. Which of the following is a type of fungal microorganism?
   A. bacterium
   B. protozoan
   C. alga
   D. yeast

14. Which of the following is not a subfield of microbiology?
   A. bacteriology
   B. botany
   C. clinical microbiology
   D. virology

15. Which of these occurs through symbiotic nitrogen fixation?
   a. The plant benefits from using an endless source of nitrogen.
   b. The soil benefits from being naturally fertilized.
   c. Bacteria benefit from using photosynthates from the plant.
   d. All of the above occur.

16. Synthetic compounds found in an organism but not normally produced or expected to be present in that organism are called _____.
   a. pesticides
   b. bioremediators
   c. recalcitrant compounds
   d. xenobiotics

17. Bioremediation includes _____.
   a. the use of prokaryotes that can fix nitrogen
   b. the use of prokaryotes to clean up pollutants
   c. the use of prokaryotes as natural fertilizers
   d. All of the above

18. In addition to providing yogurt with its unique flavor and texture, lactic acid-producing bacteria also provide which additional benefit during food production?
   a. Providing xenobiotics
   b. Lowering the pH to kill pathogenic bacteria
   c. Pasteurizing milk products
   d. Breaking down lactose for lactose-intolerant individuals

FILL IN THE BLANK

8.1 What Our Ancestors Knew

19. Thucydides is known as the father of _______________.

20. Researchers think that Ötzi the Iceman may have been infected with _____ disease.

21. The process by which microbes turn grape juice into wine is called _______________.

8.2 A Systematic Approach

22. In binomial nomenclature, an organism's scientific name includes its ________ and __________.

23. Whittaker proposed adding the kingdoms ________ and ________ to his phylogenetic tree.

24. __________ are organisms without membrane-bound nuclei.

25. ______ are microorganisms that are not included in phylogenetic trees because they are acellular.

8.3 Types of Microorganisms

26. A ________ is a disease-causing microorganism.

27. Multicellular parasitic worms studied by microbiologists are called ___________.

28. The study of viruses is ___________.

29. The cells of prokaryotic organisms lack a _______.

SHORT ANSWER

8.1 What Our Ancestors Knew

30. What did Thucydides learn by observing the Athenian plague?

31. Why was the invention of the microscope important for microbiology?

32. What are some ways people use microbes?

8.2 A Systematic Approach

33. What is a phylogenetic tree?

34. Which of the five kingdoms in Whittaker's phylogenetic tree are prokaryotic, and which are eukaryotic?

35. What molecule did Woese and Fox use to construct their phylogenetic tree?

36. Name some techniques that can be used to identify and differentiate species of bacteria.

8.3 Types of Microorganisms

37. Describe the differences between bacteria and archaea.

38. Name three structures that various protozoa use for locomotion.

39. Describe the actual and relative sizes of a virus, a bacterium, and a plant or animal cell.

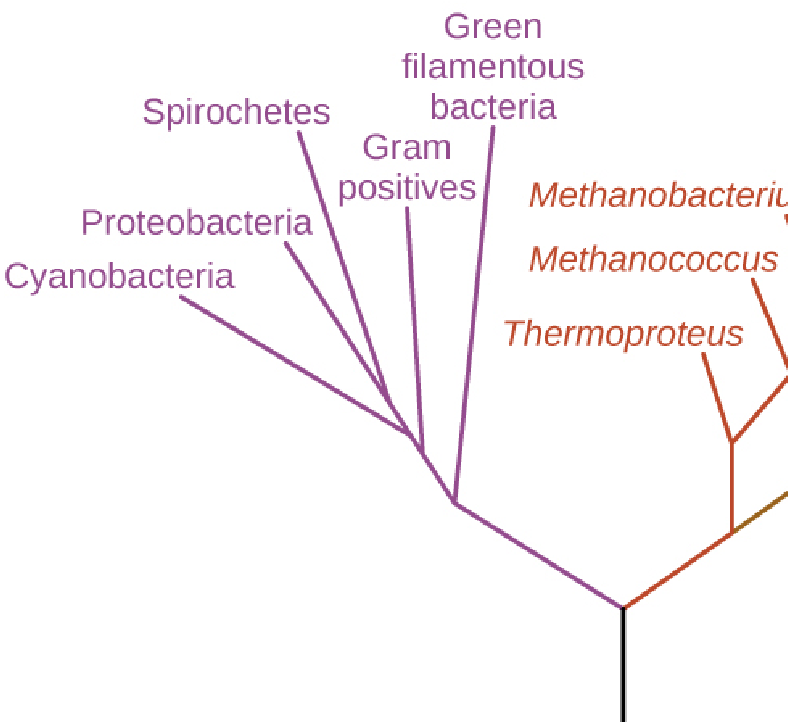
CRITICAL THINKING

40. Explain how the discovery of fermented foods likely benefited our ancestors.

41. What evidence would you use to support this statement: Ancient people thought that disease was transmitted by things they could not see.

42. Why is using binomial nomenclature more useful than using common names?

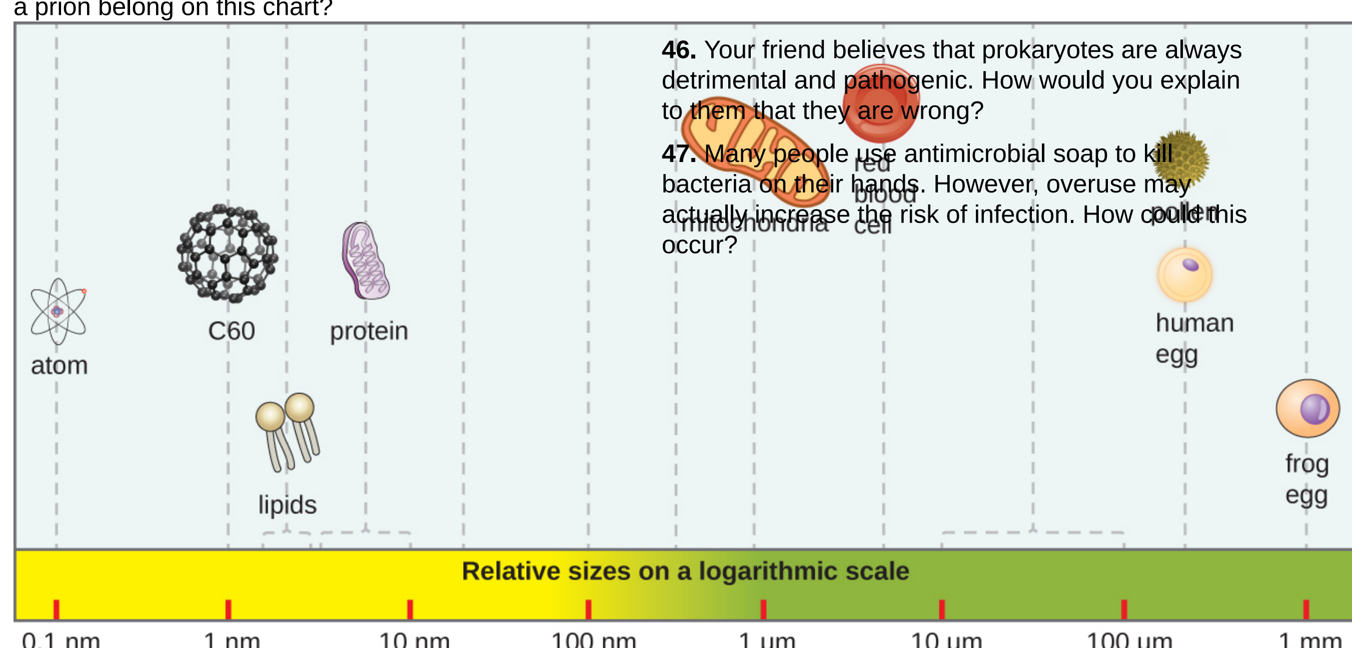
43. Label the three Domains found on modern phylogenetic trees.



44. Contrast the behavior of a virus outside versus inside a cell.

45. Where would a virus, bacterium, animal cell, and

a prion belong on this chart?



46. Your friend believes that prokaryotes are always detrimental and pathogenic. How would you explain to them that they are wrong?

47. Many people use antimicrobial soap to kill bacteria on their hands. However, overuse may actually increase the risk of infection. How could this occur?

9 | MICROBIAL TECHNIQUES

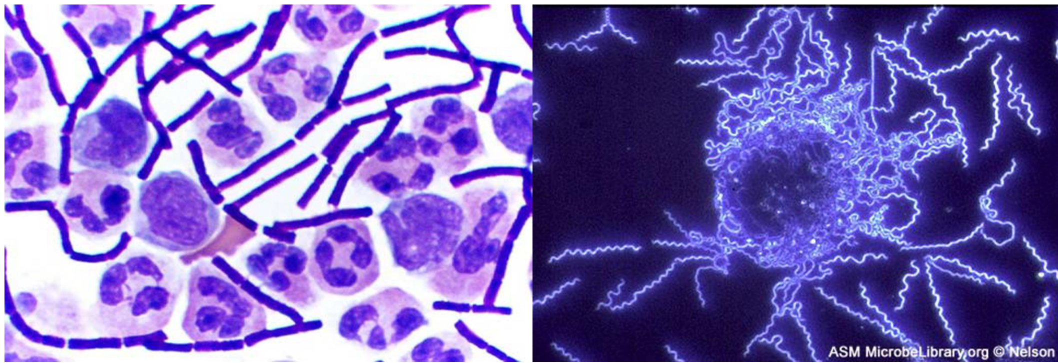


Figure 9.1 Different types of microscopy are used to visualize different structures. Brightfield microscopy (left) renders a darker image on a lighter background, producing a clear image of these *Bacillus anthracis* cells in cerebrospinal fluid (the rod-shaped bacterial cells are surrounded by larger white blood cells). Darkfield microscopy (right) increases contrast, rendering a brighter image on a darker background, as demonstrated by this image of the bacterium *Borrelia burgdorferi*, which causes Lyme disease. (credit left: modification of work by Centers for Disease Control and Prevention; credit right: modification of work by American Society for Microbiology)

Chapter Outline

9.1: The Properties of Light
9.2: Instruments of Microscopy
9.3: Staining Microscopic Specimens

Introduction

When we look at a rainbow, its colors span the full spectrum of light that the human eye can detect and differentiate. Each hue represents a different frequency of visible light, processed by our eyes and brains and rendered as red, orange, yellow, green, or one of the many other familiar colors that have always been a part of the human experience. But only recently have humans developed an understanding of the properties of light that allow us to see images in color.

Over the past several centuries, we have learned to manipulate light to peer into previously invisible worlds—those too small or too far away to be seen by the naked eye. Through a microscope, we can examine microbial cells and colonies, using various techniques to manipulate color, size, and contrast in ways that help us identify species and diagnose disease.

Figure 9.1 illustrates how we can apply the properties of light to visualize and magnify images; but these stunning micrographs are just two examples of the numerous types of images we are now able to produce with different microscopic technologies. This chapter explores how various types of microscopes manipulate light in order to provide a window into the world of microorganisms. By understanding how various kinds of microscopes work, we can produce highly detailed images of microbes that can be useful for both research and clinical applications.

9.1 | The Properties of Light

Learning Objectives

By the end of this section, you will be able to:

- Identify and define the characteristics of electromagnetic radiation (EMR) used in microscopy
- Explain how lenses are used in microscopy to manipulate visible and ultraviolet (UV) light

Part 1

Cindy, a 17-year-old counselor at a summer sports camp, scraped her knee playing basketball 2 weeks ago. At the time, she thought it was only a minor abrasion that would heal, like many others before it. Instead, the wound began to look like an insect bite and has continued to become increasingly painful and swollen.

The camp nurse examines the lesion and observes a large amount of pus oozing from the surface. Concerned that Cindy may have developed a potentially aggressive infection, she swabs the wound to collect a sample from the infection site. Then she cleans out the pus and dresses the wound, instructing Cindy to keep the area clean and to come back the next day. When Cindy leaves, the nurse sends the sample to the closest medical lab to be analyzed under a microscope.

- What are some things we can learn about these bacteria by looking at them under a microscope?

*Jump to the **next** Clinical Focus box.*

Visible light consists of electromagnetic waves that behave like other waves. Hence, many of the properties of light that are relevant to microscopy can be understood in terms of light's behavior as a wave. An important property of light waves is the **wavelength**, or the distance between one peak of a wave and the next peak. The height of each peak (or depth of each trough) is called the **amplitude**. In contrast, the **frequency** of the wave is the rate of vibration of the wave, or the number of wavelengths within a specified time period (Figure 9.2).

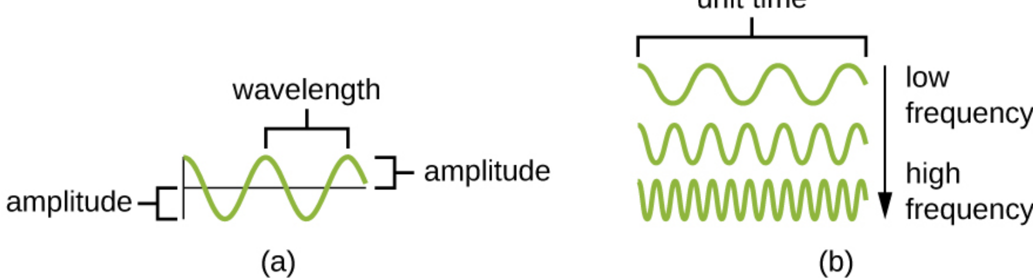
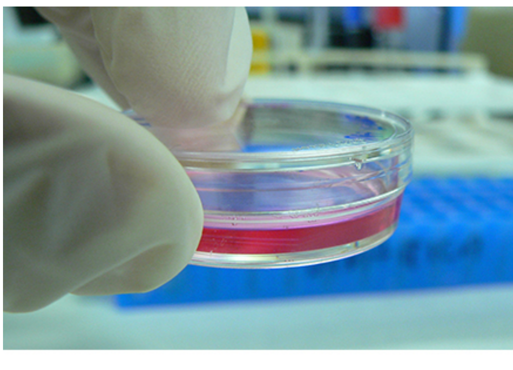


Figure 9.2 (a) The amplitude is the height of a wave, whereas the wavelength is the distance between one peak and the next. (b) These waves have different frequencies, or rates of vibration. The wave at the top has the lowest frequency, since it has the fewest peaks per unit time. The wave at the bottom has the highest frequency.

Interactions of Light

Light waves interact with materials by being reflected, absorbed, or transmitted. **Reflection** occurs when a wave bounces off of a material. For example, a red piece of cloth may reflect red light to our eyes while absorbing other colors of light. **Absorbance** occurs when a material captures the energy of a light wave. In the case of glow-in-the-dark plastics, the energy from light can be absorbed and then later re-emitted as another form of phosphorescence. Transmission occurs when a wave travels through a material, like light through glass (the process of transmission is called **transmittance**). When a material allows a large proportion of light to be transmitted, it may do so because it is thinner, or more transparent (having more **transparency** and less **opacity**). Figure 9.3 illustrates the difference between transparency and opacity.



(a) (b)

Figure 9.3 (a) A Petri dish is made of transparent plastic or glass, which allows transmission of a high proportion of light. This transparency allows us to see through the sides of the dish to view the contents. (b) This slice of an iron meteorite is opaque (i.e., it has opacity). Light is not transmitted through the material, making it impossible to see the part of the hand covered by the object. (credit a: modification of work by Umberto Salvagnin; credit b: modification of work by "Waifer X"/Flickr)

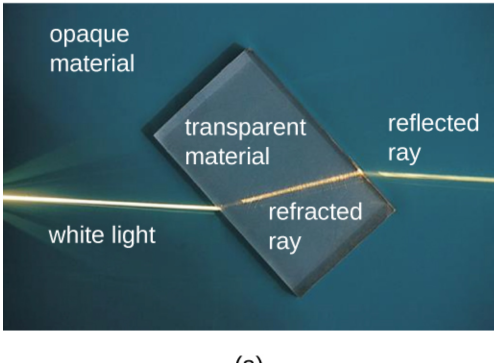
Light waves can also interact with each other by **interference**, creating complex patterns of motion. Dropping two pebbles into a puddle causes the waves on the puddle's surface to interact, creating complex interference patterns. Light waves can interact in the same way.

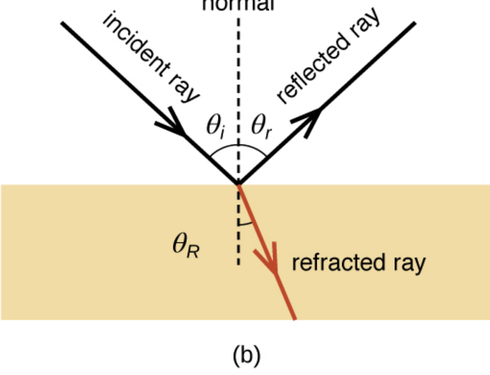
In addition to interfering with each other, light waves can also interact with small objects or openings by bending or scattering. This is called **diffraction**. Diffraction is larger when the object is smaller relative to the wavelength of the light (the distance between two consecutive peaks of a light wave). Often, when waves diffract in different directions around an obstacle or opening, they will interfere with each other.

- If a light wave has a long wavelength, is it likely to have a low or high frequency?
- If an object is transparent, does it reflect, absorb, or transmit light?

Lenses and Refraction

In the context of microscopy, **refraction** is perhaps the most important behavior exhibited by light waves. Refraction occurs when light waves change direction as they enter a new medium (Figure 9.4). Different transparent materials transmit light at different speeds; thus, light can change speed when passing from one material to another. This change in speed usually also causes a change in direction (refraction), with the degree of change dependent on the angle of the incoming light.





(a) (b)

Figure 9.4 (a) Refraction occurs when light passes from one medium, such as air, to another, such as glass, changing the direction of the light rays. (b) As shown in this diagram, light rays passing from one medium to another may be either refracted or reflected.

The extent to which a material slows transmission speed relative to empty space is called the **refractive index** of that material. Large differences between the refractive indices of two materials will result in a large amount of refraction when light passes from one material to the other. For example, light moves much more slowly through

water than through air, so light entering water from air can change direction greatly. We say that the water has a higher refractive index than air (**Figure 9.5**).

Figure 9.5 This straight pole appears to bend at an angle as it enters the water. This optical illusion is due to the large difference between the refractive indices of air and water.

When light crosses a boundary into a material with a higher refractive index, its direction turns to be closer to perpendicular to the boundary (i.e., more toward a normal to that boundary; see **Figure 9.5**). This is the principle behind lenses. We can think of a lens as an object with a curved boundary (or a collection of prisms) that collects all of the light that strikes it and refracts it so that it all meets at a single point called the **image point (focus)**. A convex lens can be used to magnify because it can focus at closer range than the human eye, producing a larger image. Concave lenses and mirrors can also be used in microscopes to redirect the light path. **Figure 9.6** shows the **focal point** (the image point when light entering the lens is parallel) and the **focal length** (the distance to the focal point) for convex and concave lenses.

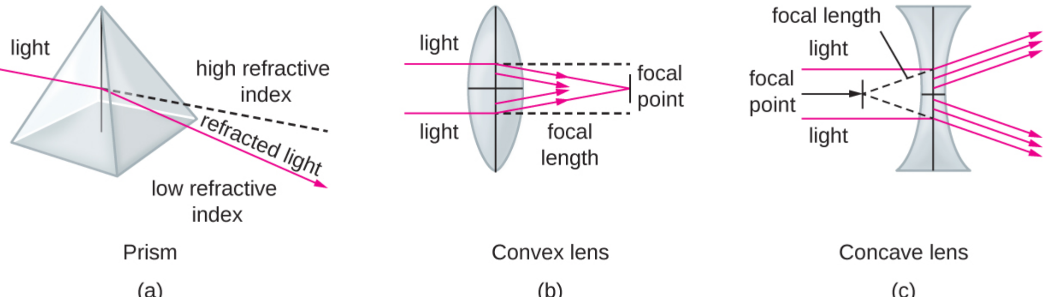


Figure 9.6 (a) A lens is like a collection of prisms, such as the one shown here. (b) When light passes through a convex lens, it is refracted toward a focal point on the other side of the lens. The focal length is the distance to the focal point. (c) Light passing through a concave lens is refracted away from a focal point in front of the lens.

The human eye contains a lens that enables us to see images. This lens focuses the light reflecting off of objects in front of the eye onto the surface of the retina, which is like a screen in the back of the eye. Artificial lenses placed in front of the eye (contact lenses, glasses, or microscopic lenses) focus light before it is focused (again) by the lens of the eye, manipulating the image that ends up on the retina (e.g., by making it appear larger).

Images are commonly manipulated by controlling the distances between the object, the lens, and the screen, as well as the curvature of the lens. For example, for a given amount of curvature, when an object is closer to the lens, the focal points are farther from the lens. As a result, it is often necessary to manipulate these distances to create a focused image on a screen. Similarly, more curvature creates image points closer to the lens and a larger image when the image is in focus. This property is often described in terms of the focal distance, or

distance to the focal point.

- Explain how a lens focuses light at the image point.
- Name some factors that affect the focal length of a lens.

Electromagnetic Spectrum and Color

Visible light is just one form of electromagnetic radiation (EMR), a type of energy that is all around us. Other forms of EMR include microwaves, X-rays, and radio waves, among others. The different types of EMR fall on the electromagnetic spectrum, which is defined in terms of wavelength and frequency. The spectrum of visible light occupies a relatively small range of frequencies between infrared and ultraviolet light (Figure 9.7).

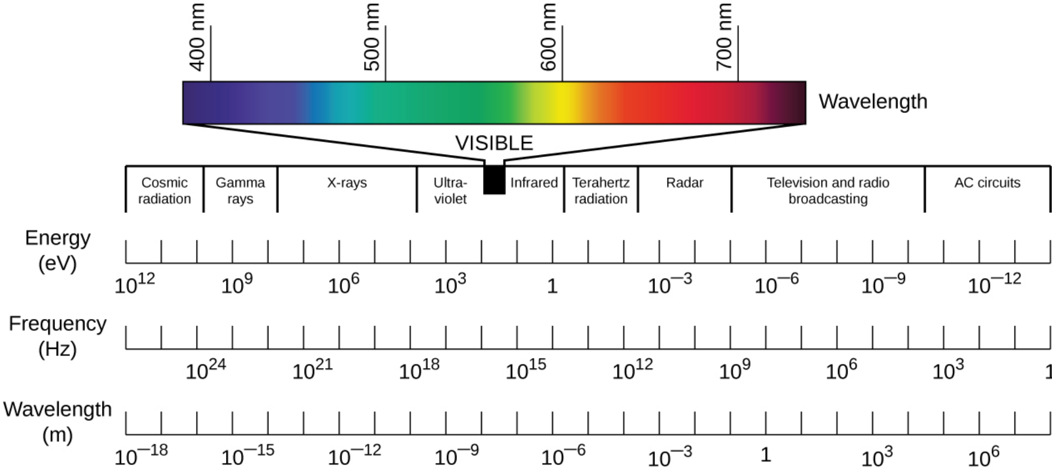


Figure 9.7 The electromagnetic spectrum ranges from high-frequency gamma rays to low-frequency radio waves. Visible light is the relatively small range of electromagnetic frequencies that can be sensed by the human eye. On the electromagnetic spectrum, visible light falls between ultraviolet and infrared light. (credit: modification of work by Johannes Ahlmann)

Whereas wavelength represents the distance between adjacent peaks of a light wave, frequency, in a simplified definition, represents the rate of oscillation. Waves with higher frequencies have shorter wavelengths and, therefore, have more oscillations per unit time than lower-frequency waves. Higher-frequency waves also contain more energy than lower-frequency waves. This energy is delivered as elementary particles called photons. Higher-frequency waves deliver more energetic photons than lower-frequency waves.

Photons with different energies interact differently with the retina. In the spectrum of visible light, each color corresponds to a particular frequency and wavelength (Figure 9.7).The lowest frequency of visible light appears as the color red, whereas the highest appears as the color violet. When the retina receives visible light of many different frequencies, we perceive this as white light. However, white light can be separated into its component colors using refraction. If we pass white light through a prism, different colors will be refracted in different directions, creating a rainbow-like spectrum on a screen behind the prism. This separation of colors is called **dispersion**, and it occurs because, for a given material, the refractive index is different for different frequencies of light.

Certain materials can refract nonvisible forms of EMR and, in effect, transform them into visible light. Certain **fluorescent** dyes, for instance, absorb ultraviolet or blue light and then use the energy to emit photons of a different color, giving off light rather than simply vibrating. This occurs because the energy absorption causes electrons to jump to higher energy states, after which they then almost immediately fall back down to their ground states, emitting specific amounts of energy as photons. Not all of the energy is emitted in a given photon, so the emitted photons will be of lower energy and, thus, of lower frequency than the absorbed ones. Thus, a dye such as Texas red may be excited by blue light, but emit red light; or a dye such as fluorescein isothiocyanate (FITC) may absorb (invisible) high-energy ultraviolet light and emit green light (Figure 9.8). In some materials, the photons may be emitted following a delay after absorption; in this case, the process is called **phosphorescence**. Glow-in-the-dark plastic works by using phosphorescent material.

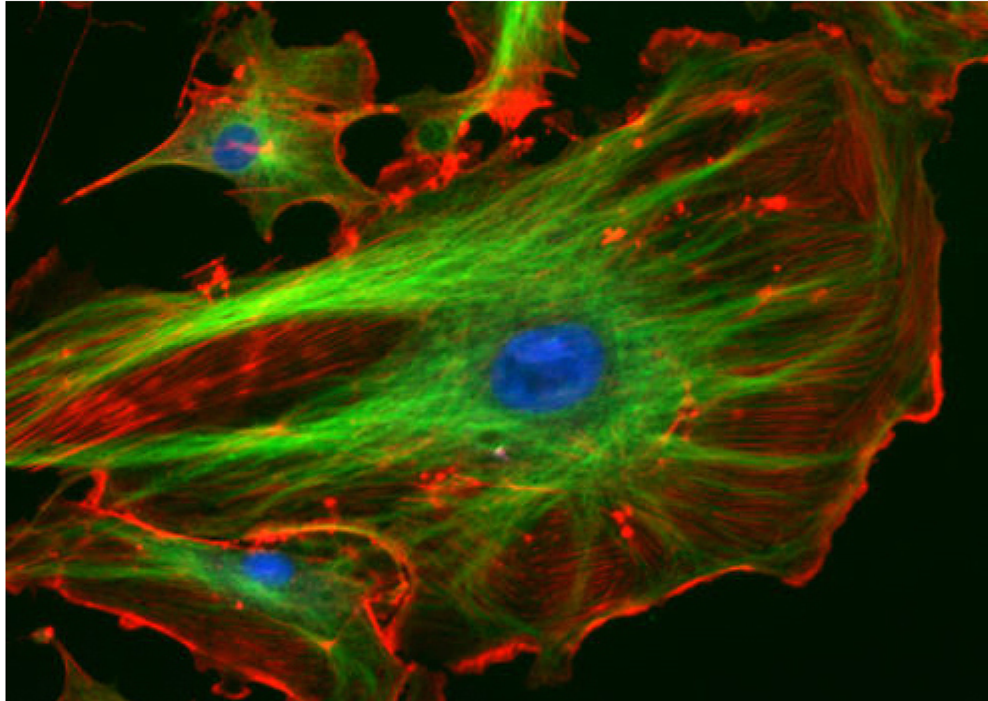


Figure 9.8 The fluorescent dyes absorbed by these bovine pulmonary artery endothelial cells emit brilliant colors when excited by ultraviolet light under a fluorescence microscope. Various cell structures absorb different dyes. The nuclei are stained blue with 4’,6-diamidino-2-phenylindole (DAPI); microtubles are marked green by an antibody bound to FITC; and actin filaments are labeled red with phalloidin bound to tetramethylrhodamine (TRITC). (credit: National Institutes of Health)

- Which has a higher frequency: red light or green light?
- Explain why dispersion occurs when white light passes through a prism.
- Why do fluorescent dyes emit a different color of light than they absorb?

Magnification, Resolution, and Contrast

Microscopes magnify images and use the properties of light to create useful images of small objects. **Magnification** is defined as the ability of a lens to enlarge the image of an object when compared to the real object. For example, a magnification of 10⨯ means that the image appears 10 times the size of the object as viewed with the naked eye.

Greater magnification typically improves our ability to see details of small objects, but magnification alone is not sufficient to make the most useful images. It is often useful to enhance the **resolution** of objects: the ability to tell that two separate points or objects are separate. A low-resolution image appears fuzzy, whereas a high-resolution image appears sharp. Two factors affect resolution. The first is wavelength. Shorter wavelengths are able to resolve smaller objects; thus, an electron microscope has a much higher resolution than a light microscope, since it uses an electron beam with a very short wavelength, as opposed to the long-wavelength visible light used by a light microscope. The second factor that affects resolution is **numerical aperture**, which is a measure of a lens’s ability to gather light. The higher the numerical aperture, the better the resolution.

Read this **article (https://www.openstax.org/l/22aperture)** to learn more about factors that can increase or decrease the numerical aperture of a lens.

Even when a microscope has high resolution, it can be difficult to distinguish small structures in many specimens because microorganisms are relatively transparent. It is often necessary to increase **contrast** to detect different structures in a specimen. Various types of microscopes use different features of light or electrons to increase contrast—visible differences between the parts of a specimen (see **Instruments of Microscopy**). Additionally, dyes that bind to some structures but not others can be used to improve the contrast between images of

relatively transparent objects (see **Staining Microscopic Specimens**).

- Explain the difference between magnification and resolution.
- Explain the difference between resolution and contrast.
- Name two factors that affect resolution.

9.2 | Instruments of Microscopy

Learning Objectives

By the end of this section, you will be able to:

- Identify and describe the parts of a brightfield microscope
- Calculate total magnification for a compound microscope
- Describe the distinguishing features and typical uses for various types of light microscopes, electron microscopes, and scanning probe microscopes

The early pioneers of microscopy opened a window into the invisible world of microorganisms. But microscopy continued to advance in the centuries that followed. In 1830, Joseph Jackson Lister created an essentially modern light microscope. The 20th century saw the development of microscopes that leveraged nonvisible light, such as fluorescence microscopy, which uses an ultraviolet light source, and electron microscopy, which uses short-wavelength electron beams. These advances led to major improvements in magnification, resolution, and contrast. By comparison, the relatively rudimentary microscopes of van Leeuwenhoek and his contemporaries were far less powerful than even the most basic microscopes in use today. In this section, we will survey the broad range of modern microscopic technology and common applications for each type of microscope.

Light Microscopy

Many types of microscopes fall under the category of light microscopes, which use light to visualize images. Examples of light microscopes include brightfield microscopes, darkfield microscopes, phase-contrast microscopes, differential interference contrast microscopes, fluorescence microscopes, confocal scanning laser microscopes, and two-photon microscopes. These various types of light microscopes can be used to complement each other in diagnostics and research.

Brightfield Microscopes

The **brightfield microscope**, perhaps the most commonly used type of microscope, is a compound microscope with two or more lenses that produce a dark image on a bright background. Some brightfield microscopes are **monocular** (having a single eyepiece), though most newer brightfield microscopes are **binocular** (having two eyepieces), like the one shown in **Figure 9.9**; in either case, each eyepiece contains a lens called an **ocular lens**. The ocular lenses typically magnify images 10 times (10⨯). At the other end of the body tube are a set of **objective lenses** on a rotating nosepiece. The magnification of these objective lenses typically ranges from 4⨯ to 100⨯, with the magnification for each lens designated on the metal casing of the lens. The ocular and objective lenses work together to create a magnified image. The **total magnification** is the product of the ocular magnification times the objective magnification:

$$\text{ocular magnification} \times \text{objective magnification}$$

For example, if a 40⨯ objective lens is selected and the ocular lens is 10⨯, the total magnification would be

$$(40\times)(10\times) = 400\times$$

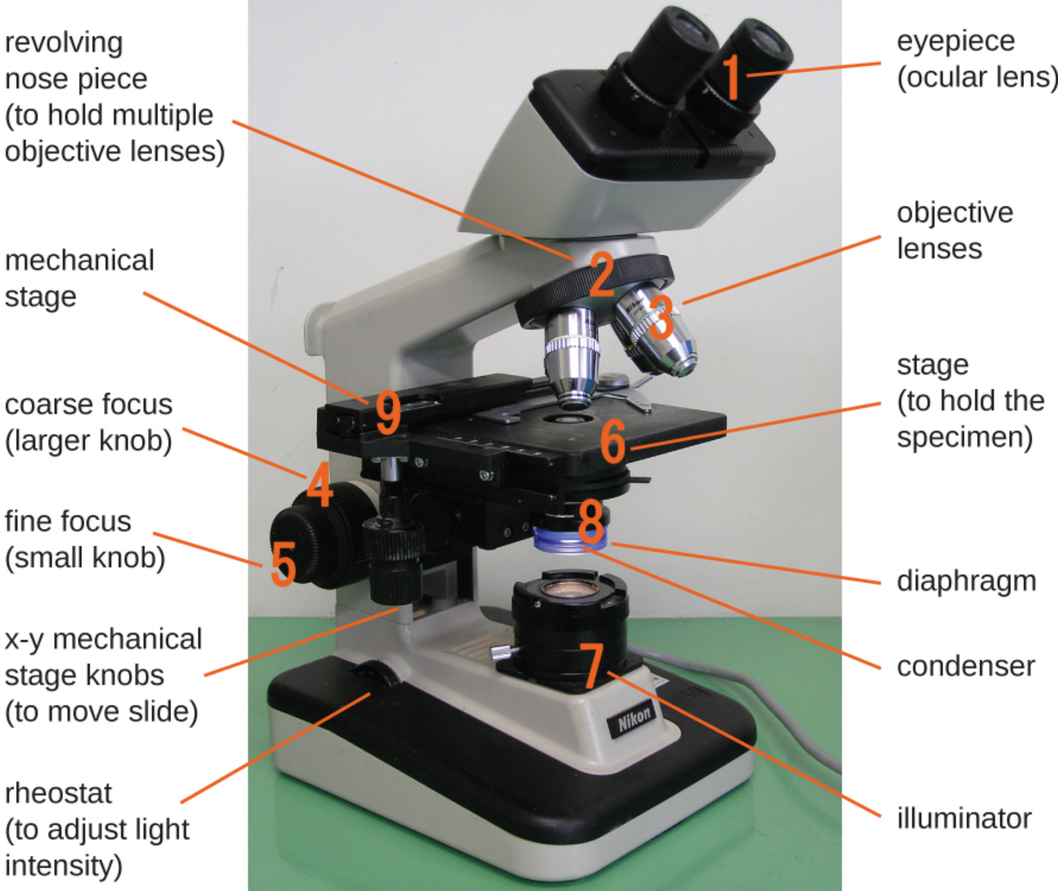


Figure 9.9 Components of a typical brightfield microscope.

The item being viewed is called a specimen. The specimen is placed on a glass slide, which is then clipped into place on the **stage** (a platform) of the microscope. Once the slide is secured, the specimen on the slide is positioned over the light using the **x-y mechanical stage knobs**. These knobs move the slide on the surface of the stage, but do not raise or lower the stage. Once the specimen is centered over the light, the stage position can be raised or lowered to focus the image. The **coarse focusing knob** is used for large-scale movements with 4× and 10× objective lenses; the **fine focusing knob** is used for small-scale movements, especially with 40× or 100× objective lenses.

When images are magnified, they become dimmer because there is less light per unit area of image. Highly magnified images produced by microscopes, therefore, require intense lighting. In a brightfield microscope, this light is provided by an **illuminator**, which is typically a high-intensity bulb below the stage. Light from the illuminator passes up through **condenser lens** (located below the stage), which focuses all of the light rays on the specimen to maximize illumination. The position of the condenser can be optimized using the attached condenser focus knob; once the optimal distance is established, the condenser should not be moved to adjust the brightness. If less-than-maximal light levels are needed, the amount of light striking the specimen can be easily adjusted by opening or closing a **diaphragm** between the condenser and the specimen. In some cases, brightness can also be adjusted using the **rheostat**, a dimmer switch that controls the intensity of the illuminator.

A brightfield microscope creates an image by directing light from the illuminator at the specimen; this light is differentially transmitted, absorbed, reflected, or refracted by different structures. Different colors can behave differently as they interact with **chromophores** (pigments that absorb and reflect particular wavelengths of light) in parts of the specimen. Often, chromophores are artificially added to the specimen using stains, which serve to increase contrast and resolution. In general, structures in the specimen will appear darker, to various extents, than the bright background, creating maximally sharp images at magnifications up to about 1000×. Further magnification would create a larger image, but without increased resolution. This allows us to see objects as small as bacteria, which are visible at about 400× or so, but not smaller objects such as viruses.

At very high magnifications, resolution may be compromised when light passes through the small amount of air between the specimen and the lens. This is due to the large difference between the refractive indices of air and

glass; the air scatters the light rays before they can be focused by the lens. To solve this problem, a drop of oil can be used to fill the space between the specimen and an **oil immersion lens**, a special lens designed to be used with immersion oils. Since the oil has a refractive index very similar to that of glass, it increases the maximum angle at which light leaving the specimen can strike the lens. This increases the light collected and, thus, the resolution of the image (Figure 9.10). A variety of oils can be used for different types of light.

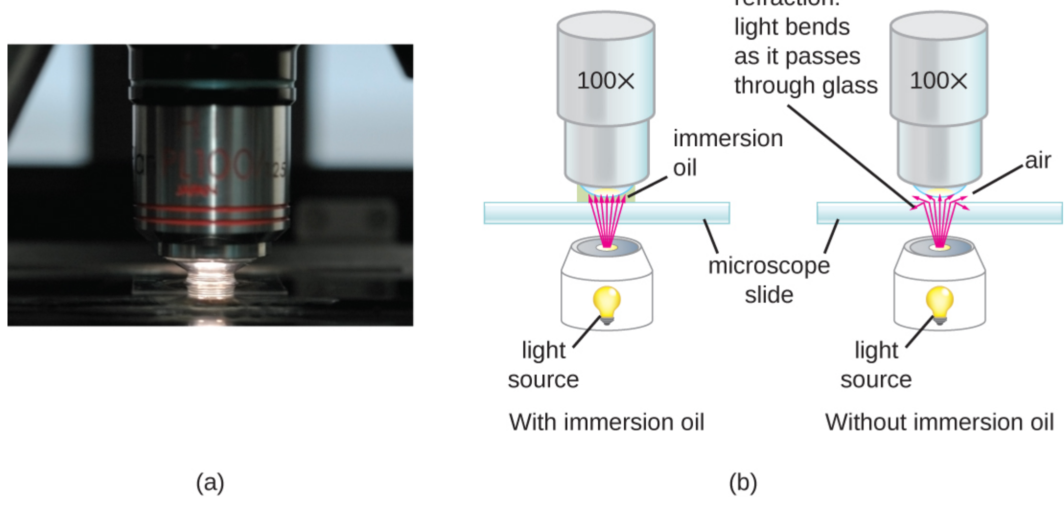


Figure 9.10 (a) Oil immersion lenses like this one are used to improve resolution. (b) Because immersion oil and glass have very similar refractive indices, there is a minimal amount of refraction before the light reaches the lens. Without immersion oil, light scatters as it passes through the air above the slide, degrading the resolution of the image.

Microscope Maintenance: Best Practices

Even a very powerful microscope cannot deliver high-resolution images if it is not properly cleaned and maintained. Since lenses are carefully designed and manufactured to refract light with a high degree of precision, even a slightly dirty or scratched lens will refract light in unintended ways, degrading the image of the specimen. In addition, microscopes are rather delicate instruments, and great care must be taken to avoid damaging parts and surfaces. Among other things, proper care of a microscope includes the following:

- cleaning the lenses with lens paper
- not allowing lenses to contact the slide (e.g., by rapidly changing the focus)
- protecting the bulb (if there is one) from breakage
- not pushing an objective into a slide
- not using the coarse focusing knob when using the 40× or greater objective lenses
- only using immersion oil with a specialized oil objective, usually the 100× objective
- cleaning oil from immersion lenses after using the microscope
- cleaning any oil accidentally transferred from other lenses
- covering the microscope or placing it in a cabinet when not in use

Visit the online resource linked below for simulations and demonstrations involving the use of microscopes. Keep in mind that execution of specific techniques and procedures can vary depending on the specific instrument you are using. Thus, it is important to learn and practice with an actual microscope in a laboratory setting under expert supervision.

- University of Delaware's Virtual Microscope (https://www.openstax.org/l/22virtualsim)

Darkfield Microscopy

A **darkfield microscope** is a brightfield microscope that has a small but significant modification to the condenser. A small, opaque disk (about 1 cm in diameter) is placed between the illuminator and the condenser

lens. This opaque light stop, as the disk is called, blocks most of the light from the illuminator as it passes through the condenser on its way to the objective lens, producing a hollow cone of light that is focused on the specimen. The only light that reaches the objective is light that has been refracted or reflected by structures in the specimen. The resulting image typically shows bright objects on a dark background (**Figure 9.11**).

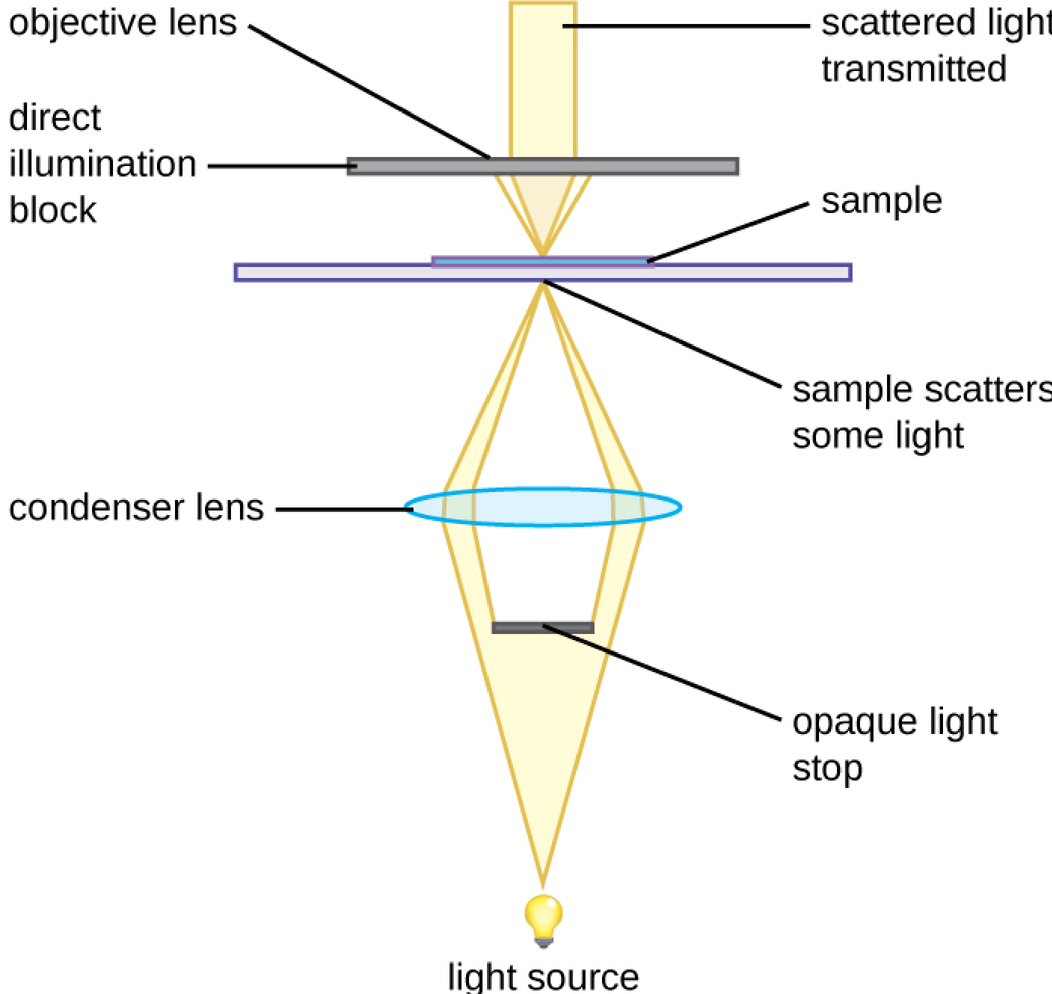


Figure 9.11 An opaque light stop inserted into a brightfield microscope is used to produce a darkfield image. The light stop blocks light traveling directly from the illuminator to the objective lens, allowing only light reflected or refracted off the specimen to reach the eye.

Darkfield microscopy can often create high-contrast, high-resolution images of specimens without the use of stains, which is particularly useful for viewing live specimens that might be killed or otherwise compromised by the stains. For example, thin spirochetes like *Treponema pallidum*, the causative agent of syphilis, can be best viewed using a darkfield microscope (**Figure 9.12**).

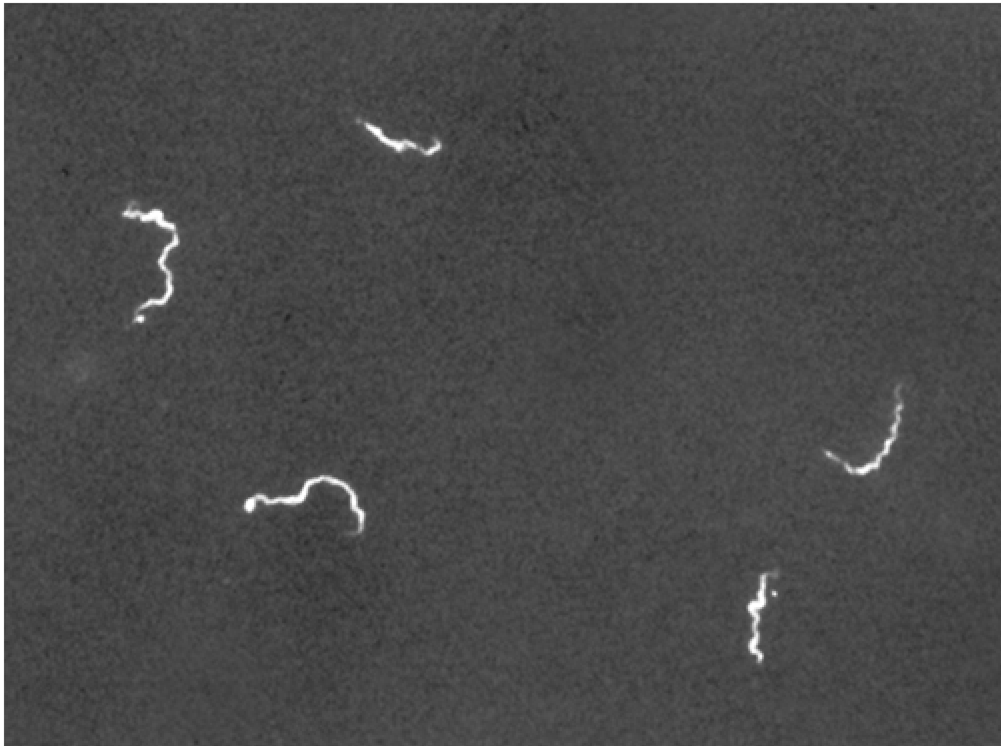


Figure 9.12 Use of a darkfield microscope allows us to view living, unstained samples of the spirochete *Treponema pallidum*. Similar to a photographic negative, the spirochetes appear bright against a dark background. (credit: Centers for Disease Control and Prevention)

- Identify the key differences between brightfield and darkfield microscopy.

Part 2

Wound infections like Cindy's can be caused by many different types of bacteria, some of which can spread rapidly with serious complications. Identifying the specific cause is very important to select a medication that can kill or stop the growth of the bacteria.

After calling a local doctor about Cindy's case, the camp nurse sends the sample from the wound to the closest medical laboratory. Unfortunately, since the camp is in a remote area, the nearest lab is small and poorly equipped. A more modern lab would likely use other methods to culture, grow, and identify the bacteria, but in this case, the technician decides to make a wet mount from the specimen and view it under a brightfield microscope. In a wet mount, a small drop of water is added to the slide, and a cover slip is placed over the specimen to keep it in place before it is positioned under the objective lens.

Under the brightfield microscope, the technician can barely see the bacteria cells because they are nearly transparent against the bright background. To increase contrast, the technician inserts an opaque light stop above the illuminator. The resulting darkfield image clearly shows that the bacteria cells are spherical and grouped in clusters, like grapes.

- Why is it important to identify the shape and growth patterns of cells in a specimen?
- What other types of microscopy could be used effectively to view this specimen?

*Jump to the **next** Clinical Focus box. Go back to the **previous** Clinical Focus box.*

Phase-Contrast Microscopes

Phase-contrast microscopes use refraction and interference caused by structures in a specimen to create high-contrast, high-resolution images without staining. It is the oldest and simplest type of microscope that creates an image by altering the wavelengths of light rays passing through the specimen. To create altered wavelength paths, an annular stop is used in the condenser. The annular stop produces a hollow cone of

light that is focused on the specimen before reaching the objective lens. The objective contains a phase plate containing a phase ring. As a result, light traveling directly from the illuminator passes through the phase ring while light refracted or reflected by the specimen passes through the plate. This causes waves traveling through the ring to be about one-half of a wavelength out of phase with those passing through the plate. Because waves have peaks and troughs, they can add together (if in phase together) or cancel each other out (if out of phase). When the wavelengths are out of phase, wave troughs will cancel out wave peaks, which is called destructive interference. Structures that refract light then appear dark against a bright background of only unrefracted light. More generally, structures that differ in features such as refractive index will differ in levels of darkness (**Figure 9.13**).

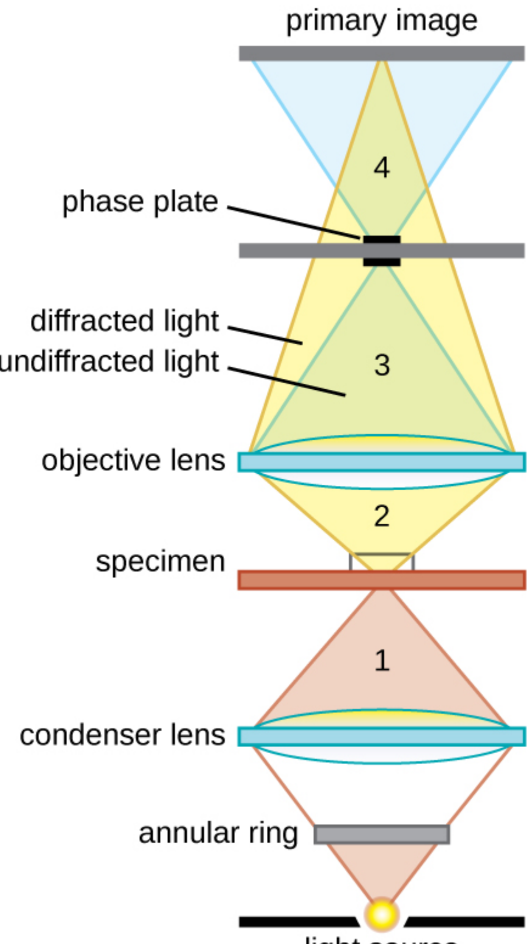


Figure 9.13 This diagram of a phase-contrast microscope illustrates phase differences between light passing through the object and background. These differences are produced by passing the rays through different parts of a phase plate. The light rays are superimposed in the image plane, producing contrast due to their interference.

Because it increases contrast without requiring stains, phase-contrast microscopy is often used to observe live specimens. Certain structures, such as organelles in eukaryotic cells and endospores in prokaryotic cells, are especially well visualized with phase-contrast microscopy (**Figure 9.14**).

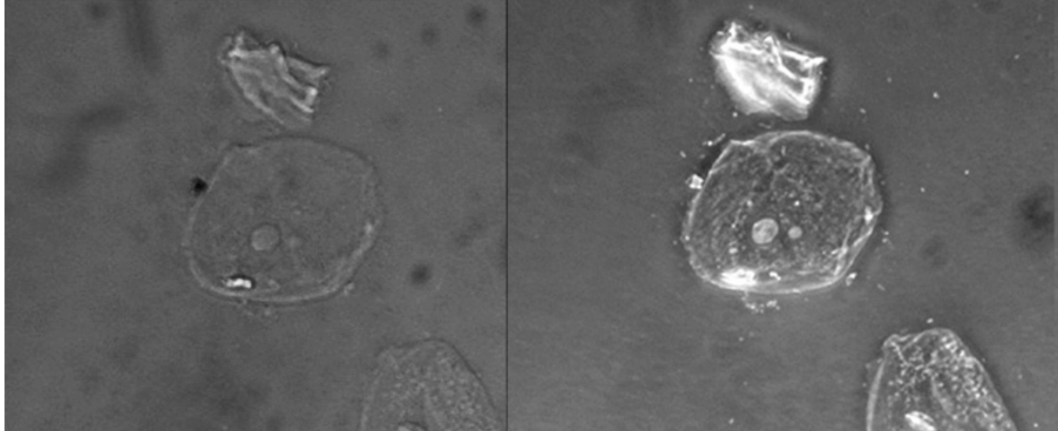


Figure 9.14 This figure compares a brightfield image (left) with a phase-contrast image (right) of the same unstained simple squamous epithelial cells. The cells are in the center and bottom right of each photograph (the irregular item above the cells is acellular debris). Notice that the unstained cells in the brightfield image are almost invisible against the background, whereas the cells in the phase-contrast image appear to glow against the background, revealing far more detail.

Differential Interference Contrast Microscopes

Differential interference contrast (DIC) microscopes (also known as Nomarski optics) are similar to phase-contrast microscopes in that they use interference patterns to enhance contrast between different features of a specimen. In a DIC microscope, two beams of light are created in which the direction of wave movement (polarization) differs. Once the beams pass through either the specimen or specimen-free space, they are recombined and effects of the specimens cause differences in the interference patterns generated by the combining of the beams. This results in high-contrast images of living organisms with a three-dimensional appearance. These microscopes are especially useful in distinguishing structures within live, unstained specimens. (**Figure 9.15**)

Figure 9.15 A DIC image of *Fonsecaea pedrosoi* grown on modified Leonian's agar. This fungus causes chromoblastomycosis, a chronic skin infection common in tropical and subtropical climates.

- What are some advantages of phase-contrast and DIC microscopy?

Fluorescence Microscopes

A **fluorescence microscope** uses fluorescent chromophores called **fluorochromes**, which are capable of absorbing energy from a light source and then emitting this energy as visible light. Fluorochromes include naturally fluorescent substances (such as chlorophylls) as well as fluorescent stains that are added to the specimen to create contrast. Dyes such as Texas red and FITC are examples of fluorochromes. Other examples include the nucleic acid dyes 4',6'-diamidino-2-phenylindole (DAPI) and acridine orange.

The microscope transmits an excitation light, generally a form of EMR with a short wavelength, such as ultraviolet or blue light, toward the specimen; the chromophores absorb the excitation light and emit visible light with longer wavelengths. The excitation light is then filtered out (in part because ultraviolet light is harmful to the eyes) so that only visible light passes through the ocular lens. This produces an image of the specimen in bright colors against a dark background.

Fluorescence microscopes are especially useful in clinical microbiology. They can be used to identify pathogens, to find particular species within an environment, or to find the locations of particular molecules and structures within a cell. Approaches have also been developed to distinguish living from dead cells using fluorescence microscopy based upon whether they take up particular fluorochromes. Sometimes, multiple fluorochromes are used on the same specimen to show different structures or features.

One of the most important applications of fluorescence microscopy is a technique called **immunofluorescence**, which is used to identify certain disease-causing microbes by observing whether antibodies bind to them. (Antibodies are protein molecules produced by the immune system that attach to specific pathogens to kill or inhibit them.) There are two approaches to this technique: direct immunofluorescence assay (DFA) and indirect immunofluorescence assay (IFA). In DFA, specific antibodies (e.g., those that the target the rabies virus) are stained with a fluorochrome. If the specimen contains the targeted pathogen, one can observe the antibodies binding to the pathogen under the fluorescent microscope. This is called a primary antibody stain because the stained antibodies attach directly to the pathogen.

In IFA, secondary antibodies are stained with a fluorochrome rather than primary antibodies. Secondary antibodies do not attach directly to the pathogen, but they do bind to primary antibodies. When the unstained primary antibodies bind to the pathogen, the fluorescent secondary antibodies can be observed binding to the primary antibodies. Thus, the secondary antibodies are attached indirectly to the pathogen. Since multiple secondary antibodies can often attach to a primary antibody, IFA increases the number of fluorescent antibodies attached to the specimen, making it easier visualize features in the specimen (**Figure 9.16**).

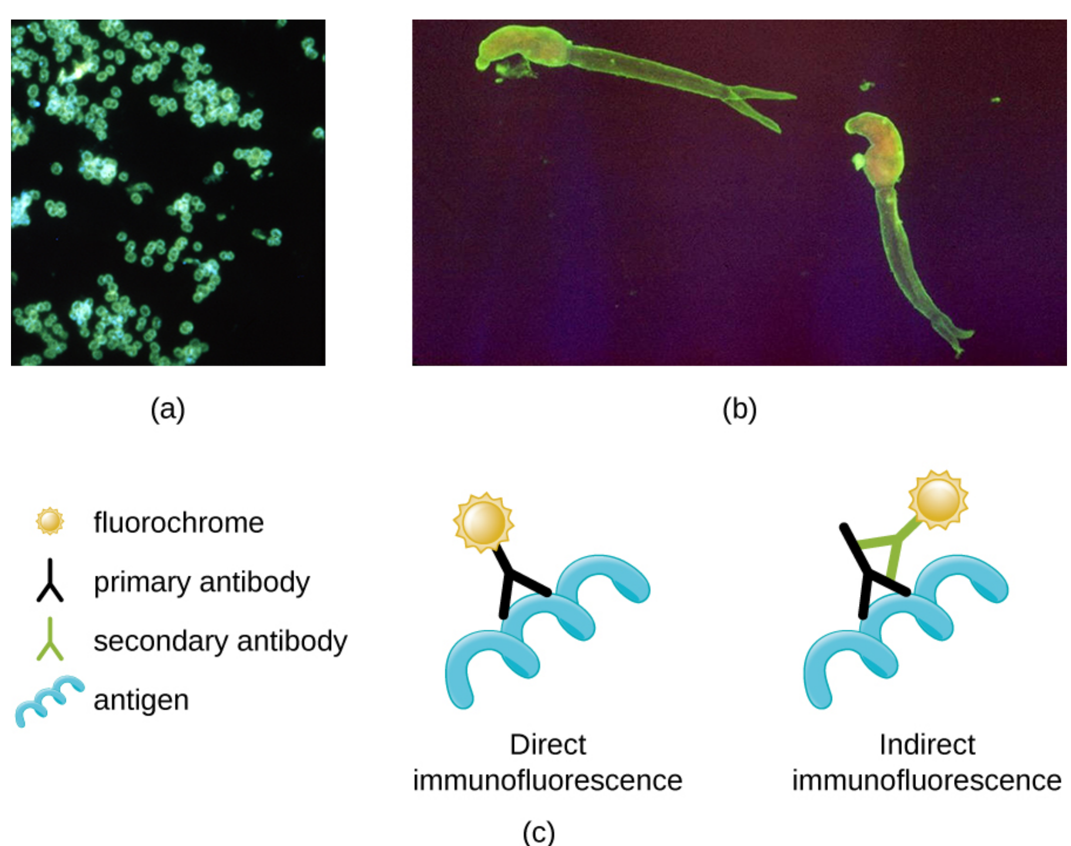


Figure 9.16 (a) A direct immunofluorescent stain is used to visualize *Neisseria gonorrhoeae*, the bacterium that causes gonorrhea. (b) An indirect immunofluorescent stain is used to visualize larvae of *Schistosoma mansoni*, a parasitic worm that causes schistosomiasis, an intestinal disease common in the tropics. (c) In direct immunofluorescence, the stain is absorbed by a primary antibody, which binds to the antigen. In indirect immunofluorescence, the stain is absorbed by a secondary antibody, which binds to a primary antibody, which, in turn, binds to the antigen. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by Centers for Disease Control and Prevention)

- Why must fluorochromes be used to examine a specimen under a fluorescence microscope?

Confocal Microscopes

Whereas other forms of light microscopy create an image that is maximally focused at a single distance from the observer (the depth, or z-plane), a **confocal microscope** uses a laser to scan multiple z-planes successively. This produces numerous two-dimensional, high-resolution images at various depths, which can be constructed into a three-dimensional image by a computer. As with fluorescence microscopes, fluorescent stains are generally used to increase contrast and resolution. Image clarity is further enhanced by a narrow aperture that eliminates any light that is not from the z-plane. Confocal microscopes are thus very useful for examining thick specimens such as biofilms, which can be examined alive and unfixed (Figure 9.17).

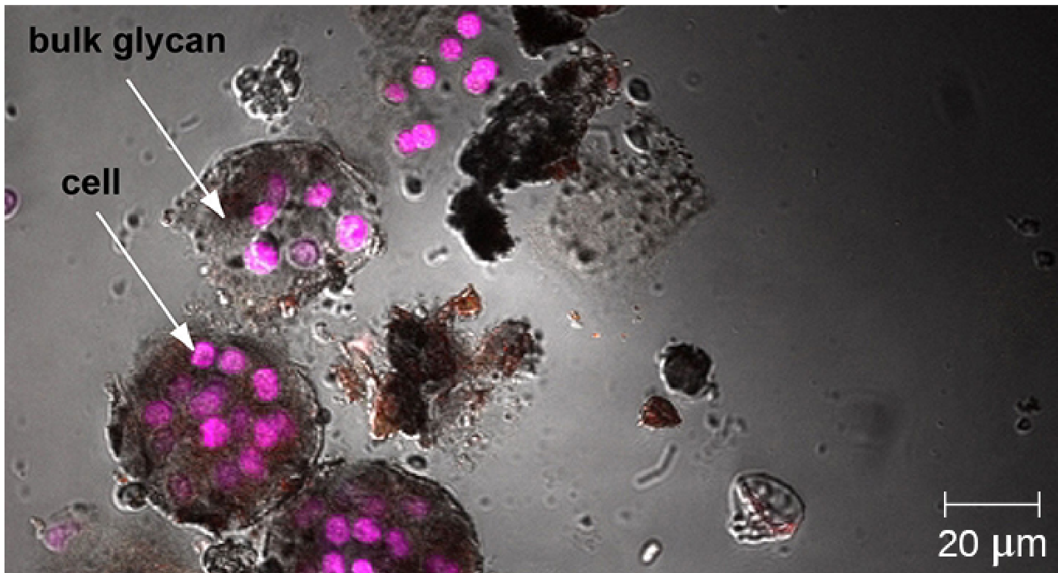


Figure 9.17 Confocal microscopy can be used to visualize structures such as this roof-dwelling cyanobacterium biofilm. (credit: modification of work by American Society for Microbiology)

Explore a rotating three-dimensional **view (https://www.openstax.org/l/22biofilm3d)** of a biofilm as observed under a confocal microscope. After navigating to the webpage, click the “play” button to launch the video.

Two-Photon Microscopes

While the original fluorescent and confocal microscopes allowed better visualization of unique features in specimens, there were still problems that prevented optimum visualization. The effective sensitivity of fluorescence microscopy when viewing thick specimens was generally limited by out-of-focus flare, which resulted in poor resolution. This limitation was greatly reduced in the confocal microscope through the use of a confocal pinhole to reject out-of-focus background fluorescence with thin (<1 μm), unblurred optical sections. However, even the confocal microscopes lacked the resolution needed for viewing thick tissue samples. These problems were resolved with the development of the **two-photon microscope**, which uses a scanning technique, fluorochromes, and long-wavelength light (such as infrared) to visualize specimens. The low energy associated with the long-wavelength light means that two photons must strike a location at the same time to excite the fluorochrome. The low energy of the excitation light is less damaging to cells, and the long wavelength of the excitation light more easily penetrates deep into thick specimens. This makes the two-photon microscope useful for examining living cells within intact tissues—brain slices, embryos, whole organs, and even entire animals.

Currently, use of two-photon microscopes is limited to advanced clinical and research laboratories because of the high costs of the instruments. A single two-photon microscope typically costs between $300,000 and $500,000, and the lasers used to excite the dyes used on specimens are also very expensive. However, as technology improves, two-photon microscopes may become more readily available in clinical settings.

- What types of specimens are best examined using confocal or two-photon microscopy?

Electron Microscopy

The maximum theoretical resolution of images created by light microscopes is ultimately limited by the wavelengths of visible light. Most light microscopes can only magnify 1000×, and a few can magnify up to 1500×, but this does not begin to approach the magnifying power of an **electron microscope (EM)**, which uses short-wavelength electron beams rather than light to increase magnification and resolution.

Electrons, like electromagnetic radiation, can behave as waves, but with wavelengths of 0.005 nm, they can produce much better resolution than visible light. An EM can produce a sharp image that is magnified up to 100,000×. Thus, EMs can resolve subcellular structures as well as some molecular structures (e.g., single

strands of DNA); however, electron microscopy cannot be used on living material because of the methods needed to prepare the specimens.

There are two basic types of EM: the **transmission electron microscope (TEM)** and the **scanning electron microscope (SEM)** (Figure 9.18). The TEM is somewhat analogous to the brightfield light microscope in terms of the way it functions. However, it uses an electron beam from above the specimen that is focused using a magnetic lens (rather than a glass lens) and projected through the specimen onto a detector. Electrons pass through the specimen, and then the detector captures the image (Figure 9.19).

Figure 9.18 A transmission electron microscope (TEM).

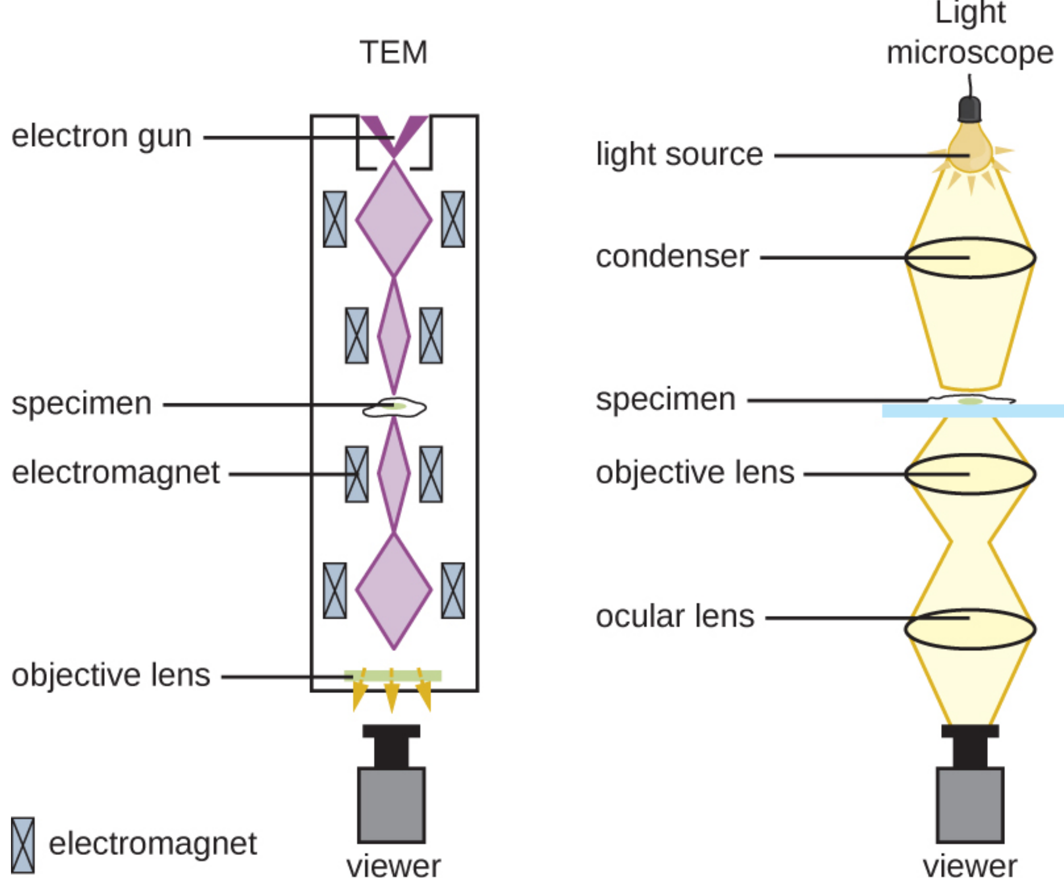


Figure 9.19 Electron microscopes use magnets to focus electron beams similarly to the way that light microscopes use lenses to focus light.

For electrons to pass through the specimen in a TEM, the specimen must be extremely thin (20–100 nm thick). The image is produced because of varying opacity in various parts of the specimen. This opacity can be enhanced by staining the specimen with materials such as heavy metals, which are electron dense. TEM requires that the beam and specimen be in a vacuum and that the specimen be very thin and dehydrated. The specific steps needed to prepare a specimen for observation under an EM are discussed in detail in the next section.

SEMs form images of surfaces of specimens, usually from electrons that are knocked off of specimens by a beam of electrons. This can create highly detailed images with a three-dimensional appearance that are displayed on a monitor (**Figure 9.20**). Typically, specimens are dried and prepared with fixatives that reduce artifacts, such as shriveling, that can be produced by drying, before being sputter-coated with a thin layer of metal such as gold. Whereas transmission electron microscopy requires very thin sections and allows one to see internal structures such as organelles and the interior of membranes, scanning electron microscopy can be used to view the surfaces of larger objects (such as a pollen grain) as well as the surfaces of very small samples (**Figure 9.21**). Some EMs can magnify an image up to 2,000,000×.[1]

1. “JEM-ARM200F Transmission Electron Microscope,” *JEOL USA Inc*, http://www.jeolusa.com/PRODUCTS/TransmissionElectronMicroscopes%28TEM%29/200kV/JEM-ARM200F/tabid/663/Default.aspx#195028-specifications. Accessed 8/28/2015.

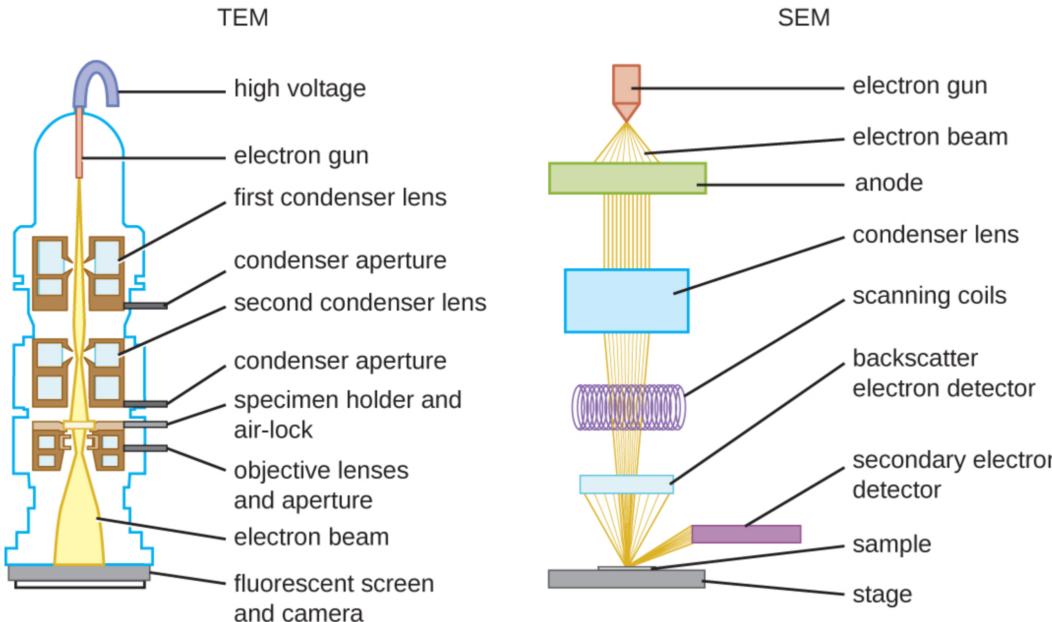


Figure 9.20 These schematic illustrations compare the components of transmission electron microscopes and scanning electron microscopes.

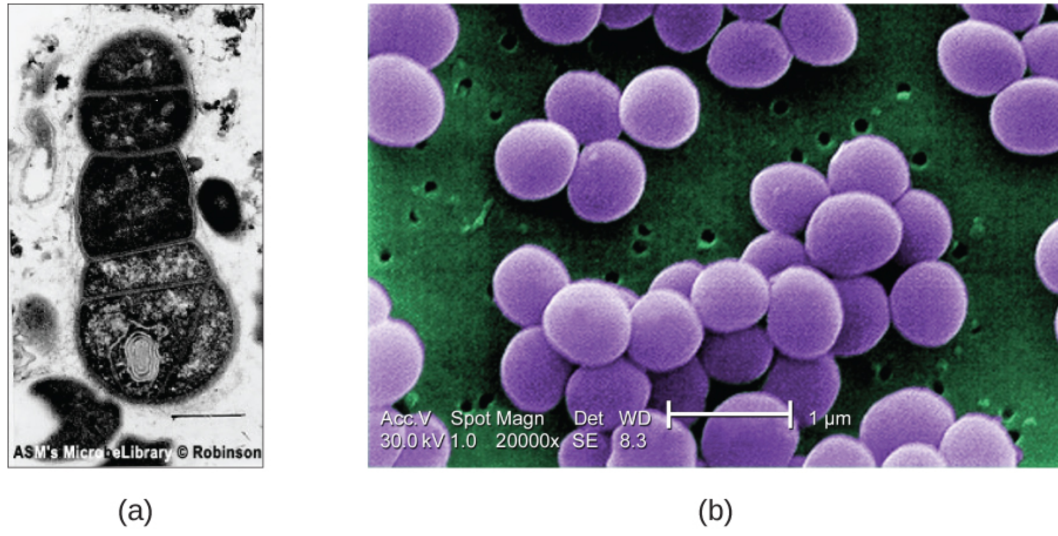


Figure 9.21 (a) This TEM image of cells in a biofilm shows well-defined internal structures of the cells because of varying levels of opacity in the specimen. (b) This color-enhanced SEM image of the bacterium *Staphylococcus aureus* illustrates the ability of scanning electron microscopy to render three-dimensional images of the surface structure of cells. (credit a: modification of work by American Society for Microbiology; credit b: modification of work by Centers for Disease Control and Prevention)

- What are some advantages and disadvantages of electron microscopy, as opposed to light microscopy, for examining microbiological specimens?
- What kinds of specimens are best examined using TEM? SEM?

Using Microscopy to Study Biofilms

A biofilm is a complex community of one or more microorganism species, typically forming as a slimy coating attached to a surface because of the production of an extrapolymeric substance (EPS) that attaches to a surface or at the interface between surfaces (e.g., between air and water). In nature, biofilms are abundant and frequently occupy complex niches within ecosystems (**Figure 9.22**). In medicine, biofilms can coat medical devices and exist within the body. Because they possess unique characteristics, such

as increased resistance against the immune system and to antimicrobial drugs, biofilms are of particular interest to microbiologists and clinicians alike.

Because biofilms are thick, they cannot be observed very well using light microscopy; slicing a biofilm to create a thinner specimen might kill or disturb the microbial community. Confocal microscopy provides clearer images of biofilms because it can focus on one z-plane at a time and produce a three-dimensional image of a thick specimen. Fluorescent dyes can be helpful in identifying cells within the matrix. Additionally, techniques such as immunofluorescence and fluorescence in situ hybridization (FISH), in which fluorescent probes are used to bind to DNA, can be used.

Electron microscopy can be used to observe biofilms, but only after dehydrating the specimen, which produces undesirable artifacts and distorts the specimen. In addition to these approaches, it is possible to follow water currents through the shapes (such as cones and mushrooms) of biofilms, using video of the movement of fluorescently coated beads (Figure 9.23).

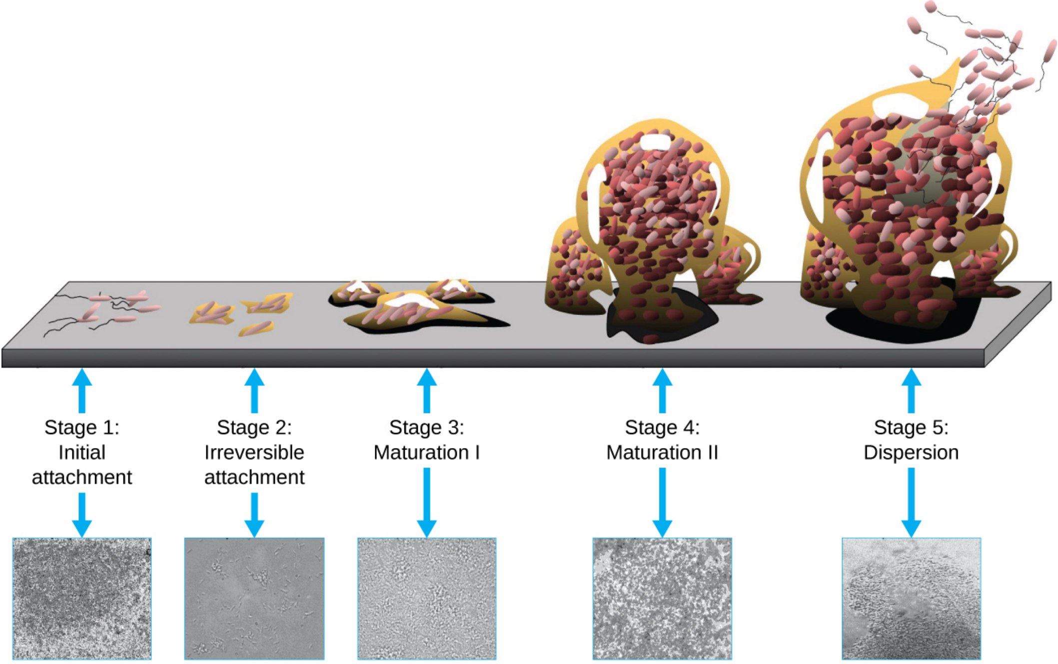


Figure 9.22 A biofilm forms when planktonic (free-floating) bacteria of one or more species adhere to a surface, produce slime, and form a colony. (credit: Public Library of Science)

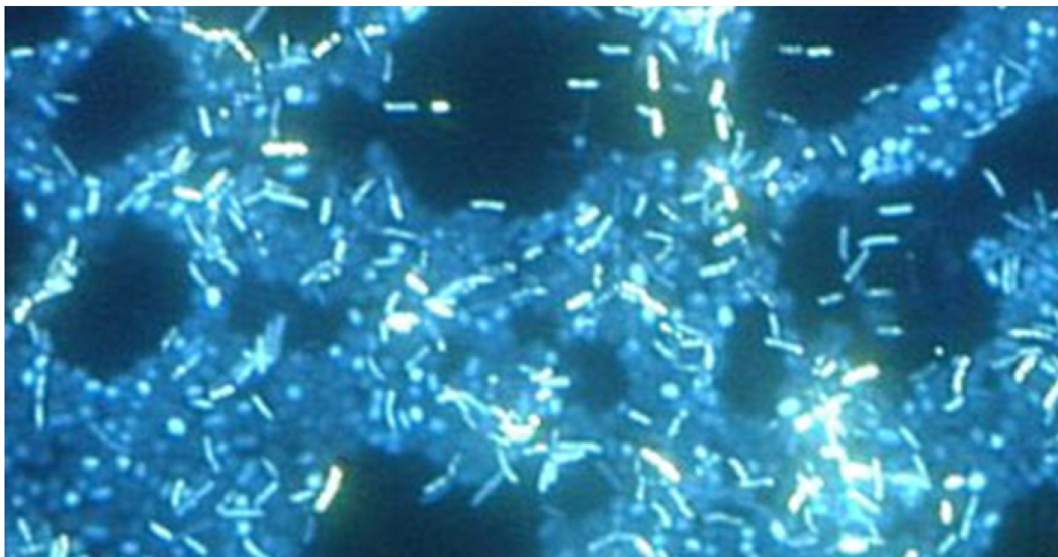


Figure 9.23 In this image, multiple species of bacteria grow in a biofilm on stainless steel (stained with DAPI for epifluorescence miscroscopy). (credit: Ricardo Murga, Rodney Donlan)

Scanning Probe Microscopy

A **scanning probe microscope** does not use light or electrons, but rather very sharp probes that are passed over the surface of the specimen and interact with it directly. This produces information that can be assembled into images with magnifications up to 100,000,000×. Such large magnifications can be used to observe individual atoms on surfaces. To date, these techniques have been used primarily for research rather than for diagnostics.

There are two types of scanning probe microscope: the **scanning tunneling microscope (STM)** and the **atomic force microscope (AFM)**. An STM uses a probe that is passed just above the specimen as a constant voltage bias creates the potential for an electric current between the probe and the specimen. This current occurs via quantum tunneling of electrons between the probe and the specimen, and the intensity of the current is dependent upon the distance between the probe and the specimen. The probe is moved horizontally above the surface and the intensity of the current is measured. Scanning tunneling microscopy can effectively map the structure of surfaces at a resolution at which individual atoms can be detected.

Similar to an STM, AFMs have a thin probe that is passed just above the specimen. However, rather than measuring variations in the current at a constant height above the specimen, an AFM establishes a constant current and measures variations in the height of the probe tip as it passes over the specimen. As the probe tip is passed over the specimen, forces between the atoms (van der Waals forces, capillary forces, chemical bonding, electrostatic forces, and others) cause it to move up and down. Deflection of the probe tip is determined and measured using Hooke's law of elasticity, and this information is used to construct images of the surface of the specimen with resolution at the atomic level (Figure 9.24).

Figure 9.25, Figure 9.26, and Figure 9.27 summarize the microscopy techniques for light microscopes, electron microscopes, and scanning probe microscopes, respectively.

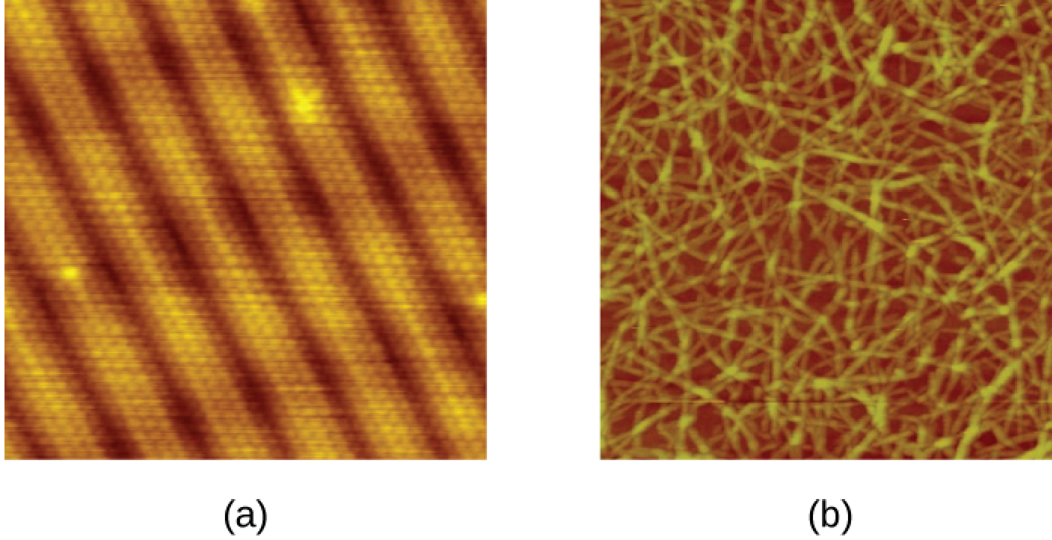


Figure 9.24 STMs and AFMs allow us to view images at the atomic level. (a) This STM image of a pure gold surface shows individual atoms of gold arranged in columns. (b) This AFM image shows long, strand-like molecules of nanocellulose, a laboratory-created substance derived from plant fibers. (credit a: modification of work by “Erwinrossen”/Wikimedia Commons)

- Which has higher magnification, a light microscope or a scanning probe microscope?
- Name one advantage and one limitation of scanning probe microscopy.

LIGHT MICROSCOPES **Magnification: up to about 1000×** Use visible or ultraviolet light to produce an image.		
Microscope Type	**Key Uses**	**Sample Images**
Brightfield	Commonly used in a wide variety of laboratory applications as the standard microscope; produces an image on a bright background. **Example:** *Bacillus* sp. showing endospores.	ASM MicrobeLibrary.org © Smith
Darkfield	Increases contrast without staining by producing a bright image on a darker background; especially useful for viewing live specimens. **Example:** *Borrelia burgdorferi*	
Phase contrast	Uses refraction and interference caused by structures in the specimen to create high-contrast, high-resolution images without staining, making it useful for viewing live specimens, and structures such as endospores and organelles. **Example:** *Pseudomonas* sp.	
Differential interference contrast (DIC)	Uses interference patterns to enhance contrast between different features of a specimen to produce high-contrast images of living organisms with a three-dimensional appearance, making it especially useful in distinguishing structures within live, unstained specimens; images viewed reveal detailed structures within cells. **Example:** *Escherichia coli* O157:H7	
Fluorescence	Uses fluorescent stains to produce an image; can be used to identify pathogens, to find particular species, to distinguish living from dead cells, or to find locations of particular molecules within a cell; also used for immunofluorescence. **Example:** *P. putida* stained with fluorescent dyes to visualize the capsule.	ASM MicrobeLibrary.org©Ghiorse
Confocal	Uses a laser to scan mutiple z-planes successively, producing numerous two-dimensional, high-resolution images at various depths that can be constructed into a three-dimensional image by a computer, making this useful for examining thick specimens such as biofilms. **Example:** *Escherichia coli* stained with acridine orange dye to show the nucleoid regions of the cells.	
Two-photon	Uses a scanning technique, fluorochromes, and long-wavelength light (such as infrared) to penetrate deep into thick specimens such as biofilms. **Example:** Mouse intestine cells stained with fluorescent dye.	

Figure 9.25 (credit "Brightfield": modification of work by American Society for Microbiology; credit "Darkfield": modification of work by American Society for Microbiology; credit "Phase contrast": modification of work by American Society for Microbiology; credit "DIC": modification of work by American Society for Microbiology; credit "Fluorescence": modification of work by American Society for Microbiology; credit "Confocal": modification of work by American Society for Microbiology; credit "Two-photon": modification of work by Alberto Diaspro, Paolo Bianchini, Giuseppe Vicidomini, Mario Faretta, Paola Ramoino, Cesare Usai)

ELECTRON MICROSCOPES Magnification: 20–100,000× or more Use electron beams focused with magnets to produce an image.		
Microscope Type	**Key Uses**	**Sample Images**
Transmission (TEM)	Uses electron beams that pass through a specimen to visualize small images; useful to observe small, thin specimens such as tissue sections and subcellular structures. **Example:** *Ebola* virus	
Scanning (SEM)	Uses electron beams to visualize surfaces; useful to observe the three-dimensional surface details of specimens. **Example:** *Campylobactor jejuni*	

Figure 9.26 (credit “TEM”: modification of work by American Society for Microbiology; credit “SEM”: modification of work by American Society for Microbiology)

SCANNING PROBE MICROSCOPES Magnification: 100–100,000,000× or more Use very short probes that are passed over the surface of the specimen and interact with it directly.		
Microscope Type	**Key Uses**	**Sample Images**
Scanning tunneling (STM)	Uses a probe passed horizontally at a constant distance just above the specimen while the intensity of the current is measured; can map the structure of surfaces at the atomic level; works best on conducting materials but can also be used to examine organic materials such as DNA, if fixed on a surface. **Example:** Image of surface reconstruction on a clean gold [Au(100)] surface, as visualized using scanning tunneling microscopy.	
Atomic force (AFM)	Can be used in several ways, including using a laser focused on a cantilever to measure the bending of the tip or a probe passed above the specimen while the height needed to maintain a constant current is measured; useful to observe specimens at the atomic level and can be more easily used with nonconducting samples. **Example:** AFM height image of carboxymethylated nanocellulose adsorbed on a silica surface.	

Figure 9.27

9.3 | Staining Microscopic Specimens

Learning Objectives

By the end of this section, you will be able to:

- Differentiate between simple and differential stains
- Describe the unique features of commonly used stains
- Explain the procedures and name clinical applications for Gram, endospore, acid-fast, negative capsule, and flagella staining

In their natural state, most of the cells and microorganisms that we observe under the microscope lack color and contrast. This makes it difficult, if not impossible, to detect important cellular structures and their distinguishing characteristics without artificially treating specimens. We have already alluded to certain techniques involving stains and fluorescent dyes, and in this section we will discuss specific techniques for sample preparation in greater detail. Indeed, numerous methods have been developed to identify specific microbes, cellular structures, DNA sequences, or indicators of infection in tissue samples, under the microscope. Here, we will focus on the

most clinically relevant techniques.

Preparing Specimens for Light Microscopy

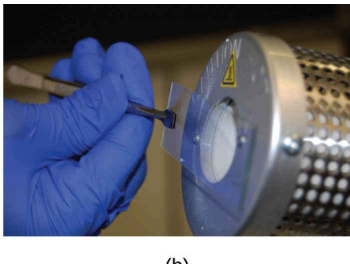
In clinical settings, light microscopes are the most commonly used microscopes. There are two basic types of preparation used to view specimens with a light microscope: wet mounts and fixed specimens.

The simplest type of preparation is the **wet mount**, in which the specimen is placed on the slide in a drop of liquid. Some specimens, such as a drop of urine, are already in a liquid form and can be deposited on the slide using a dropper. Solid specimens, such as a skin scraping, can be placed on the slide before adding a drop of liquid to prepare the wet mount. Sometimes the liquid used is simply water, but often stains are added to enhance contrast. Once the liquid has been added to the slide, a coverslip is placed on top and the specimen is ready for examination under the microscope.

The second method of preparing specimens for light microscopy is **fixation**. The “fixing” of a sample refers to the process of attaching cells to a slide. Fixation is often achieved either by heating (heat fixing) or chemically treating the specimen. In addition to attaching the specimen to the slide, fixation also kills microorganisms in the specimen, stopping their movement and metabolism while preserving the integrity of their cellular components for observation.

To heat-fix a sample, a thin layer of the specimen is spread on the slide (called a **smear**), and the slide is then briefly heated over a heat source (Figure 9.28). Chemical fixatives are often preferable to heat for tissue specimens. Chemical agents such as acetic acid, ethanol, methanol, formaldehyde (formalin), and glutaraldehyde can denature proteins, stop biochemical reactions, and stabilize cell structures in tissue samples (Figure 9.28).

(a)



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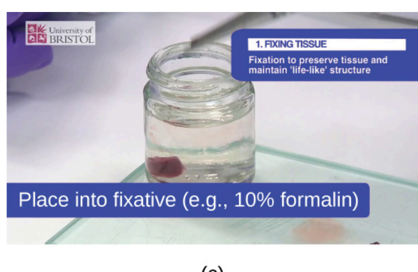
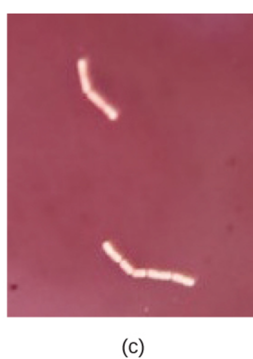


(c)

Figure 9.28 (a) A specimen can be heat-fixed by using a slide warmer like this one. (b) Another method for heat-fixing a specimen is to hold a slide with a smear over a microincinerator. (c) This tissue sample is being fixed in a solution of formalin (also known as formaldehyde). Chemical fixation kills microorganisms in the specimen, stopping degradation of the tissues and preserving their structure so that they can be examined later under the microscope. (credit a: modification of work by Nina Parker; credit b: modification of work by Nina Parker; credit c: modification of work by “University of Bristol”/YouTube)

In addition to fixation, **staining** is almost always applied to color certain features of a specimen before examining it under a light microscope. Stains, or dyes, contain salts made up of a positive ion and a negative ion. Depending on the type of dye, the positive or the negative ion may be the chromophore (the colored ion); the other, uncolored ion is called the counterion. If the chromophore is the positively charged ion, the stain is classified as a **basic dye**; if the negative ion is the chromophore, the stain is considered an **acidic dye**.

Dyes are selected for staining based on the chemical properties of the dye and the specimen being observed, which determine how the dye will interact with the specimen. In most cases, it is preferable to use a **positive stain**, a dye that will be absorbed by the cells or organisms being observed, adding color to objects of interest to make them stand out against the background. However, there are scenarios in which it is advantageous to use a **negative stain**, which is absorbed by the background but not by the cells or organisms in the specimen. Negative staining produces an outline or silhouette of the organisms against a colorful background (Figure 9.29).



(a) (b) (c)

Figure 9.29 (a) These *Bacillus anthracis* cells have absorbed crystal violet, a basic positive stain. (b) This specimen of *Spinoloricus*, a microscopic marine organism, has been stained with rose bengal, a positive acidic stain. (c) These *B. megaterium* appear to be white because they have not absorbed the negative red stain applied to the slide. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by Roberto Danovaro, Antonio Pusceddu, Cristina Gambi, Iben Heiner, Reinhardt Mobjerg Kristensen; credit c: modification of work by Anh-Hue Tu)

Because cells typically have negatively charged cell walls, the positive chromophores in basic dyes tend to stick to the cell walls, making them positive stains. Thus, commonly used basic dyes such as basic fuchsin, crystal violet, malachite green, methylene blue, and safranin typically serve as positive stains. On the other hand, the negatively charged chromophores in acidic dyes are repelled by negatively charged cell walls, making them negative stains. Commonly used acidic dyes include acid fuchsin, eosin, and rose bengal. **Figure 9.37** provides more detail.

Some staining techniques involve the application of only one dye to the sample; others require more than one dye. In **simple staining**, a single dye is used to emphasize particular structures in the specimen. A simple stain will generally make all of the organisms in a sample appear to be the same color, even if the sample contains more than one type of organism. In contrast, **differential staining** distinguishes organisms based on their interactions with multiple stains. In other words, two organisms in a differentially stained sample may appear to be different colors. Differential staining techniques commonly used in clinical settings include Gram staining, acid-fast staining, endospore staining, flagella staining, and capsule staining. **Figure 9.38** provides more detail on these differential staining techniques.

- Explain why it is important to fix a specimen before viewing it under a light microscope.
- What types of specimens should be chemically fixed as opposed to heat-fixed?
- Why might an acidic dye react differently with a given specimen than a basic dye?
- Explain the difference between a positive stain and a negative stain.
- Explain the difference between simple and differential staining.

Gram Staining

The **Gram stain procedure** is a differential staining procedure that involves multiple steps. It was developed by Danish microbiologist Hans Christian Gram in 1884 as an effective method to distinguish between bacteria with different types of cell walls, and even today it remains one of the most frequently used staining techniques. The steps of the Gram stain procedure are listed below and illustrated in **Figure 9.30**.

1. First, crystal violet, a **primary stain**, is applied to a heat-fixed smear, giving all of the cells a purple color.
2. Next, Gram's iodine, a **mordant**, is added. A mordant is a substance used to set or stabilize stains or dyes; in this case, Gram's iodine acts like a trapping agent that complexes with the crystal violet, making the crystal violet–iodine complex clump and stay contained in thick layers of peptidoglycan in the cell walls.
3. Next, a **decolorizing agent** is added, usually ethanol or an acetone/ethanol solution. Cells that have thick peptidoglycan layers in their cell walls are much less affected by the decolorizing agent; they generally retain the crystal violet dye and remain purple. However, the decolorizing agent more easily washes the dye out of cells with thinner peptidoglycan layers, making them again colorless.
4. Finally, a secondary **counterstain**, usually safranin, is added. This stains the decolorized cells pink and is

less noticeable in the cells that still contain the crystal violet dye.

Gram stain process			
Gram staining steps	**Cell effects**	**Gram-positive**	**Gram-negative**
Step 1 **Crystal violet** *primary stain added to specimen smear.*	Stains cells purple or blue.		
Step 2 **Iodine** *mordant makes dye less soluble so it adheres to cell walls.*	Cells remain purple or blue.		
Step 3 **Alcohol** *decolorizer washes away stain from gram-negative cell walls.*	Gram-positive cells remain purple or blue. Gram-negative cells are colorless.		
Step 4 **Safranin** *counterstain allows dye adherance to gram-negative cells.*	Gram-positive cells remain purple or blue. Gram-negative cells appear pink or red.		

Figure 9.30 Gram-staining is a differential staining technique that uses a primary stain and a secondary counterstain to distinguish between gram-positive and gram-negative bacteria.

The purple, crystal-violet stained cells are referred to as gram-positive cells, while the red, safranin-dyed cells are gram-negative (**Figure 9.31**). However, there are several important considerations in interpreting the results of a Gram stain. First, older bacterial cells may have damage to their cell walls that causes them to appear gram-negative even if the species is gram-positive. Thus, it is best to use fresh bacterial cultures for Gram staining. Second, errors such as leaving on decolorizer too long can affect the results. In some cases, most cells will appear gram-positive while a few appear gram-negative (as in **Figure 9.31**). This suggests damage to the individual cells or that decolorizer was left on for too long; the cells should still be classified as gram-positive if they are all the same species rather than a mixed culture.

Besides their differing interactions with dyes and decolorizing agents, the chemical differences between gram-positive and gram-negative cells have other implications with clinical relevance. For example, Gram staining can help clinicians classify bacterial pathogens in a sample into categories associated with specific properties. Gram-negative bacteria tend to be more resistant to certain antibiotics than gram-positive bacteria. We will discuss this and other applications of Gram staining in more detail in later chapters.

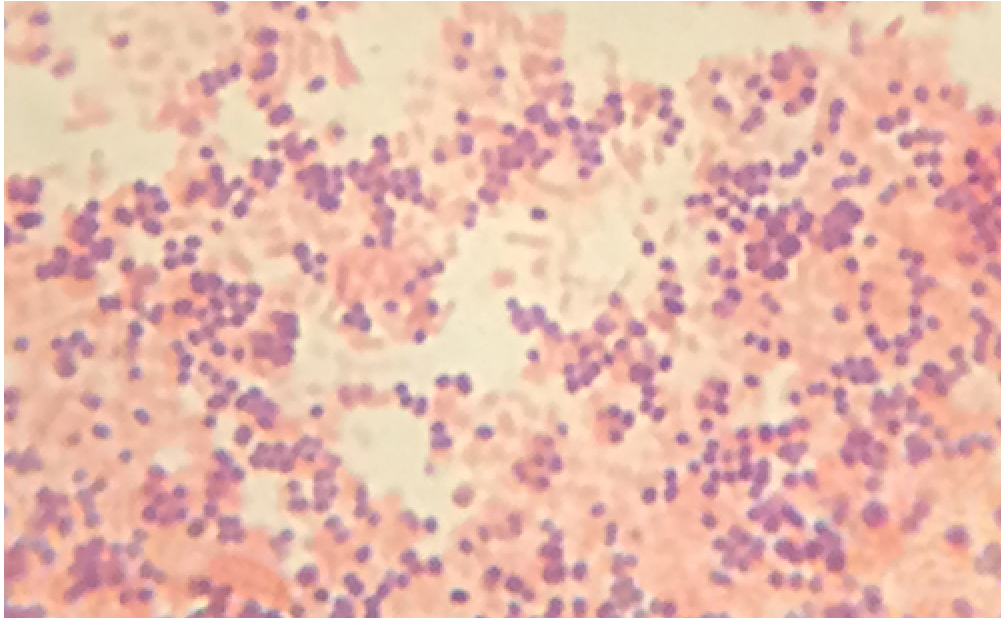


Figure 9.31 In this specimen, the gram-positive bacterium *Staphylococcus aureus* retains crystal violet dye even after the decolorizing agent is added. Gram-negative *Escherichia coli*, the most common Gram stain quality-control bacterium, is decolorized, and is only visible after the addition of the pink counterstain safranin. (credit: modification of work by Nina Parker)

- Explain the role of Gram's iodine in the Gram stain procedure.
- Explain the role of alcohol in the Gram stain procedure.
- What color are gram-positive and gram-negative cells, respectively, after the Gram stain procedure?

Part 3

Viewing Cindy's specimen under the darkfield microscope has provided the technician with some important clues about the identity of the microbe causing her infection. However, more information is needed to make a conclusive diagnosis. The technician decides to make a Gram stain of the specimen. This technique is commonly used as an early step in identifying pathogenic bacteria. After completing the Gram stain procedure, the technician views the slide under the brightfield microscope and sees purple, grape-like clusters of spherical cells (**Figure 9.32**).

- Are these bacteria gram-positive or gram-negative?
- What does this reveal about their cell walls?

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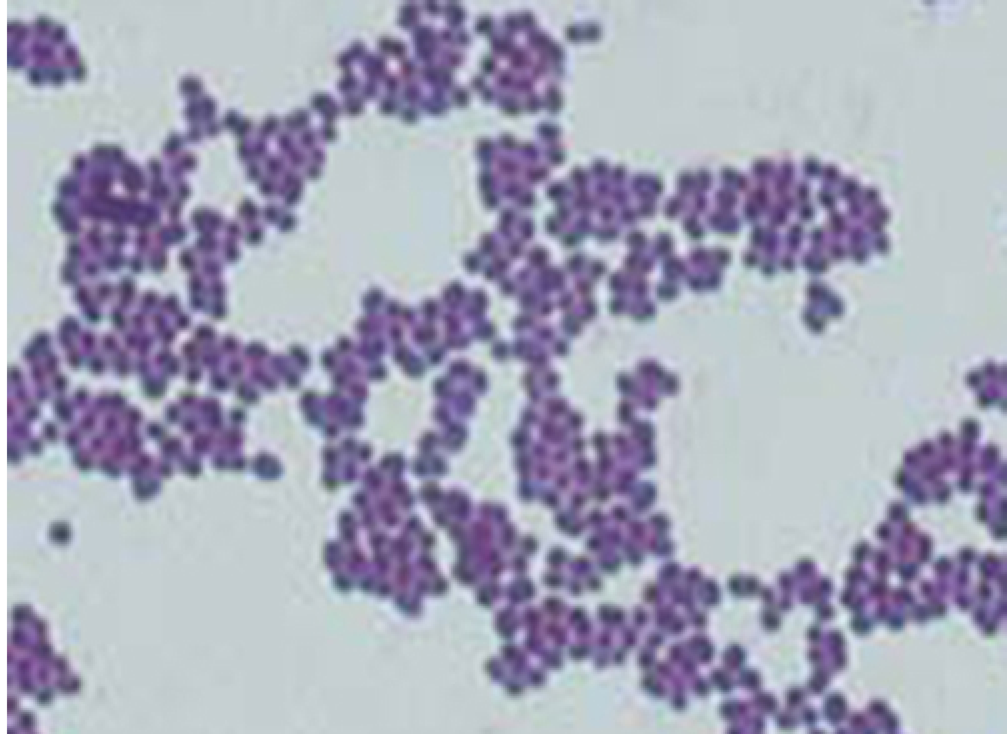


Figure 9.32 (credit: modification of work by American Society for Microbiology)

Acid-Fast Stains

Acid-fast staining is another commonly used, differential staining technique that can be an important diagnostic tool. An **acid-fast stain** is able to differentiate two types of gram-positive cells: those that have waxy mycolic acids in their cell walls, and those that do not. Two different methods for acid-fast staining are the **Ziehl-Neelsen technique** and the **Kinyoun technique**. Both use carbolfuchsin as the primary stain. The waxy, acid-fast cells retain the carbolfuchsin even after a decolorizing agent (an acid-alcohol solution) is applied. A secondary counterstain, methylene blue, is then applied, which renders non–acid-fast cells blue.

The fundamental difference between the two carbolfuchsin-based methods is whether heat is used during the primary staining process. The Ziehl-Neelsen method uses heat to infuse the carbolfuchsin into the acid-fast cells, whereas the Kinyoun method does not use heat. Both techniques are important diagnostic tools because a number of specific diseases are caused by acid-fast bacteria (AFB). If AFB are present in a tissue sample, their red or pink color can be seen clearly against the blue background of the surrounding tissue cells (Figure 9.33).

- Why are acid-fast stains useful?

Using Microscopy to Diagnose Tuberculosis

Mycobacterium tuberculosis, the bacterium that causes tuberculosis, can be detected in specimens based on the presence of acid-fast bacilli. Often, a smear is prepared from a sample of the patient's sputum and then stained using the Ziehl-Neelsen technique (Figure 9.33). If acid-fast bacteria are confirmed, they are generally cultured to make a positive identification. Variations of this approach can be used as a first step in determining whether *M. tuberculosis* or other acid-fast bacteria are present, though samples from elsewhere in the body (such as urine) may contain other *Mycobacterium* species.

An alternative approach for determining the presence of *M. tuberculosis* is immunofluorescence. In this technique, fluorochrome-labeled antibodies bind to *M. tuberculosis*, if present. Antibody-specific fluorescent dyes can be used to view the mycobacteria with a fluorescence microscope.

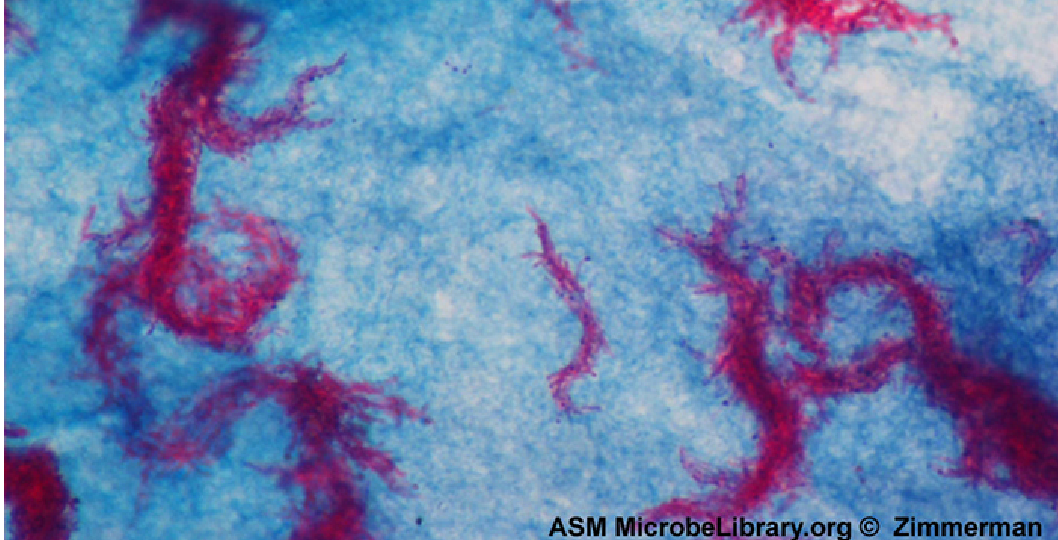
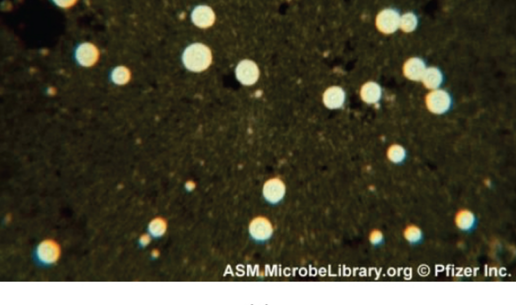


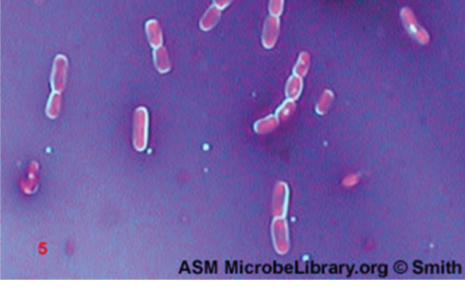
Figure 9.33 Ziehl-Neelsen staining has rendered these *Mycobacterium tuberculosis* cells red and the surrounding growth indicator medium blue. (credit: modification of work by American Society for Microbiology)

Capsule Staining

Certain bacteria and yeasts have a protective outer structure called a capsule. Since the presence of a capsule is directly related to a microbe's virulence (its ability to cause disease), the ability to determine whether cells in a sample have capsules is an important diagnostic tool. Capsules do not absorb most basic dyes; therefore, a negative staining technique (staining around the cells) is typically used for **capsule staining**. The dye stains the background but does not penetrate the capsules, which appear like halos around the borders of the cell. The specimen does not need to be heat-fixed prior to negative staining.

One common negative staining technique for identifying encapsulated yeast and bacteria is to add a few drops of India ink or nigrosin to a specimen. Other capsular stains can also be used to negatively stain encapsulated cells (**Figure 9.34**). Alternatively, positive and negative staining techniques can be combined to visualize capsules: The positive stain colors the body of the cell, and the negative stain colors the background but not the capsule, leaving halo around each cell.





(a) (b)

Figure 9.34 (a) India-ink was used to stain the background around these cells of the yeast *Cryptococcus neoformans*. The halos surrounding the cells are the polysaccharide capsules. (b) Crystal violet and copper sulfate dyes cannot penetrate the encapsulated *Bacillus* cells in this negatively stained sample. Encapsulated cells appear to have a light-blue halo. (credit a: modification of work by American Society for Microbiology; credit b: modification of work by American Society for Microbiology)

- How does negative staining help us visualize capsules?

Endospore Staining

Endospores are structures produced within certain bacterial cells that allow them to survive harsh conditions.

Gram staining alone cannot be used to visualize endospores, which appear clear when Gram-stained cells are viewed. **Endospore staining** uses two stains to differentiate endospores from the rest of the cell. The Schaeffer-Fulton method (the most commonly used endospore-staining technique) uses heat to push the primary stain (malachite green) into the endospore. Washing with water decolorizes the cell, but the endospore retains the green stain. The cell is then counterstained pink with safranin. The resulting image reveals the shape and location of endospores, if they are present. The green endospores will appear either within the pink vegetative cells or as separate from the pink cells altogether. If no endospores are present, then only the pink vegetative cells will be visible (Figure 9.35).

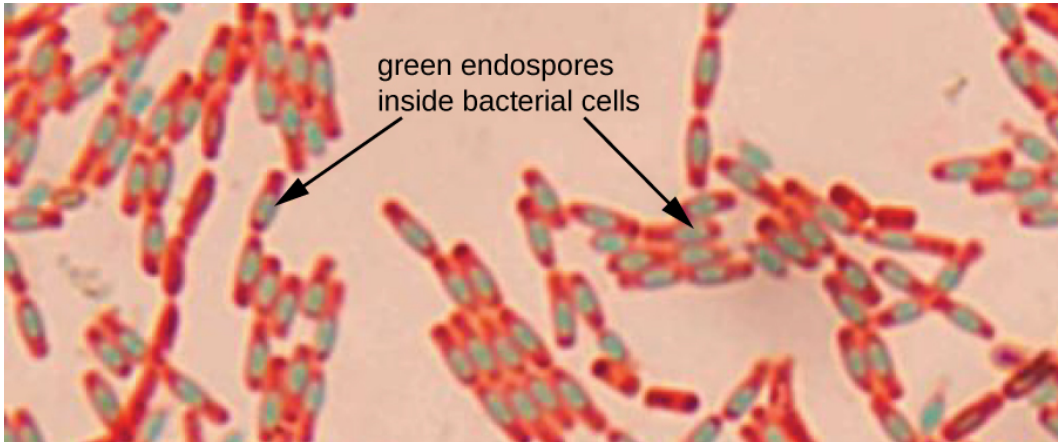


Figure 9.35 A stained preparation of *Bacillus subtilis* showing endospores as green and the vegetative cells as pink. (credit: modification of work by American Society for Microbiology)

Endospore-staining techniques are important for identifying *Bacillus* and *Clostridium*, two genera of endospore-producing bacteria that contain clinically significant species. Among others, *B. anthracis* (which causes anthrax) has been of particular interest because of concern that its spores could be used as a bioterrorism agent. *C. difficile* is a particularly important species responsible for the typically hospital-acquired infection known as "C. diff."

- Is endospore staining an example of positive, negative, or differential staining?

Flagella Staining

Flagella (singular: flagellum) are tail-like cellular structures used for locomotion by some bacteria, archaea, and eukaryotes. Because they are so thin, flagella typically cannot be seen under a light microscope without a specialized **flagella staining** technique. Flagella staining thickens the flagella by first applying mordant (generally tannic acid, but sometimes potassium alum), which coats the flagella; then the specimen is stained with pararosaniline (most commonly) or basic fuchsin (Figure 9.36).

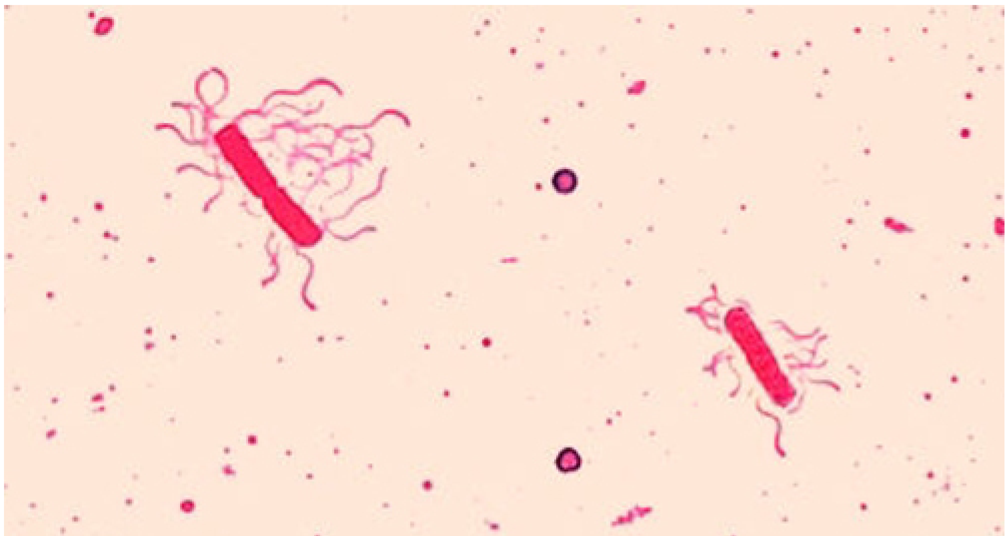


Figure 9.36 A flagella stain of *Bacillus cereus*, a common cause of foodborne illness, reveals that the cells have numerous flagella, used for locomotion. (credit: modification of work by Centers for Disease Control and Prevention)

Though flagella staining is uncommon in clinical settings, the technique is commonly used by microbiologists, since the location and number of flagella can be useful in classifying and identifying bacteria in a sample. When using this technique, it is important to handle the specimen with great care; flagella are delicate structures that can easily be damaged or pulled off, compromising attempts to accurately locate and count the number of flagella.

SIMPLE STAINS				
Stain Type	**Specific Dyes**	**Purpose**	**Outcome**	**Sample Images**
Basic stains	Methylene blue, crystal violet, malachite green, basic fuchsin, carbolfuchsin, safranin	Stain negatively charged molecules and structures, such as nucleic acids and proteins	Positive stain	
Acidic stains	Eosin, acid fuchsin, rose bengal, Congo red	Stain positively charged molecules and structures, such as proteins	Can be either a positive or negative stain, depending on the cell's chemistry.	
Negative stains	India ink, nigrosin	Stains background, not specimen	Dark background with light specimen	

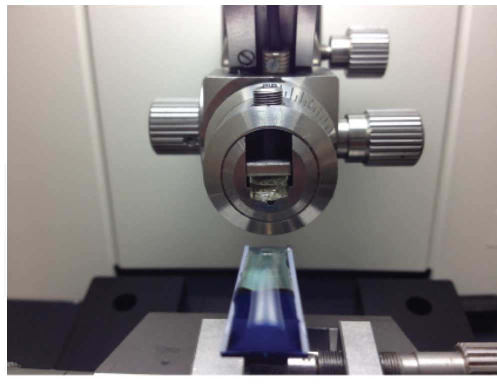
Figure 9.37 (credit "basic stains": modification of work by Centers for Disease Control and Prevention; credit "Acidic stains": modification of work by Roberto Danovaro, Antonio Dell'Anno, Antonio Pusceddu, Cristina Gambi, Iben Heiner, Reinhardt Mobjerg Kristensen; credit "Negative stains": modification of work by Anh-Hue Tu)

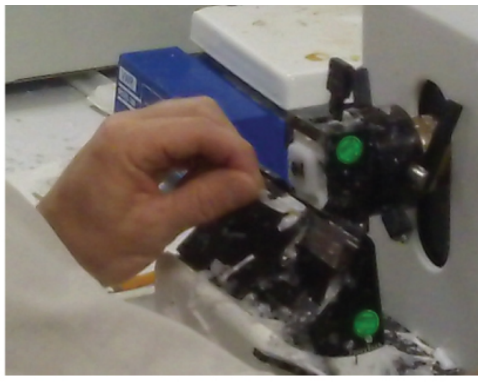
DIFFERENTIAL STAINS				
Stain Type	**Specific Dyes**	**Purpose**	**Outcome**	**Sample Images**
Gram stain	Uses crystal violet, Gram's iodine, ethanol (decolorizer), and safranin	Used to distinguish cells by cell-wall type (gram-positive, gram-negative)	Gram-positive cells stain purple/violet. Gram-negative cells stain pink.	
Acid-fast stain	After staining with basic fuchsin, acid-fast bacteria resist decolorization by acid-alcohol. Non acid-fast bacteria are counterstained with methylene blue.	Used to distinguish acid-fast bacteria such as *M. tuberculosis*, from non–acid-fast cells	Acid-fast bacteria are red; non–acid-fast cells are blue.	ASM MicrobeLibrary.org © Zimmerman
Endospore stain	Uses heat to stain endospores with malachite green (Schaeffer-Fulton procedure), then cell is washed and counterstained with safranin.	Used to distinguish organisms with endospores from those without; used to study the endospore.	Endospores appear bluish-green; other structures appear pink to red.	
Flagella stain	Flagella are coated with a tannic acid or potassium alum mordant, then stained using either pararosaline or basic fuchsin.	Used to view and study flagella in bacteria that have them.	Flagella are visible if present.	
Capsule stain	Negative staining with India ink or nigrosin is used to stain the background, leaving a clear area of the cell and the capsule. Counterstaining can be used to stain the cell while leaving the capsule clear.	Used to distinguish cells with capsules from those without.	Capsules appear clear or as halos if present.	ASM.MicrobeLibrary.org © Pfizer Inc.

Figure 9.38 (credit "Gram stain": modification of work by Nina Parker; credit "Acid-fast stain": modification of work by American Society for Microbiology; credit "Endospore stain": modification of work by American Society for Microbiology; credit "Capsule stain" : modification of work by American Society for Microbiology; credit "Flagella stain": modification of work by Centers for Disease Control and Prevention)

Preparing Specimens for Electron Microscopy

Samples to be analyzed using a TEM must have very thin sections. But cells are too soft to cut thinly, even with diamond knives. To cut cells without damage, the cells must be embedded in plastic resin and then dehydrated through a series of soaks in ethanol solutions (50%, 60%, 70%, and so on). The ethanol replaces the water in the cells, and the resin dissolves in ethanol and enters the cell, where it solidifies. Next, **thin sections** are cut using a specialized device called an **ultramicrotome** (Figure 9.39). Finally, samples are fixed to fine copper wire or carbon-fiber grids and stained—not with colored dyes, but with substances like uranyl acetate or osmium tetroxide, which contain electron-dense heavy metal atoms.





(a) (b)

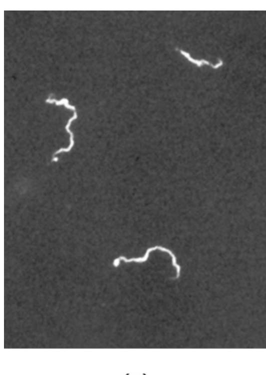
Figure 9.39 (a) An ultramicrotome used to prepare specimens for a TEM. (b) A technician uses an ultramicrotome to slice a specimen into thin sections. (credit a: modification of work by “Frost Museum”/Flickr; credit b: modification of work by U.S. Fish and Wildlife Service Northeast Region)

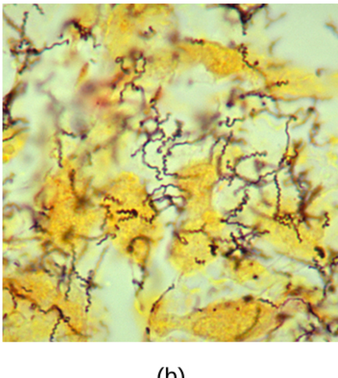
When samples are prepared for viewing using an SEM, they must also be dehydrated using an ethanol series. However, they must be even drier than is necessary for a TEM. Critical point drying with inert liquid carbon dioxide under pressure is used to displace the water from the specimen. After drying, the specimens are sputter-coated with metal by knocking atoms off of a palladium target, with energetic particles. Sputter-coating prevents specimens from becoming charged by the SEM’s electron beam.

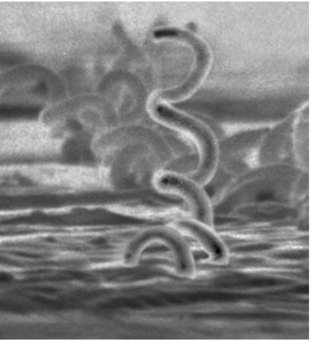
- Why is it important to dehydrate cells before examining them under an electron microscope?
- Name the device that is used to create thin sections of specimens for electron microscopy.

Using Microscopy to Diagnose Syphilis

The causative agent of syphilis is *Treponema pallidum*, a flexible, spiral cell (spirochete) that can be very thin (<0.15 μm) and match the refractive index of the medium, making it difficult to view using brightfield microscopy. Additionally, this species has not been successfully cultured in the laboratory on an artificial medium; therefore, diagnosis depends upon successful identification using microscopic techniques and serology (analysis of body fluids, often looking for antibodies to a pathogen). Since fixation and staining would kill the cells, darkfield microscopy is typically used for observing live specimens and viewing their movements. However, other approaches can also be used. For example, the cells can be thickened with silver particles (in tissue sections) and observed using a light microscope. It is also possible to use fluorescence or electron microscopy to view *Treponema* (**Figure 9.40**).







(a) (b) (c)

Figure 9.40 (a) Living, unstained *Treponema pallidum* spirochetes can be viewed under a darkfield microscope. (b) In this brightfield image, a modified Steiner silver stain is used to visualized *T. pallidum* spirochetes. Though the stain kills the cells, it increases the contrast to make them more visible. (c) While not used for standard diagnostic testing, *T. pallidum* can also be examined using scanning electron microscopy. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by Centers for Disease Control and Prevention; credit c: modification of work by Centers for Disease Control and Prevention)

In clinical settings, indirect immunofluorescence is often used to identify *Treponema.* A primary, unstained antibody attaches directly to the pathogen surface, and secondary antibodies "tagged" with a fluorescent stain attach to the primary antibody. Multiple secondary antibodies can attach to each primary antibody, amplifying the amount of stain attached to each *Treponema* cell, making them easier to spot (**Figure 9.41**).

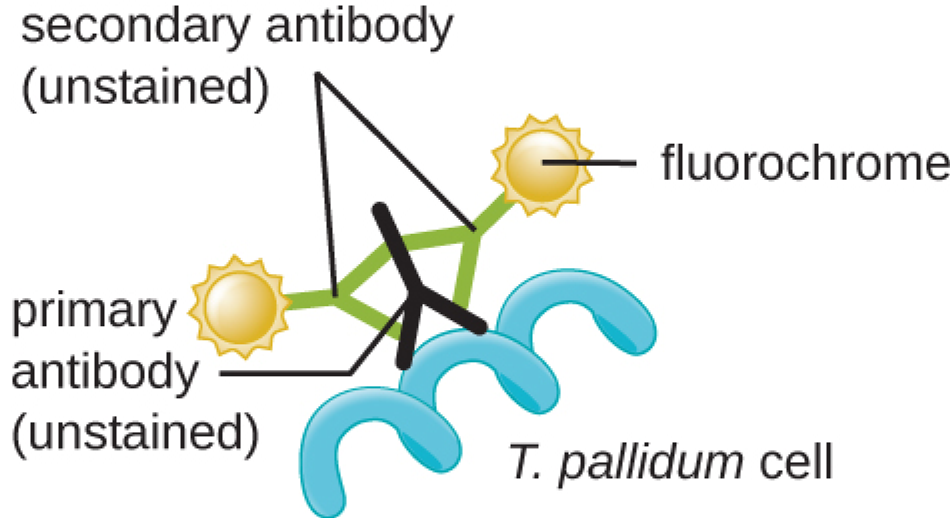


Figure 9.41 Indirect immunofluorescence can be used to identify *T. pallidum*, the causative agent of syphilis, in a specimen.

Preparation and Staining for Other Microscopes

Samples for fluorescence and confocal microscopy are prepared similarly to samples for light microscopy, except that the dyes are fluorochromes. Stains are often diluted in liquid before applying to the slide. Some dyes attach to an antibody to stain specific proteins on specific types of cells (immunofluorescence); others may attach to DNA molecules in a process called fluorescence in situ hybridization (FISH), causing cells to be stained based on whether they have a specific DNA sequence.

Sample preparation for two-photon microscopy is similar to fluorescence microscopy, except for the use of infrared dyes. Specimens for STM need to be on a very clean and atomically smooth surface. They are often mica coated with Au(111). Toluene vapor is a common fixative.

- What is the main difference between preparing a sample for fluorescence microscopy versus light microscopy?

Cornell University's **Case Studies in Microscopy (https://www.openstax.org/l/22cornellstud)** offers a series of clinical problems based on real-life events. Each case study walks you through a clinical problem using appropriate techniques in microscopy at each step.

Resolution

From the results of the Gram stain, the technician now knows that Cindy's infection is caused by spherical, gram-positive bacteria that form grape-like clusters, which is typical of staphylococcal bacteria. After some additional testing, the technician determines that these bacteria are the medically important species known as *Staphylococcus aureus*, a common culprit in wound infections. Because some strains of *S. aureus* are resistant to many antibiotics, skin infections may spread to other areas of the body and become serious, sometimes even resulting in amputations or death if the correct antibiotics are not used.

After testing several antibiotics, the lab is able to identify one that is effective against this particular strain of *S. aureus*. Cindy's doctor quickly prescribes the medication and emphasizes the importance of taking the entire course of antibiotics, even if the infection appears to clear up before the last scheduled dose. This reduces the risk that any especially resistant bacteria could survive, causing a second infection or spreading

to another person.

*Go back to the **previous** Clinical Focus box.*

Microscopy and Antibiotic Resistance

As the use of antibiotics has proliferated in medicine, as well as agriculture, microbes have evolved to become more resistant. Strains of bacteria such as methicillin-resistant *S. aureus* (MRSA), which has developed a high level of resistance to many antibiotics, are an increasingly worrying problem, so much so that research is underway to develop new and more diversified antibiotics.

Fluorescence microscopy can be useful in testing the effectiveness of new antibiotics against resistant strains like MRSA. In a test of one new antibiotic derived from a marine bacterium, MC21-A (bromophene), researchers used the fluorescent dye SYTOX Green to stain samples of MRSA. SYTOX Green is often used to distinguish dead cells from living cells, with fluorescence microscopy. Live cells will not absorb the dye, but cells killed by an antibiotic will absorb the dye, since the antibiotic has damaged the bacterial cell membrane. In this particular case, MRSA bacteria that had been exposed to MC21-A did, indeed, appear green under the fluorescence microscope, leading researchers to conclude that it is an effective antibiotic against MRSA.

Of course, some argue that developing new antibiotics will only lead to even more antibiotic-resistant microbes, so-called superbugs that could spawn epidemics before new treatments can be developed. For this reason, many health professionals are beginning to exercise more discretion in prescribing antibiotics. Whereas antibiotics were once routinely prescribed for common illnesses without a definite diagnosis, doctors and hospitals are much more likely to conduct additional testing to determine whether an antibiotic is necessary and appropriate before prescribing.

A sick patient might reasonably object to this stingy approach to prescribing antibiotics. To the patient who simply wants to feel better as quickly as possible, the potential benefits of taking an antibiotic may seem to outweigh any immediate health risks that might occur if the antibiotic is ineffective. But at what point do the risks of widespread antibiotic use supersede the desire to use them in individual cases?

CHAPTER SUMMARY

9.1 The Properties of Light

- Light waves interacting with materials may be **reflected**, **absorbed**, or **transmitted**, depending on the properties of the material.
- Light waves can interact with each other (**interference**) or be distorted by interactions with small objects or openings (**diffraction**).
- **Refraction** occurs when light waves change speed and direction as they pass from one medium to another. Differences in the **refraction indices** of two materials determine the magnitude of directional changes when light passes from one to the other.
- A **lens** is a medium with a curved surface that refracts and focuses light to produce an image.
- Visible light is part of the **electromagnetic spectrum**; light waves of different frequencies and wavelengths are distinguished as colors by the human eye.
- A prism can separate the colors of white light (**dispersion**) because different frequencies of light have different refractive indices for a given material.
- **Fluorescent dyes** and **phosphorescent** materials can effectively transform nonvisible electromagnetic radiation into visible light.
- The power of a microscope can be described in terms of its **magnification** and **resolution**.
- Resolution can be increased by shortening wavelength, increasing the **numerical aperture** of the lens, or using stains that enhance contrast.

9.2 Instruments of Microscopy

- Numerous types of microscopes use various technologies to generate micrographs. Most are useful for a particular type of specimen or application.
- **Light microscopy** uses lenses to focus light on a specimen to produce an image. Commonly used light microscopes include **brightfield**, **darkfield**, **phase-contrast**, **differential interference contrast**, **fluorescence**, **confocal**, and **two-photon** microscopes.
- **Electron microscopy** focuses electrons on the specimen using magnets, producing much greater magnification than light microscopy. The **transmission electron microscope (TEM)** and **scanning electron microscope (SEM)** are two common forms.
- **Scanning probe microscopy** produces images of even greater magnification by measuring feedback from sharp probes that interact with the specimen. Probe microscopes include the **scanning tunneling microscope (STM)** and the **atomic force microscope (AFM)**.

9.3 Staining Microscopic Specimens

- Samples must be properly prepared for microscopy. This may involve **staining**, **fixation**, and/or cutting **thin sections**.
- A variety of staining techniques can be used with light microscopy, including **Gram staining, acid-fast staining**, **capsule staining**, **endospore staining,** and **flagella staining**.
- Samples for TEM require very thin sections, whereas samples for SEM require sputter-coating.
- Preparation for fluorescence microscopy is similar to that for light microscopy, except that fluorochromes are used.

REVIEW QUESTIONS

1. Which of the following has the highest energy?

A. light with a long wavelength
B. light with an intermediate wavelength
C. light with a short wavelength
D. It is impossible to tell from the information given.

2. You place a specimen under the microscope and notice that parts of the specimen begin to emit light immediately. These materials can be described as _____________.

A. fluorescent
B. phosphorescent
C. transparent
D. opaque

3. Which would be the best choice for viewing internal structures of a living protist such as a *Paramecium*?

a. a brightfield microscope with a stain
b. a brightfield microscope without a stain
c. a darkfield microscope
d. a transmission electron microscope

4. Which type of microscope is especially useful for viewing thick structures such as biofilms?

a. a transmission electron microscope
b. a scanning electron microscopes
c. a phase-contrast microscope
d. a confocal scanning laser microscope
e. an atomic force microscope

5. Which type of microscope would be the best choice for viewing very small surface structures of a cell?

a. a transmission electron microscope
b. a scanning electron microscope
c. a brightfield microscope
d. a darkfield microscope
e. a phase-contrast microscope

6. What type of microscope uses an annular stop?

a. a transmission electron microscope
b. a scanning electron microscope
c. a brightfield microscope
d. a darkfield microscope
e. a phase-contrast microscope

7. What type of microscope uses a cone of light so that light only hits the specimen indirectly, producing a darker image on a brighter background?

a. a transmission electron microscope
b. a scanning electron microscope
c. a brightfield microscope
d. a darkfield microscope
e. a phase-contrast microscope

8. What mordant is used in Gram staining?
A. crystal violet
B. safranin
C. acid-alcohol
D. iodine

9. What is one difference between specimen preparation for a transmission electron microscope (TEM) and preparation for a scanning electron microscope (SEM)?
A. Only the TEM specimen requires sputter coating.
B. Only the SEM specimen requires sputter-coating.
C. Only the TEM specimen must be dehydrated.
D. Only the SEM specimen must be dehydrated.

FILL IN THE BLANK

9.1 The Properties of Light

10. When you see light bend as it moves from air into water, you are observing _________.

9.2 Instruments of Microscopy

11. Chromophores that absorb and then emit light are called __________.

12. In a(n) _______ microscope, a probe located just above the specimen moves up and down in response to forces between the atoms and the tip of the probe.

13. What is the total magnification of a specimen that is being viewed with a standard ocular lens and a 40× objective lens?

9.3 Staining Microscopic Specimens

14. Ziehl-Neelsen staining, a type of _______ staining, is diagnostic for *Mycobacterium tuberculosis.*

15. The _______ is used to differentiate bacterial cells based on the components of their cell walls.

SHORT ANSWER

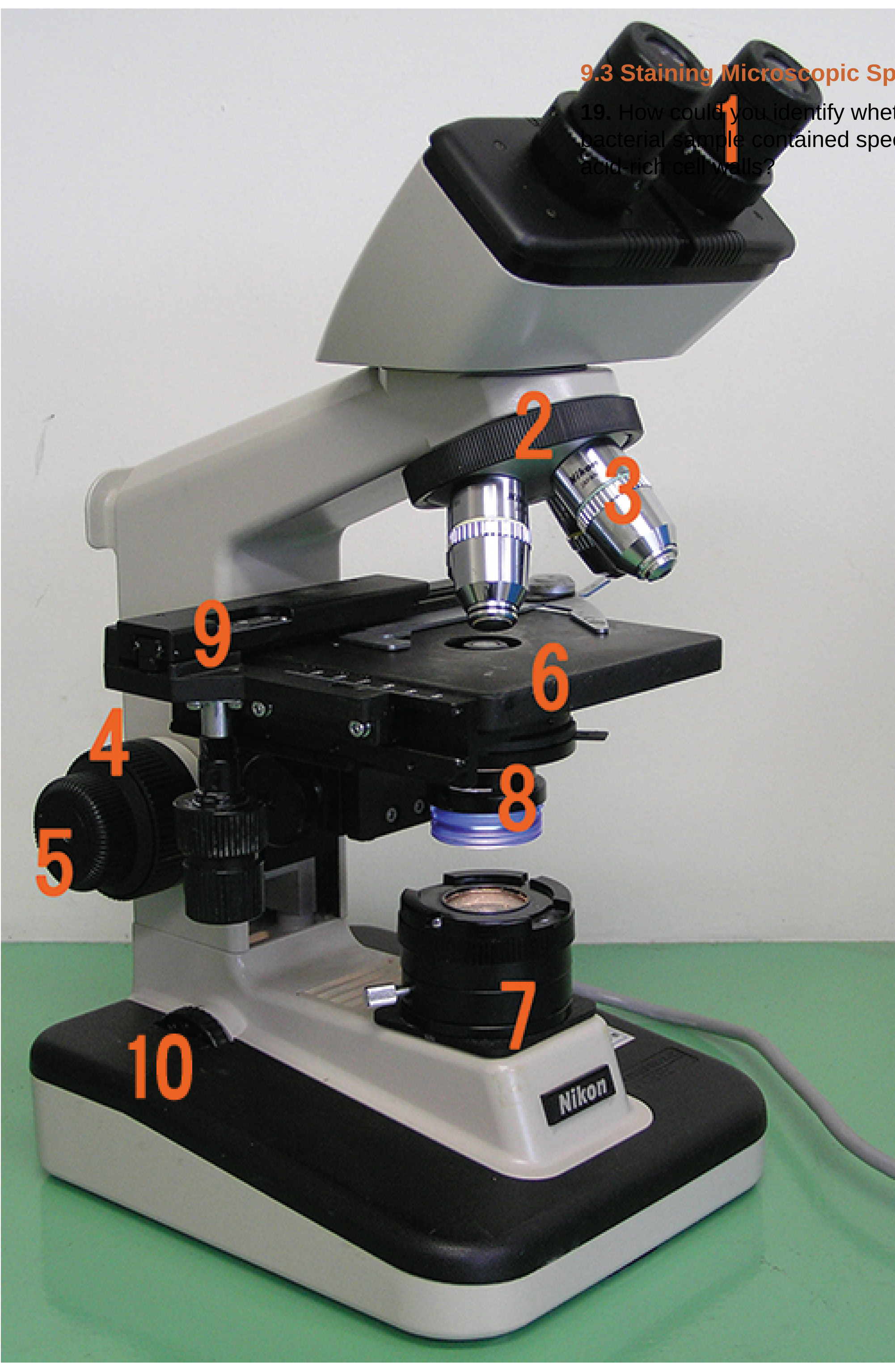
9.1 The Properties of Light

16. Explain how a prism separates white light into different colors.

9.2 Instruments of Microscopy

17. What is the function of the condenser in a brightfield microscope?

18. Art Connection: Label each component of the brightfield microscope.



9.3 Staining Microscopic Specimens

19. How could you identify whether a particular bacterial sample contained specimens with mycolic acid-rich cell walls?

CRITICAL THINKING

20. In Figure 9.7, which of the following has the lowest energy?

A. visible light
B. X-rays
C. ultraviolet rays
D. infrared rays

21. When focusing a light microscope, why is it best to adjust the focus using the coarse focusing knob before using the fine focusing knob?

22. You need to identify structures within a cell using a microscope. However, the image appears very

blurry even though you have a high magnification. What are some things that you could try to improve the resolution of the image? Describe the most basic factors that affect resolution when you first put the slide onto the stage; then consider more specific factors that could affect resolution for 40× and 100× lenses.

23. You use the Gram staining procedure to stain an L-form bacterium (a bacterium that lacks a cell wall). What color will the bacterium be after the staining procedure is finished?

10 | MICROBIAL GROWTH

Figure 10.1 Medical devices that are inserted into a patient's body often become contaminated with a thin biofilm of microorganisms enmeshed in the sticky material they secrete. The electron micrograph (left) shows the inside walls of an in-dwelling catheter. Arrows point to the round cells of *Staphylococcus aureus* bacteria attached to the layers of extracellular substrate. The garbage can (right) served as a rain collector. The arrow points to a green biofilm on the sides of the container. (credit left: modification of work by Centers for Disease Control and Prevention; credit right: modification of work by NASA)

Chapter Outline

10.1: How Microbes Grow
10.2: Oxygen Requirements for Microbial Growth
10.3: The Effects of pH on Microbial Growth
10.4: Temperature and Microbial Growth
10.5: Other Environmental Conditions that Affect Growth
10.6: Media Used for Bacterial Growth

Introduction

We are all familiar with the slimy layer on a pond surface or that makes rocks slippery. These are examples of biofilms—microorganisms embedded in thin layers of matrix material (**Figure 10.1**). Biofilms were long considered random assemblages of cells and had little attention from researchers. Recently, progress in visualization and biochemical methods has revealed that biofilms are an organized ecosystem within which many cells, usually of different species of bacteria, fungi, and algae, interact through cell signaling and coordinated responses. The biofilm provides a protected environment in harsh conditions and aids colonization by microorganisms. Biofilms also have clinical importance. They form on medical devices, resist routine cleaning and sterilization, and cause health-acquired infections. Within the body, biofilms form on the teeth as plaque, in the lungs of patients with cystic fibrosis, and on the cardiac tissue of patients with endocarditis. The slime layer helps protect the cells from host immune defenses and antibiotic treatments.

Studying biofilms requires new approaches. Because of the cells' adhesion properties, many of the methods for culturing and counting cells that are explored in this chapter are not easily applied to biofilms. This is the beginning of a new era of challenges and rewarding insight into the ways that microorganisms grow and thrive in nature.

10.1 | How Microbes Grow

Learning Objectives

By the end of this section, you will be able to:

- Define the generation time for growth based on binary fission
- Identify and describe the activities of microorganisms undergoing typical phases of binary fission (simple cell division) in a growth curve
- Explain several laboratory methods used to determine viable and total cell counts in populations undergoing exponential growth
- Describe examples of cell division not involving binary fission, such as budding or fragmentation
- Describe the formation and characteristics of biofilms
- Identify health risks associated with biofilms and how they are addressed
- Describe quorum sensing and its role in cell-to-cell communication and coordination of cellular activities

Part 1

Jeni, a 24-year-old pregnant woman in her second trimester, visits a clinic with complaints of high fever, 38.9 °C (102 °F), fatigue, and muscle aches—typical flu-like signs and symptoms. Jeni exercises regularly and follows a nutritious diet with emphasis on organic foods, including raw milk that she purchases from a local farmer's market. All of her immunizations are up to date. However, the health-care provider who sees Jeni is concerned and orders a blood sample to be sent for testing by the microbiology laboratory.

- Why is the health-care provider concerned about Jeni's signs and symptoms?

*Jump to the **next** Clinical Focus box*

The bacterial cell cycle involves the formation of new cells through the replication of DNA and partitioning of cellular components into two daughter cells. In prokaryotes, reproduction is always asexual, although extensive genetic recombination in the form of horizontal gene transfer takes place, as will be explored in a different chapter. Most bacteria have a single circular chromosome; however, some exceptions exist. For example, *Borrelia burgdorferi*, the causative agent of Lyme disease, has a linear chromosome.

Binary Fission

The most common mechanism of cell replication in bacteria is a process called **binary fission**, which is depicted in **Figure 10.2**. Before dividing, the cell grows and increases its number of cellular components. Next, the replication of DNA starts at a location on the circular chromosome called the origin of replication, where the chromosome is attached to the inner cell membrane. Replication continues in opposite directions along the chromosome until the terminus is reached.

The center of the enlarged cell constricts until two daughter cells are formed, each offspring receiving a complete copy of the parental genome and a division of the cytoplasm (cytokinesis). This process of cytokinesis and cell division is directed by a protein called FtsZ. FtsZ assembles into a Z ring on the cytoplasmic membrane (**Figure 10.3**). The Z ring is anchored by FtsZ-binding proteins and defines the division plane between the two daughter cells. Additional proteins required for cell division are added to the Z ring to form a structure called the divisome. The divisome activates to produce a peptidoglycan cell wall and build a **septum** that divides the two daughter cells. The daughter cells are separated by the division septum, where all of the cells' outer layers (the cell wall and outer membranes, if present) must be remodeled to complete division. For example, we know that specific enzymes break bonds between the monomers in peptidoglycans and allow addition of new subunits along the division septum.

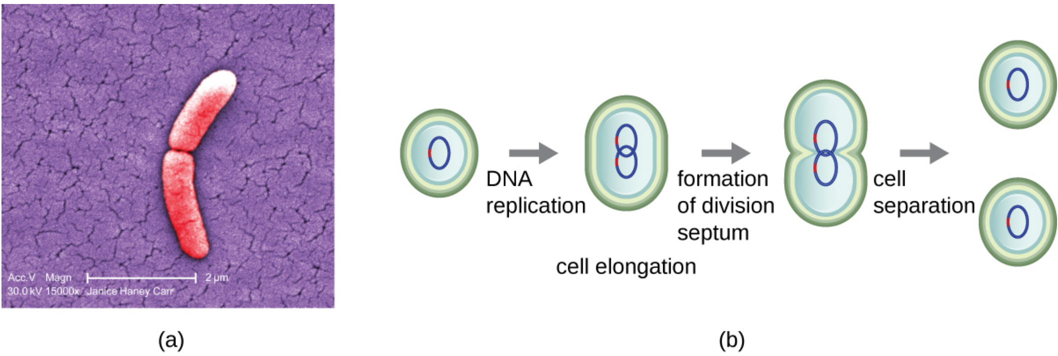
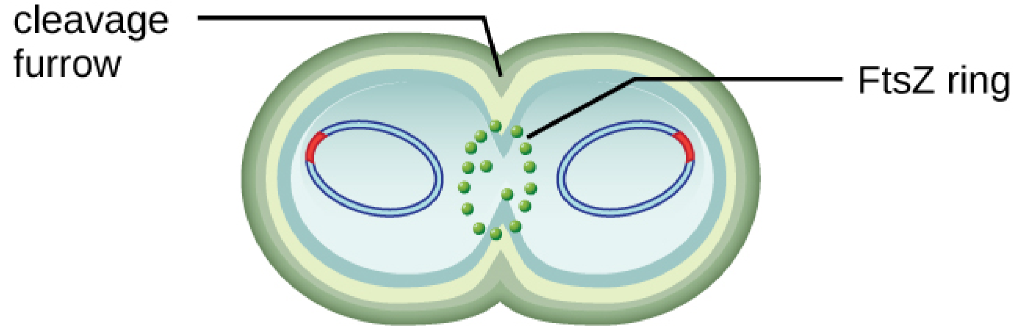
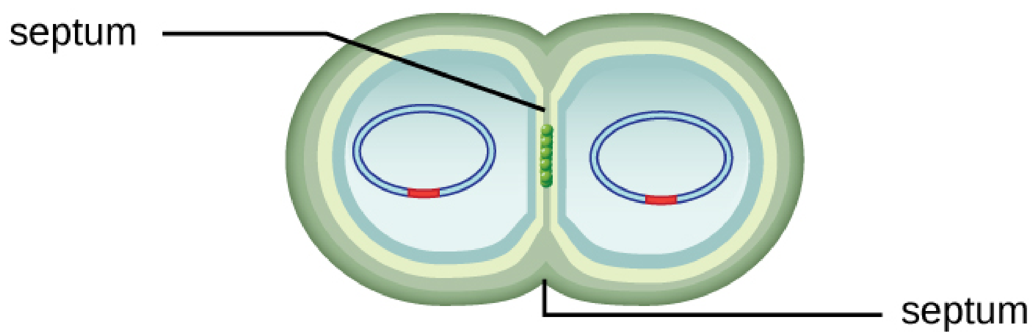


Figure 10.2 (a) The electron micrograph depicts two cells of *Salmonella typhimurium* after a binary fission event. (b) Binary fission in bacteria starts with the replication of DNA as the cell elongates. A division septum forms in the center of the cell. Two daughter cells of similar size form and separate, each receiving a copy of the original chromosome. (credit a: modification of work by Centers for Disease Control and Prevention)





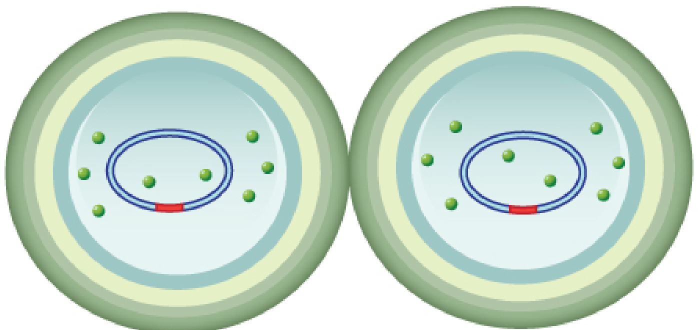


Figure 10.3 FtsZ proteins assemble to form a Z ring that is anchored to the plasma membrane. The Z ring pinches the cell envelope to separate the cytoplasm of the new cells.

- What is the name of the protein that assembles into a Z ring to initiate cytokinesis and cell division?

Generation Time

In eukaryotic organisms, the generation time is the time between the same points of the life cycle in two successive generations. For example, the typical generation time for the human population is 25 years. This definition is not practical for bacteria, which may reproduce rapidly or remain dormant for thousands of years. In prokaryotes (Bacteria and Archaea), the **generation time** is also called the **doubling time** and is defined as the time it takes for the population to double through one round of binary fission. Bacterial doubling times vary enormously. Whereas *Escherichia coli* can double in as little as 20 minutes under optimal growth conditions in the laboratory, bacteria of the same species may need several days to double in especially harsh environments. Most pathogens grow rapidly, like *E. coli*, but there are exceptions. For example, *Mycobacterium tuberculosis*,

the causative agent of tuberculosis, has a generation time of between 15 and 20 hours. On the other hand, *M. leprae*, which causes Hansen's disease (leprosy), grows much more slowly, with a doubling time of 14 days.

Calculating Number of Cells

It is possible to predict the number of cells in a population when they divide by binary fission at a constant rate. As an example, consider what happens if a single cell divides every 30 minutes for 24 hours. The diagram in Figure 10.4 shows the increase in cell numbers for the first three generations.

The number of cells increases exponentially and can be expressed as 2^n, where n is the number of generations. If cells divide every 30 minutes, after 24 hours, 48 divisions would have taken place. If we apply the formula 2^n, where n is equal to 48, the single cell would give rise to 2^{48} or 281,474,976,710,656 cells at 48 generations (24 hours). When dealing with such huge numbers, it is more practical to use scientific notation. Therefore, we express the number of cells as 2.8×10^{14} cells.

In our example, we used one cell as the initial number of cells. For any number of starting cells, the formula is adapted as follows:

$$N_n = N_0 2^n$$

N_n is the number of cells at any generation n, N_0 is the initial number of cells, and n is the number of generations.

Number of generations (n)	Number of cells	Each division adds two new cells
0	1	
1	2	
2	4	
3	8	

Figure 10.4 The parental cell divides and gives rise to two daughter cells. Each of the daughter cells, in turn, divides, giving a total of four cells in the second generation and eight cells in the third generation. Each division doubles the number of cells.

- With a doubling time of 30 minutes and a starting population size of 1×10^5 cells, how many cells will be present after 2 hours, assuming no cell death?

The Growth Curve

Microorganisms grown in closed culture (also known as a batch culture), in which no nutrients are added and most waste is not removed, follow a reproducible growth pattern referred to as the **growth curve**. An example of a batch culture in nature is a pond in which a small number of cells grow in a closed environment. The **culture density** is defined as the number of cells per unit volume. In a closed environment, the culture density is also a measure of the number of cells in the population. Infections of the body do not always follow the growth curve, but correlations can exist depending upon the site and type of infection. When the number of live cells is plotted against time, distinct phases can be observed in the curve (Figure 10.5).

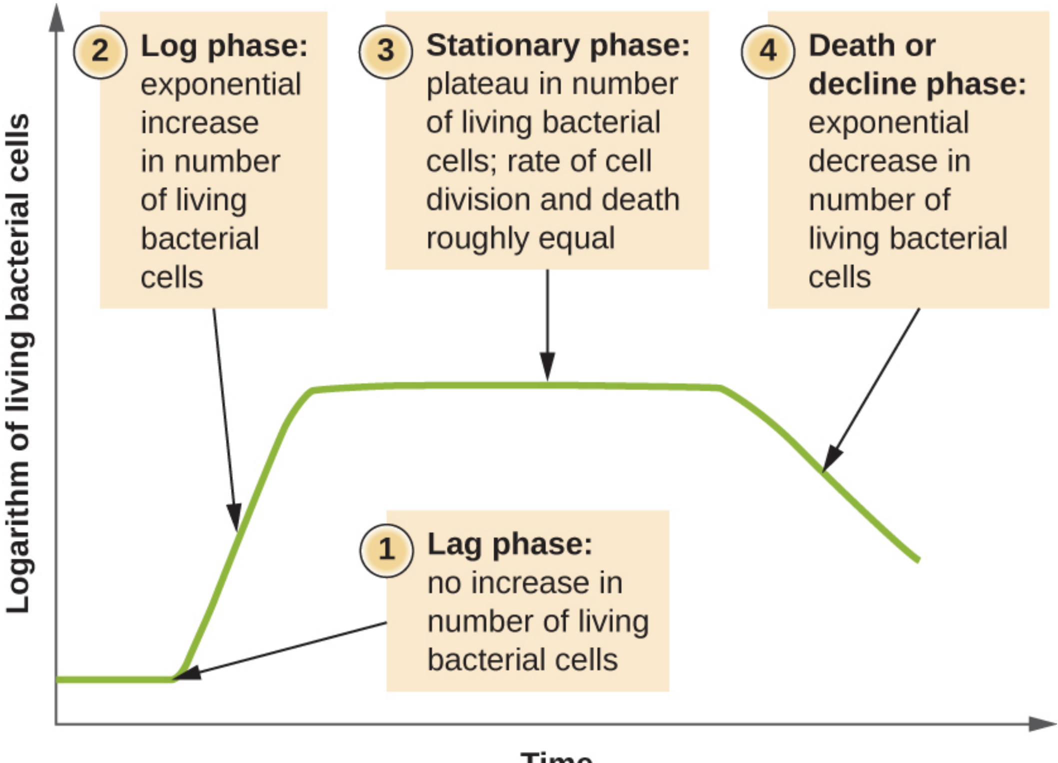


Figure 10.5 The growth curve of a bacterial culture is represented by the logarithm of the number of live cells plotted as a function of time. The graph can be divided into four phases according to the slope, each of which matches events in the cell. The four phases are lag, log, stationary, and death.

The Lag Phase

The beginning of the growth curve represents a small number of cells, referred to as an **inoculum**, that are added to a fresh **culture medium**, a nutritional broth that supports growth. The initial phase of the growth curve is called the **lag phase**, during which cells are gearing up for the next phase of growth. The number of cells does not change during the lag phase; however, cells grow larger and are metabolically active, synthesizing proteins needed to grow within the medium. If any cells were damaged or shocked during the transfer to the new medium, repair takes place during the lag phase. The duration of the lag phase is determined by many factors, including the species and genetic make-up of the cells, the composition of the medium, and the size of the original inoculum.

The Log Phase

In the **logarithmic (log) growth phase**, sometimes called exponential growth phase, the cells are actively dividing by binary fission and their number increases exponentially. For any given bacterial species, the generation time under specific growth conditions (nutrients, temperature, pH, and so forth) is genetically determined, and this generation time is called the **intrinsic growth rate**. During the log phase, the relationship between time and number of cells is not linear but exponential; however, the growth curve is often plotted on a semilogarithmic graph, as shown in Figure 10.6, which gives the appearance of a linear relationship.

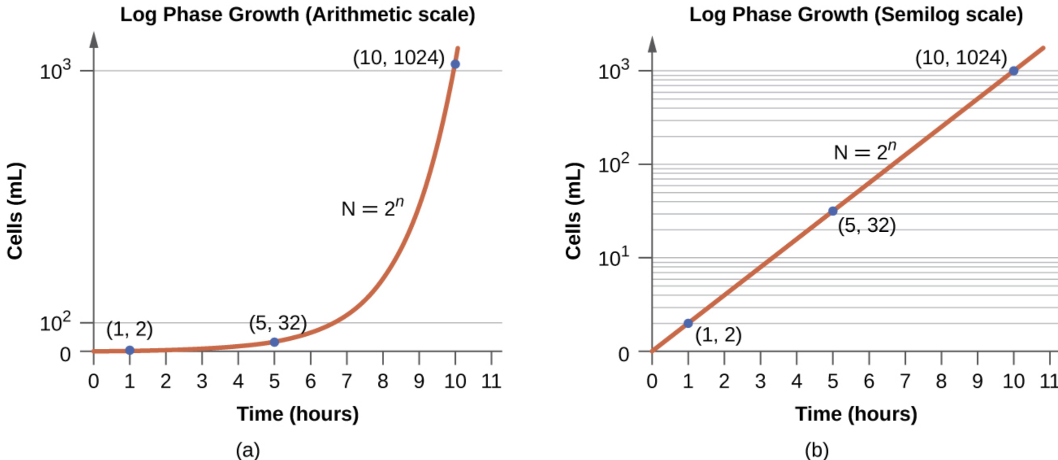


Figure 10.6 Both graphs illustrate population growth during the log phase for a bacterial sample with an initial population of one cell and a doubling time of 1 hour. (a) When plotted on an arithmetic scale, the growth rate resembles a curve. (b) When plotted on a semilogarithmic scale (meaning the values on the *y*-axis are logarithmic), the growth rate appears linear.

Cells in the log phase show constant growth rate and uniform metabolic activity. For this reason, cells in the log phase are preferentially used for industrial applications and research work. The log phase is also the stage where bacteria are the most susceptible to the action of disinfectants and common antibiotics that affect protein, DNA, and cell-wall synthesis.

Stationary Phase

As the number of cells increases through the log phase, several factors contribute to a slowing of the growth rate. Waste products accumulate and nutrients are gradually used up. In addition, gradual depletion of oxygen begins to limit aerobic cell growth. This combination of unfavorable conditions slows and finally stalls population growth. The total number of live cells reaches a plateau referred to as the **stationary phase** (**Figure 10.5**). In this phase, the number of new cells created by cell division is now equivalent to the number of cells dying; thus, the total population of living cells is relatively stagnant. The culture density in a stationary culture is constant. The culture's carrying capacity, or maximum culture density, depends on the types of microorganisms in the culture and the specific conditions of the culture; however, carrying capacity is constant for a given organism grown under the same conditions.

During the stationary phase, cells switch to a survival mode of metabolism. As growth slows, so too does the synthesis of peptidoglycans, proteins, and nucleic-acids; thus, stationary cultures are less susceptible to antibiotics that disrupt these processes. In bacteria capable of producing endospores, many cells undergo sporulation during the stationary phase. Secondary metabolites, including antibiotics, are synthesized in the stationary phase. In certain pathogenic bacteria, the stationary phase is also associated with the expression of virulence factors, products that contribute to a microbe's ability to survive, reproduce, and cause disease in a host organism. For example, quorum sensing in *Staphylococcus aureus* initiates the production of enzymes that can break down human tissue and cellular debris, clearing the way for bacteria to spread to new tissue where nutrients are more plentiful.

The Death Phase

As a culture medium accumulates toxic waste and nutrients are exhausted, cells die in greater and greater numbers. Soon, the number of dying cells exceeds the number of dividing cells, leading to an exponential decrease in the number of cells (**Figure 10.5**). This is the aptly named **death phase**, sometimes called the decline phase. Many cells lyse and release nutrients into the medium, allowing surviving cells to maintain viability and form endospores. A few cells, the so-called **persisters**, are characterized by a slow metabolic rate. Persister cells are medically important because they are associated with certain chronic infections, such as tuberculosis, that do not respond to antibiotic treatment.

Sustaining Microbial Growth

The growth pattern shown in **Figure 10.5** takes place in a closed environment; nutrients are not added and waste and dead cells are not removed. In many cases, though, it is advantageous to maintain cells in the logarithmic phase of growth. One example is in industries that harvest microbial products. A chemostat (**Figure 10.7**) is used to maintain a continuous culture in which nutrients are supplied at a steady rate. A controlled amount of

air is mixed in for aerobic processes. Bacterial suspension is removed at the same rate as nutrients flow in to maintain an optimal growth environment.

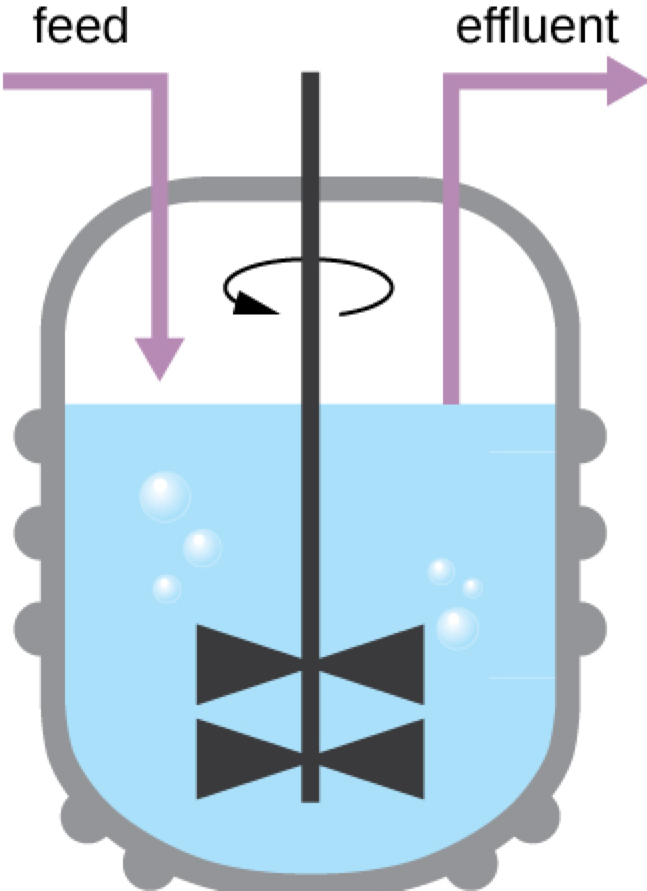


Figure 10.7 A chemostat is a culture vessel fitted with an opening to add nutrients (feed) and an outlet to remove contents (effluent), effectively diluting toxic wastes and dead cells. The addition and removal of fluids is adjusted to maintain the culture in the logarithmic phase of growth. If aerobic bacteria are grown, suitable oxygen levels are maintained.

- During which phase does growth occur at the fastest rate?
- Name two factors that limit microbial growth.

Measurement of Bacterial Growth

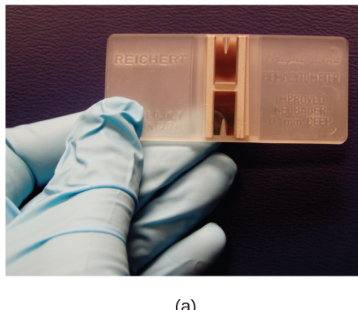
Estimating the number of bacterial cells in a sample, known as a bacterial count, is a common task performed by microbiologists. The number of bacteria in a clinical sample serves as an indication of the extent of an infection. Quality control of drinking water, food, medication, and even cosmetics relies on estimates of bacterial counts to detect contamination and prevent the spread of disease. Two major approaches are used to measure cell number. The direct methods involve counting cells, whereas the indirect methods depend on the measurement of cell presence or activity without actually counting individual cells. Both direct and indirect methods have advantages and disadvantages for specific applications.

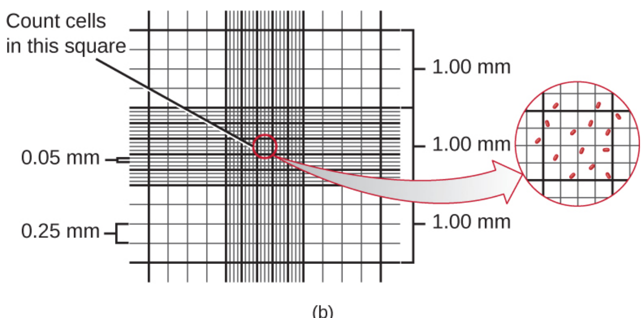
Direct Cell Count

Direct cell count refers to counting the cells in a liquid culture or colonies on a plate. It is a direct way of estimating how many organisms are present in a sample. Let's look first at a simple and fast method that requires only a specialized slide and a compound microscope.

The simplest way to count bacteria is called the **direct microscopic cell count**, which involves transferring a known volume of a culture to a calibrated slide and counting the cells under a light microscope. The calibrated slide is called a **Petroff-Hausser chamber** (Figure 10.8) and is similar to a hemocytometer used to count red

blood cells. The central area of the counting chamber is etched into squares of various sizes. A sample of the culture suspension is added to the chamber under a coverslip that is placed at a specific height from the surface of the grid. It is possible to estimate the concentration of cells in the original sample by counting individual cells in a number of squares and determining the volume of the sample observed. The area of the squares and the height at which the coverslip is positioned are specified for the chamber. The concentration must be corrected for dilution if the sample was diluted before enumeration.





(a) (b)

Figure 10.8 (a) A Petroff-Hausser chamber is a special slide designed for counting the bacterial cells in a measured volume of a sample. A grid is etched on the slide to facilitate precision in counting. (b) This diagram illustrates the grid of a Petroff-Hausser chamber, which is made up of squares of known areas. The enlarged view shows the square within which bacteria (red cells) are counted. If the coverslip is 0.2 mm above the grid and the square has an area of 0.04 mm^2, then the volume is 0.008 mm^3, or 0.000008 mL. Since there are 10 cells inside the square, the density of bacteria is 10 cells/0.000008 mL, which equates to 1,250,000 cells/mL. (credit a: modification of work by Jeffrey M. Vinocur)

Cells in several small squares must be counted and the average taken to obtain a reliable measurement. The advantages of the chamber are that the method is easy to use, relatively fast, and inexpensive. On the downside, the counting chamber does not work well with dilute cultures because there may not be enough cells to count.

Using a counting chamber does not necessarily yield an accurate count of the number of live cells because it is not always possible to distinguish between live cells, dead cells, and debris of the same size under the microscope. However, newly developed fluorescence staining techniques make it possible to distinguish viable and dead bacteria. These viability stains (or live stains) bind to nucleic acids, but the primary and secondary stains differ in their ability to cross the cytoplasmic membrane. The primary stain, which fluoresces green, can penetrate intact cytoplasmic membranes, staining both live and dead cells. The secondary stain, which fluoresces red, can stain a cell only if the cytoplasmic membrane is considerably damaged. Thus, live cells fluoresce green because they only absorb the green stain, whereas dead cells appear red because the red stain displaces the green stain on their nucleic acids (**Figure 10.9**).

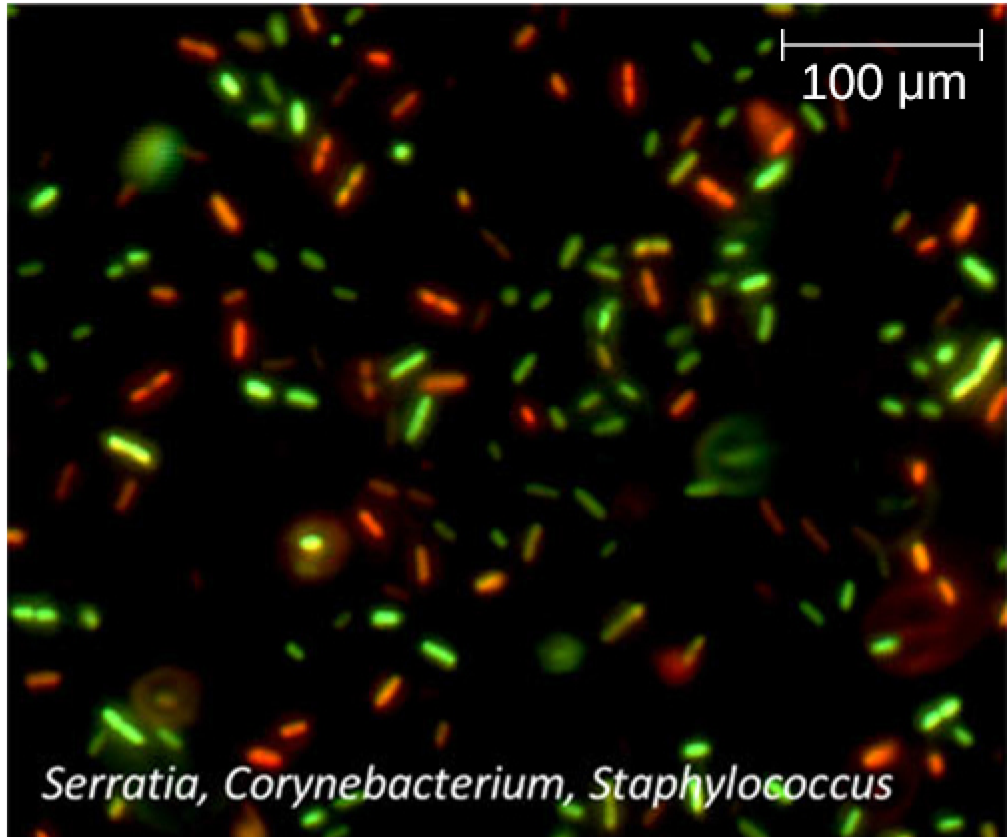


Figure 10.9 Fluorescence staining can be used to differentiate between viable and dead bacterial cells in a sample for purposes of counting. Viable cells are stained green, whereas dead cells are stained red. (credit: modification of work by Emerson J, Adams R, Bentancourt Román C, Brooks B, Coil D, Dahlhousen K, Ganz H, et al.)

Another technique uses an electronic cell counting device (Coulter counter) to detect and count the changes in electrical resistance in a saline solution. A glass tube with a small opening is immersed in an electrolyte solution. A first electrode is suspended in the glass tube. A second electrode is located outside of the tube. As cells are drawn through the small aperture in the glass tube, they briefly change the resistance measured between the two electrodes and the change is recorded by an electronic sensor (Figure 10.10); each resistance change represents a cell. The method is rapid and accurate within a range of concentrations; however, if the culture is too concentrated, more than one cell may pass through the aperture at any given time and skew the results. This method also does not differentiate between live and dead cells.

Direct counts provide an estimate of the total number of cells in a sample. However, in many situations, it is important to know the number of live, or **viable**, cells. Counts of live cells are needed when assessing the extent of an infection, the effectiveness of antimicrobial compounds and medication, or contamination of food and water.

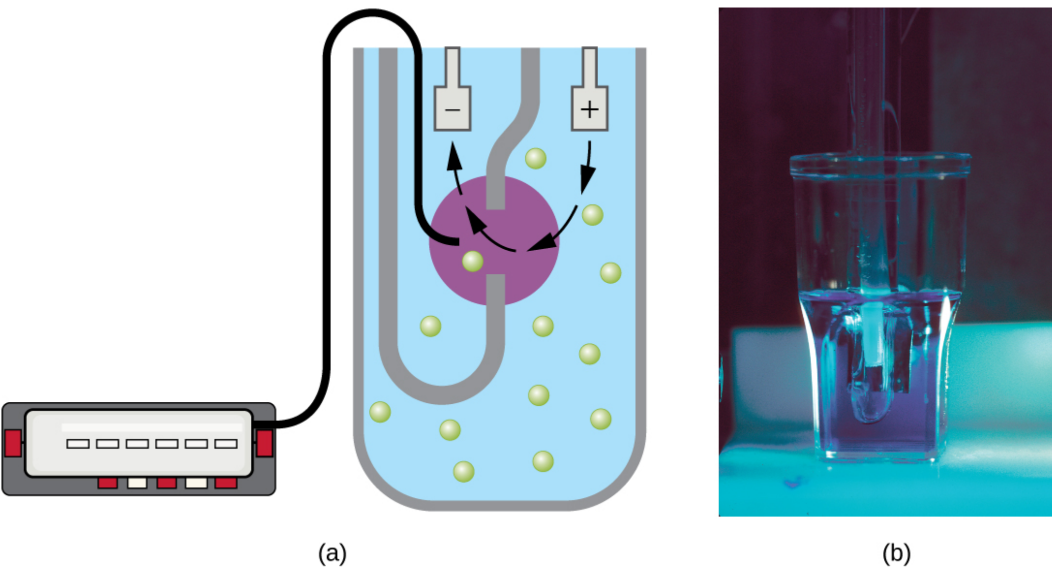


Figure 10.10 A Coulter counter is an electronic device that counts cells. It measures the change in resistance in an electrolyte solution that takes place when a cell passes through a small opening in the inside container wall. A detector automatically counts the number of cells passing through the opening. (credit b: modification of work by National Institutes of Health)

- Why would you count the number of cells in more than one square in the Petroff-Hausser chamber to estimate cell numbers?
- In the viability staining method, why do dead cells appear red?

Plate Count

The **viable plate count**, or simply plate count, is a count of viable or live cells. It is based on the principle that viable cells replicate and give rise to visible colonies when incubated under suitable conditions for the specimen. The results are usually expressed as **colony-forming unit**s per milliliter (CFU/mL) rather than cells per milliliter because more than one cell may have landed on the same spot to give rise to a single colony. Furthermore, samples of bacteria that grow in clusters or chains are difficult to disperse and a single colony may represent several cells. Some cells are described as viable but nonculturable and will not form colonies on solid media. For all these reasons, the viable plate count is considered a low estimate of the actual number of live cells. These limitations do not detract from the usefulness of the method, which provides estimates of live bacterial numbers.

Microbiologists typically count plates with 30–300 colonies. Samples with too few colonies (<30) do not give statistically reliable numbers, and overcrowded plates (>300 colonies) make it difficult to accurately count individual colonies. Also, counts in this range minimize occurrences of more than one bacterial cell forming a single colony. Thus, the calculated CFU is closer to the true number of live bacteria in the population.

There are two common approaches to inoculating plates for viable counts: the pour plate and the spread plate methods. Although the final inoculation procedure differs between these two methods, they both start with a serial dilution of the culture.

Serial Dilution

The **serial dilution** of a culture is an important first step before proceeding to either the pour plate or spread plate method. The goal of the serial dilution process is to obtain plates with CFUs in the range of 30–300, and the process usually involves several dilutions in multiples of 10 to simplify calculation. The number of serial dilutions is chosen according to a preliminary estimate of the culture density. **Figure 10.11** illustrates the serial dilution method.

A fixed volume of the original culture, 1.0 mL, is added to and thoroughly mixed with the first dilution tube solution, which contains 9.0 mL of sterile broth. This step represents a dilution factor of 10, or 1:10, compared with the original culture. From this first dilution, the same volume, 1.0 mL, is withdrawn and mixed with a fresh tube of 9.0 mL of dilution solution. The dilution factor is now 1:100 compared with the original culture. This process continues until a series of dilutions is produced that will bracket the desired cell concentration for

accurate counting. From each tube, a sample is plated on solid medium using either the **pour plate method** (Figure 10.12) or the **spread plate method** (Figure 10.13). The plates are incubated until colonies appear. Two to three plates are usually prepared from each dilution and the numbers of colonies counted on each plate are averaged. In all cases, thorough mixing of samples with the dilution medium (to ensure the cell distribution in the tube is random) is paramount to obtaining reliable results.

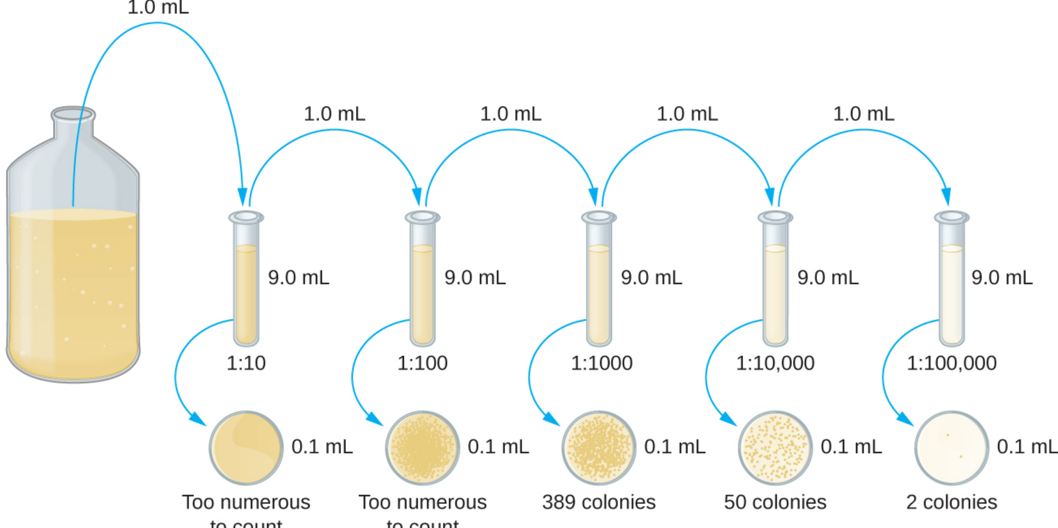


Figure 10.11 Serial dilution involves diluting a fixed volume of cells mixed with dilution solution using the previous dilution as an inoculum. The result is dilution of the original culture by an exponentially growing factor. (credit: modification of work by "Leberechtc"/Wikimedia Commons)

The dilution factor is used to calculate the number of cells in the original cell culture. In our example, an average of 50 colonies was counted on the plates obtained from the 1:10,000 dilution. Because only 0.1 mL of suspension was pipetted on the plate, the multiplier required to reconstitute the original concentration is 10 × 10,000. The number of CFU per mL is equal to 50 × 10 × 10,000 = 5,000,000. The number of bacteria in the culture is estimated as 5 million cells/mL. The colony count obtained from the 1:1000 dilution was 389, well below the expected 500 for a 10-fold difference in dilutions. This highlights the issue of inaccuracy when colony counts are greater than 300 and more than one bacterial cell grows into a single colony.

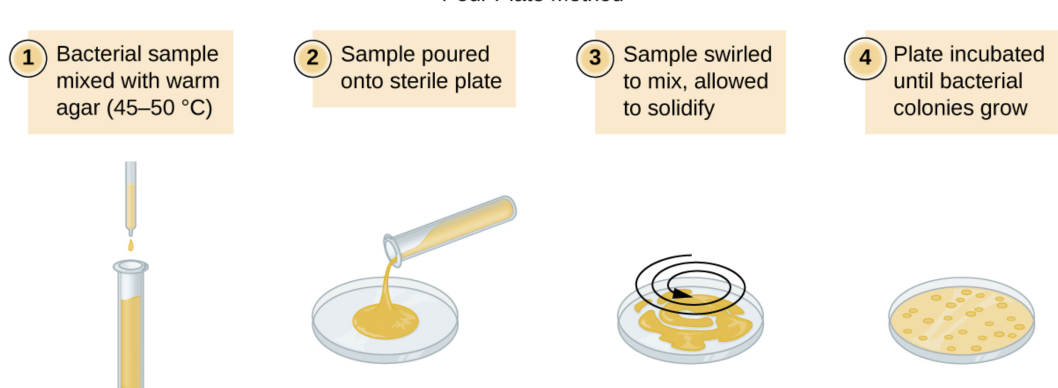


Figure 10.12 In the pour plate method of cell counting, the sample is mixed in liquid warm agar (45–50 °C) poured into a sterile Petri dish and further mixed by swirling. This process is repeated for each serial dilution prepared. The resulting colonies are counted and provide an estimate of the number of cells in the original volume sampled.

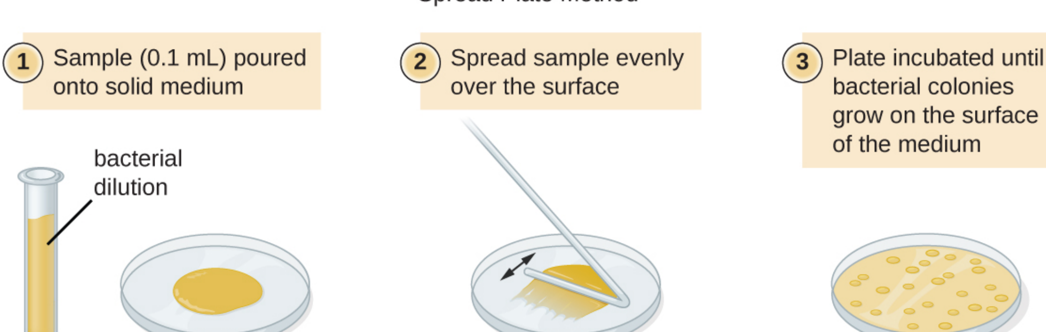


Figure 10.13 In the spread plate method of cell counting, the sample is poured onto solid agar and then spread using a sterile spreader. This process is repeated for each serial dilution prepared. The resulting colonies are counted and provide an estimate of the number of cells in the original volume samples.

A very dilute sample—drinking water, for example—may not contain enough organisms to use either of the plate count methods described. In such cases, the original sample must be concentrated rather than diluted before plating. This can be accomplished using a modification of the plate count technique called the **membrane filtration technique**. Known volumes are vacuum-filtered aseptically through a membrane with a pore size small enough to trap microorganisms. The membrane is transferred to a Petri plate containing an appropriate growth medium. Colonies are counted after incubation. Calculation of the cell density is made by dividing the cell count by the volume of filtered liquid.

Watch this **video (https://openstax.org/l/22serdilpltcvid)** for demonstrations of serial dilutions and spread plate techniques.

The Most Probable Number

The number of microorganisms in dilute samples is usually too low to be detected by the plate count methods described thus far. For these specimens, microbiologists routinely use the **most probable number (MPN) method**, a statistical procedure for estimating of the number of viable microorganisms in a sample. Often used for water and food samples, the MPN method evaluates detectable growth by observing changes in turbidity or color due to metabolic activity.

A typical application of MPN method is the estimation of the number of coliforms in a sample of pond water. Coliforms are gram-negative rod bacteria that ferment lactose. The presence of coliforms in water is considered a sign of contamination by fecal matter. For the method illustrated in **Figure 10.14**, a series of three dilutions of the water sample is tested by inoculating five lactose broth tubes with 10 mL of sample, five lactose broth tubes with 1 mL of sample, and five lactose broth tubes with 0.1 mL of sample. The lactose broth tubes contain a pH indicator that changes color from red to yellow when the lactose is fermented. After inoculation and incubation, the tubes are examined for an indication of coliform growth by a color change in media from red to yellow. The first set of tubes (10-mL sample) showed growth in all the tubes; the second set of tubes (1 mL) showed growth in two tubes out of five; in the third set of tubes, no growth is observed in any of the tubes (0.1-mL dilution). The numbers 5, 2, and 0 are compared with **Figure B1** in **Appendix B**, which has been constructed using a probability model of the sampling procedure. From our reading of the table, we conclude that 49 is the most probable number of bacteria per 100 mL of pond water.no lo

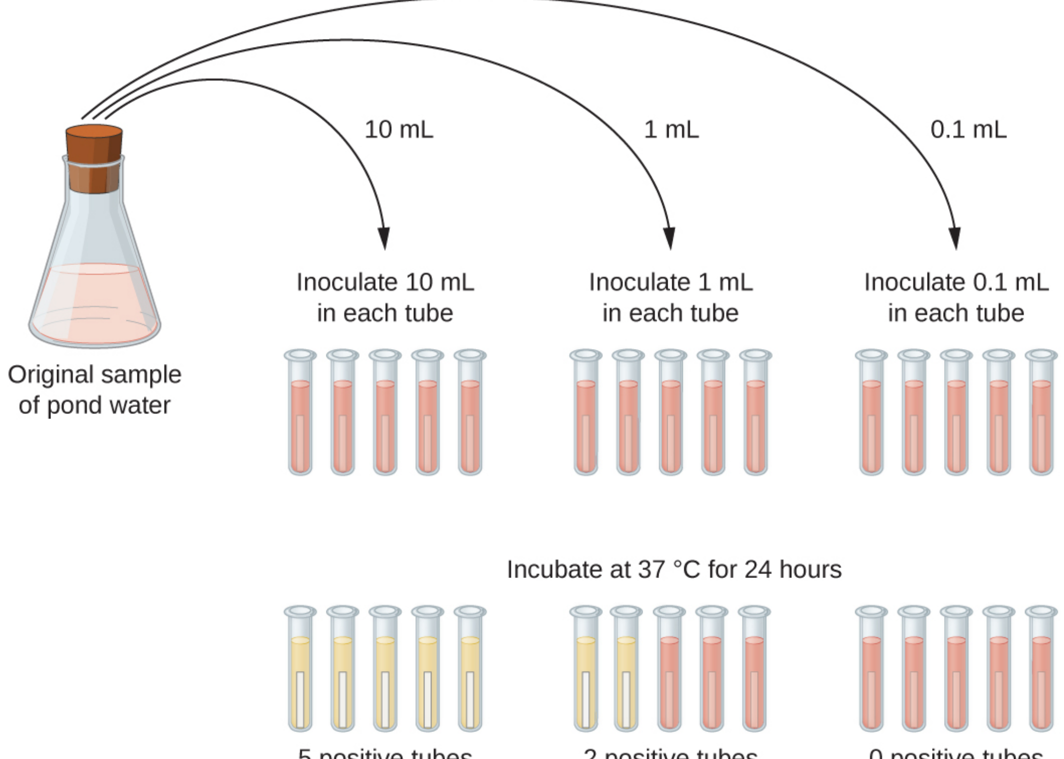


Figure 10.14 In the most probable number method, sets of five lactose broth tubes are inoculated with three different volumes of pond water: 10 mL, 1 mL, and 0.1mL. Bacterial growth is assessed through a change in the color of the broth from red to yellow as lactose is fermented.

- What is a colony-forming unit?
- What two methods are frequently used to estimate bacterial numbers in water samples?

Indirect Cell Counts

Besides direct methods of counting cells, other methods, based on an indirect detection of cell density, are commonly used to estimate and compare cell densities in a culture. The foremost approach is to measure the **turbidity** (cloudiness) of a sample of bacteria in a liquid suspension. The laboratory instrument used to measure turbidity is called a spectrophotometer (**Figure 10.15**). In a spectrophotometer, a light beam is transmitted through a bacterial suspension, the light passing through the suspension is measured by a detector, and the amount of light passing through the sample and reaching the detector is converted to either percent transmission or a logarithmic value called absorbance (optical density). As the numbers of bacteria in a suspension increase, the turbidity also increases and causes less light to reach the detector. The decrease in light passing through the sample and reaching the detector is associated with a decrease in percent transmission and increase in absorbance measured by the spectrophotometer.

Measuring turbidity is a fast method to estimate cell density as long as there are enough cells in a sample to produce turbidity. It is possible to correlate turbidity readings to the actual number of cells by performing a viable plate count of samples taken from cultures having a range of absorbance values. Using these values, a calibration curve is generated by plotting turbidity as a function of cell density. Once the calibration curve has been produced, it can be used to estimate cell counts for all samples obtained or cultured under similar conditions and with densities within the range of values used to construct the curve.

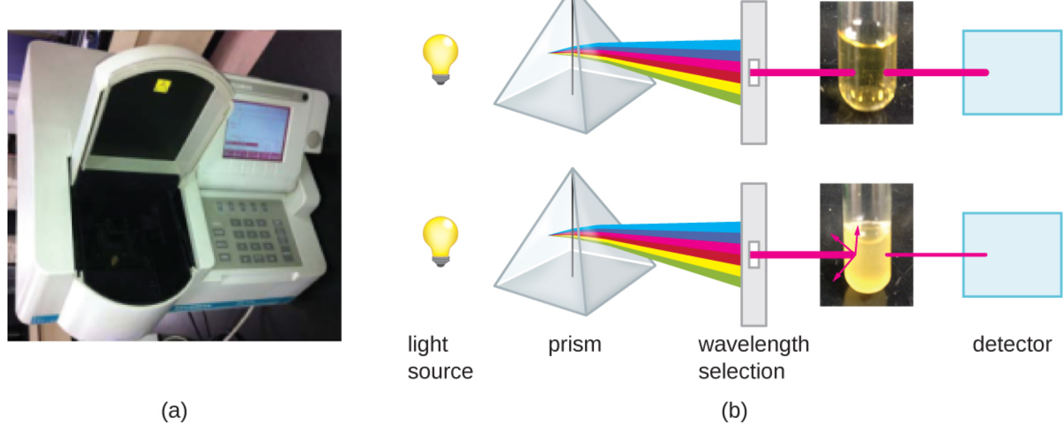


Figure 10.15 (a) A spectrophotometer is commonly used to measure the turbidity of a bacterial cell suspension as an indirect measure of cell density. (b) A spectrophotometer works by splitting white light from a source into a spectrum. The spectrophotometer allows choice of the wavelength of light to use for the measurement. The optical density (turbidity) of the sample will depend on the wavelength, so once one wavelength is chosen, it must be used consistently. The filtered light passes through the sample (or a control with only medium) and the light intensity is measured by a detector. The light passing into a suspension of bacteria is scattered by the cells in such a way that some fraction of it never reaches the detector. This scattering happens to a far lesser degree in the control tube with only the medium. (credit a: modification of work by Hwang HS, Kim MS; credit b "test tube photos": modification of work by Suzanne Wakim)

Measuring dry weight of a culture sample is another indirect method of evaluating culture density without directly measuring cell counts. The cell suspension used for weighing must be concentrated by filtration or centrifugation, washed, and then dried before the measurements are taken. The degree of drying must be standardized to account for residual water content. This method is especially useful for filamentous microorganisms, which are difficult to enumerate by direct or viable plate count.

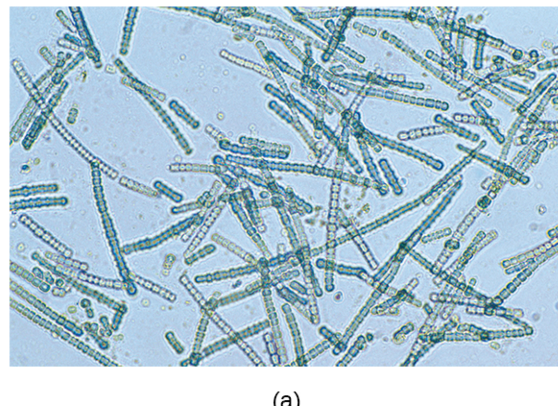
As we have seen, methods to estimate viable cell numbers can be labor intensive and take time because cells must be grown. Recently, indirect ways of measuring live cells have been developed that are both fast and easy to implement. These methods measure cell activity by following the production of metabolic products or disappearance of reactants. Adenosine triphosphate (ATP) formation, biosynthesis of proteins and nucleic acids, and consumption of oxygen can all be monitored to estimate the number of cells.

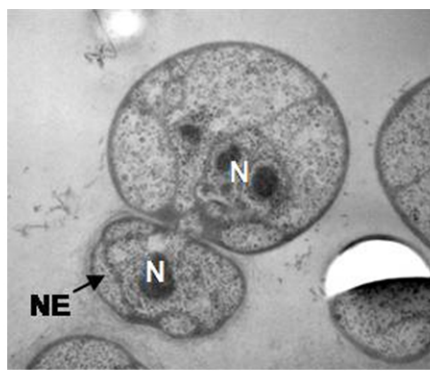
- What is the purpose of a calibration curve when estimating cell count from turbidity measurements?
- What are the newer indirect methods of counting live cells?

Alternative Patterns of Cell Division

Binary fission is the most common pattern of cell division in prokaryotes, but it is not the only one. Other mechanisms usually involve asymmetrical division (as in budding) or production of spores in aerial filaments.

In some cyanobacteria, many nucleoids may accumulate in an enlarged round cell or along a filament, leading to the generation of many new cells at once. The new cells often split from the parent filament and float away in a process called **fragmentation** (**Figure 10.16**). Fragmentation is commonly observed in the Actinomycetes, a group of gram-positive, anaerobic bacteria commonly found in soil. Another curious example of cell division in prokaryotes, reminiscent of live birth in animals, is exhibited by the giant bacterium *Epulopiscium*. Several daughter cells grow fully in the parent cell, which eventually disintegrates, releasing the new cells to the environment. Other species may form a long narrow extension at one pole in a process called **budding**. The tip of the extension swells and forms a smaller cell, the bud that eventually detaches from the parent cell. Budding is most common in yeast (**Figure 10.16**), but it is also observed in prosthecate bacteria and some cyanobacteria.





(a) (b)

Figure 10.16 (a) Filamentous cyanobacteria, like those pictured here, replicate by fragmentation. (b) In this electron micrograph, cells of the bacterium *Gemmata obscuriglobus* are budding. The larger cell is the mother cell. Labels indicate the nucleoids (N) and the still-forming nuclear envelope (NE) of the daughter cell. (credit a: modification of work by CSIRO; credit b: modification of work by Kuo-Chang Lee, Rick I Webb and John A Fuerst)

The soil bacteria *Actinomyces* grow in long filaments divided by septa, similar to the mycelia seen in fungi, resulting in long cells with multiple nucleoids. Environmental signals, probably related to low nutrient availability, lead to the formation of aerial filaments. Within these aerial filaments, elongated cells divide simultaneously. The new cells, which contain a single nucleoid, develop into spores that give rise to new colonies.

- Identify at least one difference between fragmentation and budding.

Biofilms

In nature, microorganisms grow mainly in **biofilms**, complex and dynamic ecosystems that form on a variety of environmental surfaces, from industrial conduits and water treatment pipelines to rocks in river beds. Biofilms are not restricted to solid surface substrates, however. Almost any surface in a liquid environment containing some minimal nutrients will eventually develop a biofilm. Microbial mats that float on water, for example, are biofilms that contain large populations of photosynthetic microorganisms. Biofilms found in the human mouth may contain hundreds of bacterial species. Regardless of the environment where they occur, biofilms are not random collections of microorganisms; rather, they are highly structured communities that provide a selective advantage to their constituent microorganisms.

Biofilm Structure

Observations using confocal microscopy have shown that environmental conditions influence the overall structure of biofilms. Filamentous biofilms called streamers form in rapidly flowing water, such as freshwater streams, eddies, and specially designed laboratory flow cells that replicate growth conditions in fast-moving fluids. The streamers are anchored to the substrate by a “head” and the “tail” floats downstream in the current. In still or slow-moving water, biofilms mainly assume a mushroom-like shape. The structure of biofilms may also change with other environmental conditions such as nutrient availability.

Detailed observations of biofilms under confocal laser and scanning electron microscopes reveal clusters of microorganisms embedded in a matrix interspersed with open water channels. The extracellular matrix consists of **extracellular polymeric substances (EPS)** secreted by the organisms in the biofilm. The extracellular matrix represents a large fraction of the biofilm, accounting for 50%–90% of the total dry mass. The properties of the EPS vary according to the resident organisms and environmental conditions.

EPS is a hydrated gel composed primarily of polysaccharides and containing other macromolecules such as proteins, nucleic acids, and lipids. It plays a key role in maintaining the integrity and function of the biofilm. Channels in the EPS allow movement of nutrients, waste, and gases throughout the biofilm. This keeps the cells hydrated, preventing desiccation. EPS also shelters organisms in the biofilm from predation by other microbes or cells (e.g., protozoans, white blood cells in the human body).

Biofilm Formation

Free-floating microbial cells that live in an aquatic environment are called **planktonic** cells. The formation of a biofilm essentially involves the attachment of planktonic cells to a substrate, where they become **sessile**

(attached to a surface). This occurs in stages, as depicted in **Figure 10.17**. The first stage involves the attachment of planktonic cells to a surface coated with a conditioning film of organic material. At this point, attachment to the substrate is reversible, but as cells express new phenotypes that facilitate the formation of EPS, they transition from a planktonic to a sessile lifestyle. The biofilm develops characteristic structures, including an extensive matrix and water channels. Appendages such as fimbriae, pili, and flagella interact with the EPS, and microscopy and genetic analysis suggest that such structures are required for the establishment of a mature biofilm. In the last stage of the biofilm life cycle, cells on the periphery of the biofilm revert to a planktonic lifestyle, sloughing off the mature biofilm to colonize new sites. This stage is referred to as dispersal.

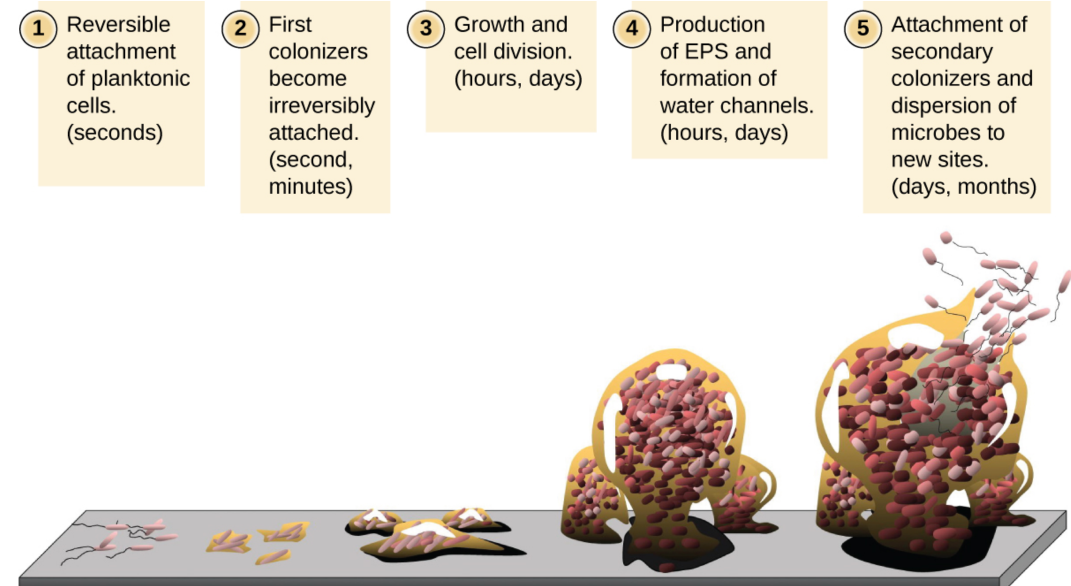


Figure 10.17 Stages in the formation and life cycle of a biofilm. (credit: modification of work by Public Library of Science and American Society for Microbiology)

Within a biofilm, different species of microorganisms establish metabolic collaborations in which the waste product of one organism becomes the nutrient for another. For example, aerobic microorganisms consume oxygen, creating anaerobic regions that promote the growth of anaerobes. This occurs in many polymicrobial infections that involve both aerobic and anaerobic pathogens.

The mechanism by which cells in a biofilm coordinate their activities in response to environmental stimuli is called **quorum sensing**. Quorum sensing—which can occur between cells of different species within a biofilm—enables microorganisms to detect their cell density through the release and binding of small, diffusible molecules called **autoinducers**. When the cell population reaches a critical threshold (a quorum), these autoinducers initiate a cascade of reactions that activate genes associated with cellular functions that are beneficial only when the population reaches a critical density. For example, in some pathogens, synthesis of virulence factors only begins when enough cells are present to overwhelm the immune defenses of the host. Although mostly studied in bacterial populations, quorum sensing takes place between bacteria and eukaryotes and between eukaryotic cells such as the fungus *Candida albicans*, a common member of the human microbiota that can cause infections in immunocompromised individuals.

The signaling molecules in quorum sensing belong to two major classes. Gram-negative bacteria communicate mainly using N-acylated homoserine lactones, whereas gram-positive bacteria mostly use small peptides (**Figure 10.18**). In all cases, the first step in quorum sensing consists of the binding of the autoinducer to its specific receptor only when a threshold concentration of signaling molecules is reached. Once binding to the receptor takes place, a cascade of signaling events leads to changes in gene expression. The result is the activation of biological responses linked to quorum sensing, notably an increase in the production of signaling molecules themselves, hence the term autoinducer.

Figure 10.18 Short peptides in gram-positive bacteria and N-acetylated homoserine lactones in gram-negative bacteria act as autoinducers in quorum sensing and mediate the coordinated response of bacterial cells. The R side chain of the N-acetylated homoserine lactone is specific for the species of gram-negative bacteria. Some secreted homoserine lactones are recognized by more than one species.

Biofilms and Human Health

The human body harbors many types of biofilms, some beneficial and some harmful. For example, the layers of normal microbiota lining the intestinal and respiratory mucosa play a role in warding off infections by pathogens. However, other biofilms in the body can have a detrimental effect on health. For example, the plaque that forms on teeth is a biofilm that can contribute to dental and periodontal disease. Biofilms can also form in wounds, sometimes causing serious infections that can spread. The bacterium *Pseudomonas aeruginosa* often colonizes biofilms in the airways of patients with cystic fibrosis, causing chronic and sometimes fatal infections of the lungs. Biofilms can also form on medical devices used in or on the body, causing infections in patients with in-dwelling catheters, artificial joints, or contact lenses.

Pathogens embedded within biofilms exhibit a higher resistance to antibiotics than their free-floating counterparts. Several hypotheses have been proposed to explain why. Cells in the deep layers of a biofilm are metabolically inactive and may be less susceptible to the action of antibiotics that disrupt metabolic activities. The EPS may also slow the diffusion of antibiotics and antiseptics, preventing them from reaching cells in the deeper layers of the biofilm. Phenotypic changes may also contribute to the increased resistance exhibited by bacterial cells in biofilms. For example, the increased production of efflux pumps, membrane-embedded proteins that actively extrude antibiotics out of bacterial cells, have been shown to be an important mechanism of antibiotic resistance among biofilm-associated bacteria. Finally, biofilms provide an ideal environment for the exchange of extrachromosomal DNA, which often includes genes that confer antibiotic resistance.

- What is the matrix of a biofilm composed of?
- What is the role of quorum sensing in a biofilm?

10.2 | Oxygen Requirements for Microbial Growth

Learning Objectives

By the end of this section, you will be able to:

- Interpret visual data demonstrating minimum, optimum, and maximum oxygen or carbon dioxide requirements for growth
- Identify and describe different categories of microbes with requirements for growth with or without oxygen: obligate aerobe, obligate anaerobe, facultative anaerobe, aerotolerant anaerobe, microaerophile, and

capnophile

- Give examples of microorganisms for each category of growth requirements

Ask most people “What are the major requirements for life?” and the answers are likely to include water and oxygen. Few would argue about the need for water, but what about oxygen? Can there be life without oxygen?

The answer is that molecular oxygen (O_2) is not always needed. The earliest signs of life are dated to a period when conditions on earth were highly reducing and free oxygen gas was essentially nonexistent. Only after cyanobacteria started releasing oxygen as a byproduct of photosynthesis and the capacity of iron in the oceans for taking up oxygen was exhausted did oxygen levels increase in the atmosphere. This event, often referred to as the Great Oxygenation Event or the Oxygen Revolution, caused a massive extinction. Most organisms could not survive the powerful oxidative properties of **reactive oxygen species** (ROS), highly unstable ions and molecules derived from partial reduction of oxygen that can damage virtually any macromolecule or structure with which they come in contact. Singlet oxygen (O_2•), superoxide $(O_2^{\,-})$, peroxides (H_2O_2), hydroxyl radical (OH•), and hypochlorite ion (OCl^-), the active ingredient of household bleach, are all examples of ROS. The organisms that were able to detoxify reactive oxygen species harnessed the high electronegativity of oxygen to produce free energy for their metabolism and thrived in the new environment.

Oxygen Requirements of Microorganisms

Many ecosystems are still free of molecular oxygen. Some are found in extreme locations, such as deep in the ocean or in earth’s crust; others are part of our everyday landscape, such as marshes, bogs, and sewers. Within the bodies of humans and other animals, regions with little or no oxygen provide an anaerobic environment for microorganisms. (**Figure 10.19**).

(a) (b)

Figure 10.19 Anaerobic environments are still common on earth. They include environments like (a) a bog where undisturbed dense sediments are virtually devoid of oxygen, and (b) the rumen (the first compartment of a cow’s stomach), which provides an oxygen-free incubator for methanogens and other obligate anaerobic bacteria. (credit a: modification of work by National Park Service; credit b: modification of work by US Department of Agriculture)

We can easily observe different requirements for molecular oxygen by growing bacteria in **thioglycolate tube culture**s. A test-tube culture starts with autoclaved **thioglycolate medium** containing a low percentage of agar to allow motile bacteria to move throughout the medium. Thioglycolate has strong reducing properties and autoclaving flushes out most of the oxygen. The tubes are inoculated with the bacterial cultures to be tested and incubated at an appropriate temperature. Over time, oxygen slowly diffuses throughout the thioglycolate tube culture from the top. Bacterial density increases in the area where oxygen concentration is best suited for the growth of that particular organism.

The growth of bacteria with varying oxygen requirements in thioglycolate tubes is illustrated in **Figure 10.20**. In tube A, all the growth is seen at the top of the tube. The bacteria are **obligate (strict) aerobe**s that cannot grow without an abundant supply of oxygen. Tube B looks like the opposite of tube A. Bacteria grow at the bottom of tube B. Those are **obligate anaerobe**s, which are killed by oxygen. Tube C shows heavy growth at the top of the tube and growth throughout the tube, a typical result with **facultative anaerobe**s. Facultative anaerobes are organisms that thrive in the presence of oxygen but also grow in its absence by relying on fermentation or anaerobic respiration, if there is a suitable electron acceptor other than oxygen and the organism is able to perform anaerobic respiration. The **aerotolerant anaerobe**s in tube D are indifferent to the presence of oxygen. They do not use oxygen because they usually have a fermentative metabolism, but they are not harmed by the presence of oxygen as obligate anaerobes are. Tube E on the right shows a “Goldilocks” culture. The oxygen

level has to be just right for growth, not too much and not too little. These **microaerophile**s are bacteria that require a minimum level of oxygen for growth, about 1%–10%, well below the 21% found in the atmosphere.

Examples of obligate aerobes are *Mycobacterium tuberculosis*, the causative agent of tuberculosis and *Micrococcus luteus*, a gram-positive bacterium that colonizes the skin. *Neisseria meningitidis*, the causative agent of severe bacterial meningitis, and *N. gonorrhoeae*, the causative agent of sexually transmitted gonorrhea, are also obligate aerobes.

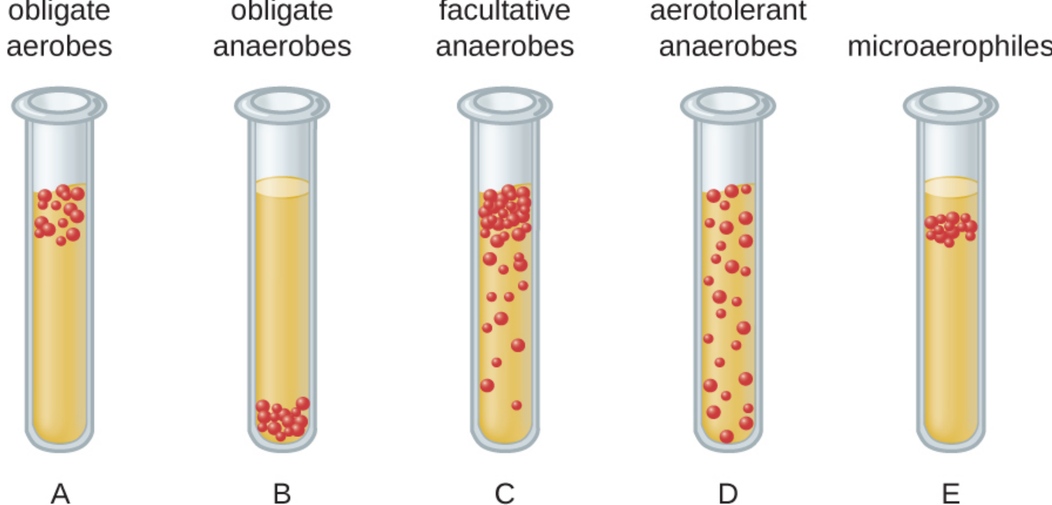
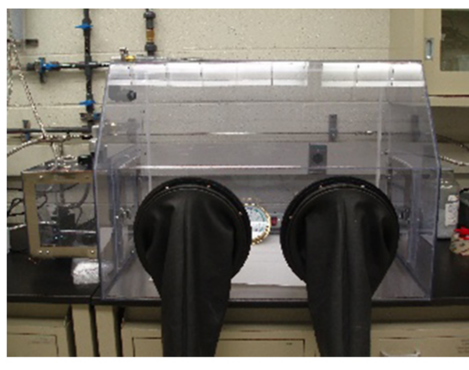


Figure 10.20 Diagram of bacterial cell distribution in thioglycolate tubes.

Many obligate anaerobes are found in the environment where anaerobic conditions exist, such as in deep sediments of soil, still waters, and at the bottom of the deep ocean where there is no photosynthetic life. Anaerobic conditions also exist naturally in the intestinal tract of animals. Obligate anaerobes, mainly *Bacteroidetes*, represent a large fraction of the microbes in the human gut. Transient anaerobic conditions exist when tissues are not supplied with blood circulation; they die and become an ideal breeding ground for obligate anaerobes. Another type of obligate anaerobe encountered in the human body is the gram-positive, rod-shaped *Clostridium* spp. Their ability to form endospores allows them to survive in the presence of oxygen. One of the major causes of health-acquired infections is *C. difficile*, known as C. diff. Prolonged use of antibiotics for other infections increases the probability of a patient developing a secondary *C. difficile* infection. Antibiotic treatment disrupts the balance of microorganisms in the intestine and allows the colonization of the gut by *C. difficile*, causing a significant inflammation of the colon.

Other clostridia responsible for serious infections include *C. tetani*, the agent of tetanus, and *C. perfringens*, which causes gas gangrene. In both cases, the infection starts in necrotic tissue (dead tissue that is not supplied with oxygen by blood circulation). This is the reason that deep puncture wounds are associated with tetanus. When tissue death is accompanied by lack of circulation, gangrene is always a danger.

The study of obligate anaerobes requires special equipment. Obligate anaerobic bacteria must be grown under conditions devoid of oxygen. The most common approach is culture in an **anaerobic jar** (**Figure 10.21**). Anaerobic jars include chemical packs that remove oxygen and release carbon dioxide (CO_2). An **anaerobic chamber** is an enclosed box from which all oxygen is removed. Gloves sealed to openings in the box allow handling of the cultures without exposing the culture to air (**Figure 10.21**).



(a) (b)

Figure 10.21 (a) An anaerobic jar is pictured that is holding nine Petri plates supporting cultures. (b) Openings in the side of an anaerobic box are sealed by glove-like sleeves that allow for the handling of cultures inside the box. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by NIST)

Staphylococci and Enterobacteriaceae are examples of facultative anaerobes. Staphylococci are found on the skin and upper respiratory tract. Enterobacteriaceae are found primarily in the gut and upper respiratory tract but can sometimes spread to the urinary tract, where they are capable of causing infections. It is not unusual to see mixed bacterial infections in which the facultative anaerobes use up the oxygen, creating an environment for the obligate anaerobes to flourish.

Examples of aerotolerant anaerobes include lactobacilli and streptococci, both found in the oral microbiota. *Campylobacter jejuni*, which causes gastrointestinal infections, is an example of a microaerophile and is grown under low-oxygen conditions.

The **optimum oxygen concentration**, as the name implies, is the ideal concentration of oxygen for a particular microorganism. The lowest concentration of oxygen that allows growth is called the **minimum permissive oxygen concentration**. The highest tolerated concentration of oxygen is the **maximum permissive oxygen concentration**. The organism will not grow outside the range of oxygen levels found between the minimum and maximum permissive oxygen concentrations.

- Would you expect the oldest bacterial lineages to be aerobic or anaerobic?
- Which bacteria grow at the top of a thioglycolate tube, and which grow at the bottom of the tube?

An Unwelcome Anaerobe

Charles is a retired bus driver who developed type 2 diabetes over 10 years ago. Since his retirement, his lifestyle has become very sedentary and he has put on a substantial amount of weight. Although he has felt tingling and numbness in his left foot for a while, he has not been worried because he thought his foot was simply “falling asleep.” Recently, a scratch on his foot does not seem to be healing and is becoming increasingly ugly. Because the sore did not bother him much, Charles figured it could not be serious until his daughter noticed a purplish discoloration spreading on the skin and oozing (**Figure 10.22**). When he was finally seen by his physician, Charles was rushed to the operating room. His open sore, or ulcer, is the result of a diabetic foot.

The concern here is that gas gangrene may have taken hold in the dead tissue. The most likely agent of gas gangrene is *Clostridium perfringens*, an endospore-forming, gram-positive bacterium. It is an obligate anaerobe that grows in tissue devoid of oxygen. Since dead tissue is no longer supplied with oxygen by the circulatory system, the dead tissue provides pockets of ideal environment for the growth of *C. perfringens*.

A surgeon examines the ulcer and radiographs of Charles’s foot and determines that the bone is not yet infected. The wound will have to be surgically debrided (debridement refers to the removal of dead and infected tissue) and a sample sent for microbiological lab analysis, but Charles will not have to have his foot amputated. Many diabetic patients are not so lucky. In 2008, nearly 70,000 diabetic patients in the United States lost a foot or limb to amputation, according to statistics from the Centers for Disease Control and Prevention.[1]

1. Centers for Disease Control and Prevention. “Living With Diabetes: Keep Your Feet Healthy.” http://www.cdc.gov/Features/DiabetesFootHealth/

- Which growth conditions would you recommend for the detection of *C. perfringens*?

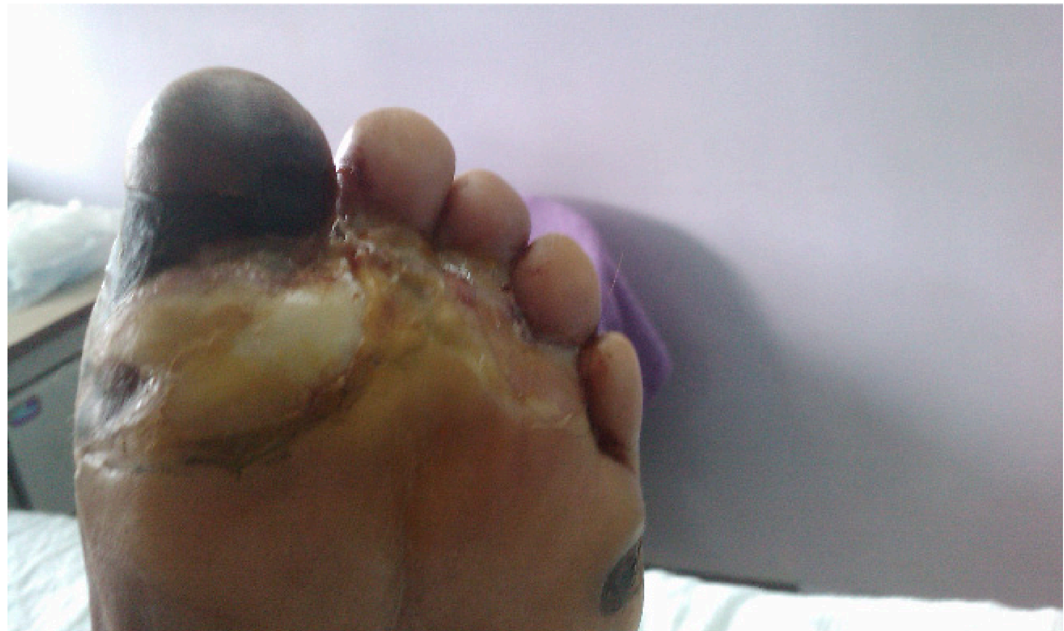


Figure 10.22 This clinical photo depicts ulcers on the foot of a diabetic patient. Dead tissue accumulating in ulcers can provide an ideal growth environment for the anaerobe *C. perfringens*, a causative agent of gas gangrene. (Credit: Phalinn Ooi / Wikimedia Commons (CC-BY))

Detoxification of Reactive Oxygen Species

Aerobic respiration constantly generates reactive oxygen species (ROS), byproducts that must be detoxified. Even organisms that do not use aerobic respiration need some way to break down some of the ROS that may form from atmospheric oxygen. Three main enzymes break down those toxic byproducts: superoxide dismutase, peroxidase, and catalase. Each one catalyzes a different reaction. Reactions of type seen in Reaction 1 are catalyzed by **peroxidase**s.

$$(1) \qquad X - (2H^+) + H_2O_2 \rightarrow \text{oxidized-}X + 2H_2O$$

In these reactions, an electron donor (reduced compound; e.g., reduced nicotinamide adenine dinucleotide [NADH]) oxidizes hydrogen peroxide, or other peroxides, to water. The enzymes play an important role by limiting the damage caused by peroxidation of membrane lipids. Reaction 2 is mediated by the enzyme **superoxide dismutase** (SOD) and breaks down the powerful superoxide anions generated by aerobic metabolism:

$$(2) \qquad 2{O_2}^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The enzyme **catalase** converts hydrogen peroxide to water and oxygen as shown in Reaction 3.

$$(3) \qquad 2H_2O_2 \rightarrow 2H_2O + O_2$$

Obligate anaerobes usually lack all three enzymes. Aerotolerant anaerobes do have SOD but no catalase. Reaction 3, shown occurring in Figure 10.23, is the basis of a useful and rapid test to distinguish streptococci, which are aerotolerant and do not possess catalase, from staphylococci, which are facultative anaerobes. A sample of culture rapidly mixed in a drop of 3% hydrogen peroxide will release bubbles if the culture is catalase positive.

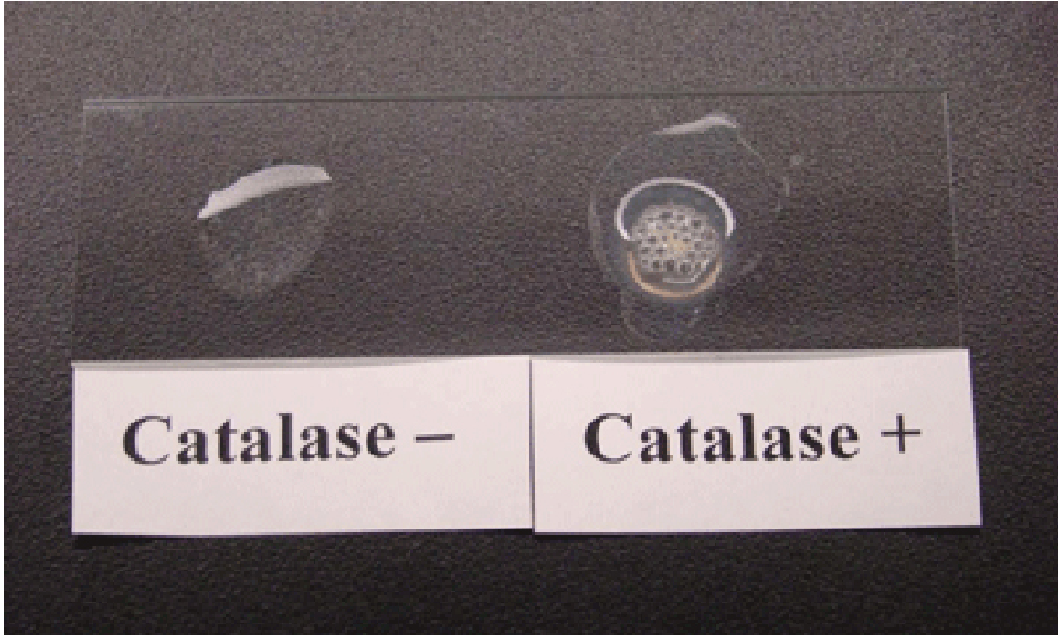


Figure 10.23 The catalase test detects the presence of the enzyme catalase by noting whether bubbles are released when hydrogen peroxide is added to a culture sample. Compare the positive result (right) with the negative result (left). (credit: Centers for Disease Control and Prevention)

Bacteria that grow best in a higher concentration of CO_2 and a lower concentration of oxygen than present in the atmosphere are called **capnophiles**. One common approach to grow capnophiles is to use a **candle jar**. A candle jar consists of a jar with a tight-fitting lid that can accommodate the cultures and a candle. After the cultures are added to the jar, the candle is lit and the lid closed. As the candle burns, it consumes most of the oxygen present and releases CO_2.

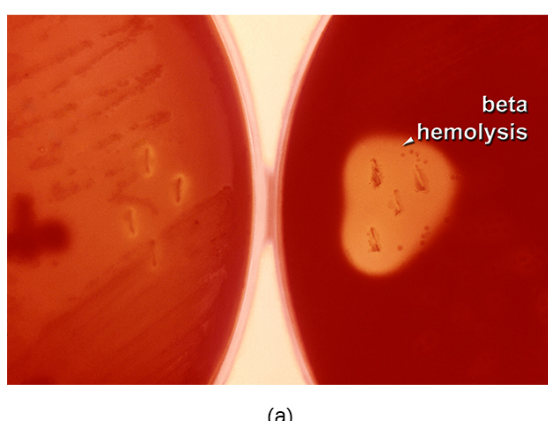
- What substance is added to a sample to detect catalase?
- What is the function of the candle in a candle jar?

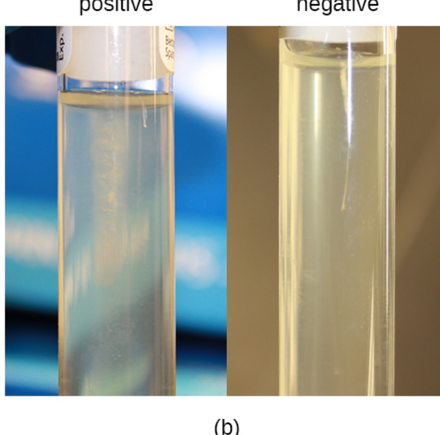
Part 2

The health-care provider who saw Jeni was concerned primarily because of her pregnancy. Her condition enhances the risk for infections and makes her more vulnerable to those infections. The immune system is downregulated during pregnancy, and pathogens that cross the placenta can be very dangerous for the fetus. A note on the provider's order to the microbiology lab mentions a suspicion of infection by *Listeria monocytogenes*, based on the signs and symptoms exhibited by the patient.

Jeni's blood samples are streaked directly on sheep blood agar, a medium containing tryptic soy agar enriched with 5% sheep blood. (Blood is considered sterile; therefore, competing microorganisms are not expected in the medium.) The inoculated plates are incubated at 37 °C for 24 to 48 hours. Small grayish colonies surrounded by a clear zone emerge. Such colonies are typical of *Listeria* and other pathogens such as streptococci; the clear zone surrounding the colonies indicates complete lysis of blood in the medium, referred to as beta-hemolysis (**Figure 10.24**). When tested for the presence of catalase, the colonies give a positive response, eliminating *Streptococcus* as a possible cause. Furthermore, a Gram stain shows short gram-positive bacilli. Cells from a broth culture grown at room temperature displayed the tumbling motility characteristic of *Listeria* (**Figure 10.24**). All of these clues lead the lab to positively confirm the presence of *Listeria* in Jeni's blood samples.

- How serious is Jeni's condition and what is the appropriate treatment?





(a) (b)

Figure 10.24 (a) A sample blood agar test showing beta-hemolysis. (b) A sample motility test showing both positive and negative results. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by “VeeDunn”/Flickr)

*Jump to the **next** Clinical Focus box. Go back to the **previous** Clinical Focus box.*

10.3 | The Effects of pH on Microbial Growth

Learning Objectives

By the end of this section, you will be able to:

- Illustrate and briefly describe minimum, optimum, and maximum pH requirements for growth
- Identify and describe the different categories of microbes with pH requirements for growth: acidophiles, neutrophiles, and alkaliphiles
- Give examples of microorganisms for each category of pH requirement

Yogurt, pickles, sauerkraut, and lime-seasoned dishes all owe their tangy taste to a high acid content (**Figure 10.25**). Recall that acidity is a function of the concentration of hydrogen ions [H^+] and is measured as pH. Environments with pH values below 7.0 are considered acidic, whereas those with pH values above 7.0 are considered basic. Extreme pH affects the structure of all macromolecules. The hydrogen bonds holding together strands of DNA break up at high pH. Lipids are hydrolyzed by an extremely basic pH. The proton motive force responsible for production of ATP in cellular respiration depends on the concentration gradient of H^+ across the plasma membrane (see **Cellular Respiration (https://legacy.cnx.org/content/m58821/latest/)**). If H^+ ions are neutralized by hydroxide ions, the concentration gradient collapses and impairs energy production. But the component most sensitive to pH in the cell is its workhorse, the protein. Moderate changes in pH modify the ionization of amino-acid functional groups and disrupt hydrogen bonding, which, in turn, promotes changes in the folding of the molecule, promoting denaturation and destroying activity.

Figure 10.25 Lactic acid bacteria that ferment milk into yogurt or transform vegetables in pickles thrive at a pH close to 4.0. Sauerkraut and dishes such as pico de gallo owe their tangy flavor to their acidity. Acidic foods have been a mainstay of the human diet for centuries, partly because most microbes that cause food spoilage grow best at a near neutral pH and do not tolerate acidity well. (credit "yogurt": modification of work by "nina.jsc"/Flickr; credit "pickles": modification of work by Noah Sussman; credit "sauerkraut": modification of work by Jesse LaBuff; credit "pico de gallo": modification of work by "regan76"/Flickr)

The **optimum growth pH** is the most favorable pH for the growth of an organism. The lowest pH value that an organism can tolerate is called the **minimum growth pH** and the highest pH is the **maximum growth pH**. These values can cover a wide range, which is important for the preservation of food and to microorganisms' survival in the stomach. For example, the optimum growth pH of *Salmonella* spp. is 7.0–7.5, but the minimum growth pH is closer to 4.2.

Most bacteria are **neutrophile**s, meaning they grow optimally at a pH within one or two pH units of the neutral pH of 7 (see **Figure 10.26**). Most familiar bacteria, like *Escherichia coli*, staphylococci, and *Salmonella* spp. are neutrophiles and do not fare well in the acidic pH of the stomach. However, there are pathogenic strains of *E. coli, S. typhi,* and other species of intestinal pathogens that are much more resistant to stomach acid. In comparison, fungi thrive at slightly acidic pH values of 5.0–6.0.

Microorganisms that grow optimally at pH less than 5.55 are called **acidophile**s. For example, the sulfur-oxidizing *Sulfolobus* spp. isolated from sulfur mud fields and hot springs in Yellowstone National Park are extreme acidophiles. These archaea survive at pH values of 2.5–3.5. Species of the archaean genus *Ferroplasma* live in acid mine drainage at pH values of 0–2.9. *Lactobacillus* bacteria, which are an important part of the normal microbiota of the vagina, can tolerate acidic environments at pH values 3.5–6.8 and also contribute to the acidity of the vagina (pH of 4, except at the onset of menstruation) through their metabolic production of lactic acid. The vagina's acidity plays an important role in inhibiting other microbes that are less tolerant of acidity. Acidophilic microorganisms display a number of adaptations to survive in strong acidic environments. For example, proteins show increased negative surface charge that stabilizes them at low pH. Pumps actively eject H^+ ions out of the cells. The changes in the composition of membrane phospholipids probably reflect the need to maintain membrane fluidity at low pH.

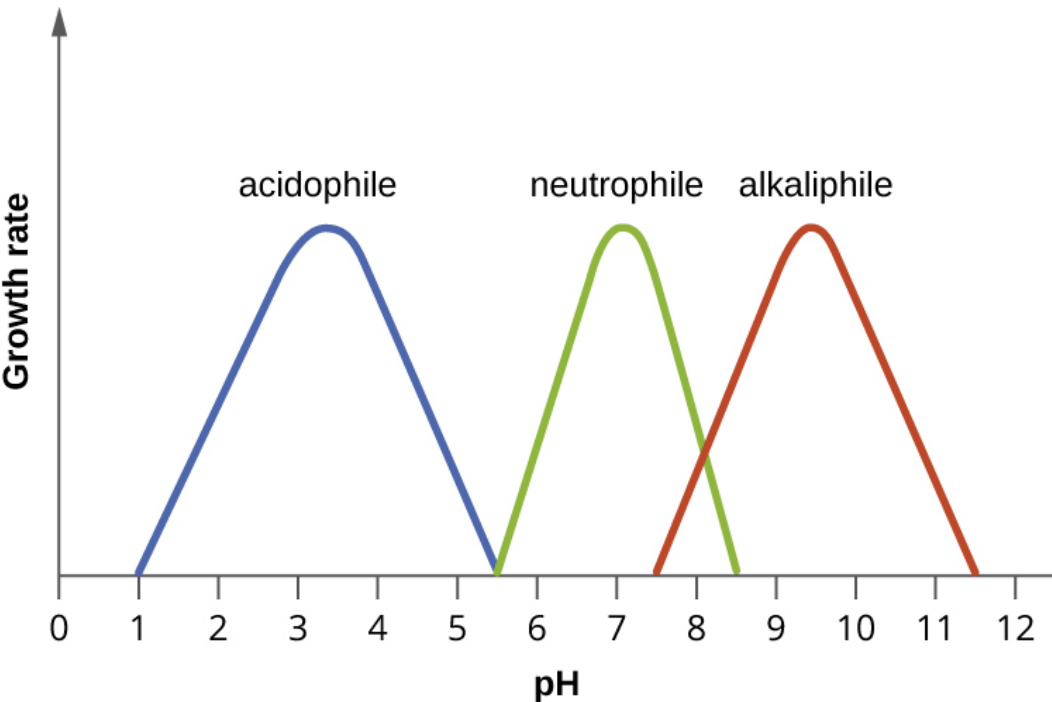


Figure 10.26 The curves show the approximate pH ranges for the growth of the different classes of pH-specific prokaryotes. Each curve has an optimal pH and extreme pH values at which growth is much reduced. Most bacteria are neutrophiles and grow best at near-neutral pH (center curve). Acidophiles have optimal growth at pH values near 3 and alkaliphiles have optimal growth at pH values above 9.

At the other end of the spectrum are **alkaliphile**s, microorganisms that grow best at pH between 8.0 and 10.5. *Vibrio cholerae*, the pathogenic agent of cholera, grows best at the slightly basic pH of 8.0; it can survive pH values of 11.0 but is inactivated by the acid of the stomach. When it comes to survival at high pH, the bright pink archaean *Natronobacterium*, found in the soda lakes of the African Rift Valley, may hold the record at a pH of 10.5 (**Figure 10.27**). Extreme alkaliphiles have adapted to their harsh environment through evolutionary modification of lipid and protein structure and compensatory mechanisms to maintain the proton motive force in an alkaline environment. For example, the alkaliphile *Bacillus firmus* derives the energy for transport reactions and motility from a Na^+ ion gradient rather than a proton motive force. Many enzymes from alkaliphiles have a higher isoelectric point, due to an increase in the number of basic amino acids, than homologous enzymes from neutrophiles.

Figure 10.27 View from space of Lake Natron in Tanzania. The pink color is due to the pigmentation of the extreme alkaliphilic and halophilic microbes that colonize the lake. (credit: NASA)

Survival at the Low pH of the Stomach

Peptic ulcers (or stomach ulcers) are painful sores on the stomach lining. Until the 1980s, they were believed to be caused by spicy foods, stress, or a combination of both. Patients were typically advised to eat bland foods, take anti-acid medications, and avoid stress. These remedies were not particularly effective, and the condition often recurred. This all changed dramatically when the real cause of most peptic ulcers was discovered to be a slim, corkscrew-shaped bacterium, *Helicobacter pylori*. This organism was identified and isolated by Barry Marshall and Robin Warren, whose discovery earned them the Nobel Prize in Medicine in 2005.

The ability of *H. pylori* to survive the low pH of the stomach would seem to suggest that it is an extreme acidophile. As it turns out, this is not the case. In fact, *H. pylori* is a neutrophile. So, how does it survive in the stomach? Remarkably, *H. pylori* creates a microenvironment in which the pH is nearly neutral. It achieves this by producing large amounts of the enzyme urease, which breaks down urea to form NH_4^+ and CO_2. The ammonium ion raises the pH of the immediate environment.

This metabolic capability of *H. pylori* is the basis of an accurate, noninvasive test for infection. The patient is given a solution of urea containing radioactively labeled carbon atoms. If *H. pylori* is present in the stomach, it will rapidly break down the urea, producing radioactive CO_2 that can be detected in the patient's breath. Because peptic ulcers may lead to gastric cancer, patients who are determined to have *H. pylori* infections are treated with antibiotics.

- What effect do extremes of pH have on proteins?
- What pH-adaptive type of bacteria would most human pathogens be?

10.4 | Temperature and Microbial Growth

Learning Objectives

By the end of this section, you will be able to:

- Illustrate and briefly describe minimum, optimum, and maximum temperature requirements for growth
- Identify and describe different categories of microbes with temperature requirements for growth: psychrophile, psychrotrophs, mesophile, thermophile, hyperthermophile
- Give examples of microorganisms in each category of temperature tolerance

When the exploration of Lake Whillans started in Antarctica, researchers did not expect to find much life. Constant subzero temperatures and lack of obvious sources of nutrients did not seem to be conditions that would support a thriving ecosystem. To their surprise, the samples retrieved from the lake showed abundant microbial life. In a different but equally harsh setting, bacteria grow at the bottom of the ocean in sea vents (**Figure 10.28**), where temperatures can reach 340 °C (700 °F).

Microbes can be roughly classified according to the range of temperature at which they can grow. The growth rates are the highest at the **optimum growth temperature** for the organism. The lowest temperature at which the organism can survive and replicate is its **minimum growth temperature**. The highest temperature at which growth can occur is its **maximum growth temperature**. The following ranges of permissive growth temperatures are approximate only and can vary according to other environmental factors.

Organisms categorized as **mesophile**s ("middle loving") are adapted to moderate temperatures, with optimal growth temperatures ranging from room temperature (about 20 °C) to about 45 °C. As would be expected from the core temperature of the human body, 37 °C (98.6 °F), normal human microbiota and pathogens (e.g., *E. coli*, *Salmonella* spp., and *Lactobacillus* spp.) are mesophiles.

Organisms called **psychrotrophs**, also known as psychrotolerant, prefer cooler environments, from a high temperature of 25 °C to refrigeration temperature about 4 °C. They are found in many natural environments in temperate climates. They are also responsible for the spoilage of refrigerated food.

Resolution

The presence of *Listeria* in Jeni's blood suggests that her symptoms are due to listeriosis, an infection caused by *L. monocytogenes*. Listeriosis is a serious infection with a 20% mortality rate and is a particular risk to Jeni's fetus. A sample from the amniotic fluid cultured for the presence of *Listeria* gave negative results. Because the absence of organisms does not rule out the possibility of infection, a molecular test based on the nucleic acid amplification of the 16S ribosomal RNA of *Listeria* was performed to confirm that no bacteria crossed the placenta. Fortunately, the results from the molecular test were also negative.

Jeni was admitted to the hospital for treatment and recovery. She received a high dose of two antibiotics intravenously for 2 weeks. The preferred drugs for the treatment of listeriosis are ampicillin or penicillin G with an aminoglycoside antibiotic. Resistance to common antibiotics is still rare in *Listeria* and antibiotic treatment is usually successful. She was released to home care after a week and fully recovered from her infection.

L. monocytogenes is a gram-positive short rod found in soil, water, and food. It is classified as a psychrophile and is halotolerant. Its ability to multiply at refrigeration temperatures (4–10 °C) and its tolerance for high concentrations of salt (up to 10% sodium chloride [NaCl]) make it a frequent source of food poisoning. Because *Listeria* can infect animals, it often contaminates food such as meat, fish, or dairy products. Contamination of commercial foods can often be traced to persistent biofilms that form on manufacturing equipment that is not sufficiently cleaned.

Listeria infection is relatively common among pregnant women because the elevated levels of progesterone downregulate the immune system, making them more vulnerable to infection. The pathogen can cross the placenta and infect the fetus, often resulting in miscarriage, stillbirth, or fatal neonatal infection. Pregnant women are thus advised to avoid consumption of soft cheeses, refrigerated cold cuts, smoked seafood, and unpasteurized dairy products. Because *Listeria* bacteria can easily be confused with diphtheroids, another common group of gram-positive rods, it is important to alert the laboratory when listeriosis is suspected.

*Go back to the **previous** Clinical Focus box.*

The organisms retrieved from arctic lakes such as Lake Whillans are considered extreme **psychrophiles** (cold loving). Psychrophiles are microorganisms that can grow at 0 °C and below, have an optimum growth

temperature close to
15 °C, and usually do not survive at temperatures above 20 °C. They are found in permanently cold environments such as the deep waters of the oceans. Because they are active at low temperature, psychrophiles and psychrotrophs are important decomposers in cold climates.

Organisms that grow at optimum temperatures of 50 °C to a maximum of 80 °C are called **thermophiles** ("heat loving"). They do not multiply at room temperature. Thermophiles are widely distributed in hot springs, geothermal soils, and manmade environments such as garden compost piles where the microbes break down kitchen scraps and vegetal material. Examples of thermophiles include *Thermus aquaticus* and *Geobacillus* spp. Higher up on the extreme temperature scale we find the **hyperthermophiles**, which are characterized by growth ranges from 80 °C to a maximum of 110 °C, with some extreme examples that survive temperatures above 121 °C, the average temperature of an autoclave. The hydrothermal vents at the bottom of the ocean are a prime example of extreme environments, with temperatures reaching an estimated 340 °C (Figure 10.28). Microbes isolated from the vents achieve optimal growth at temperatures higher than 100 °C. Noteworthy examples are *Pyrobolus* and *Pyrodictium*, archaea that grow at 105 °C and survive autoclaving. Figure 10.29 shows the typical skewed curves of temperature-dependent growth for the categories of microorganisms we have discussed.

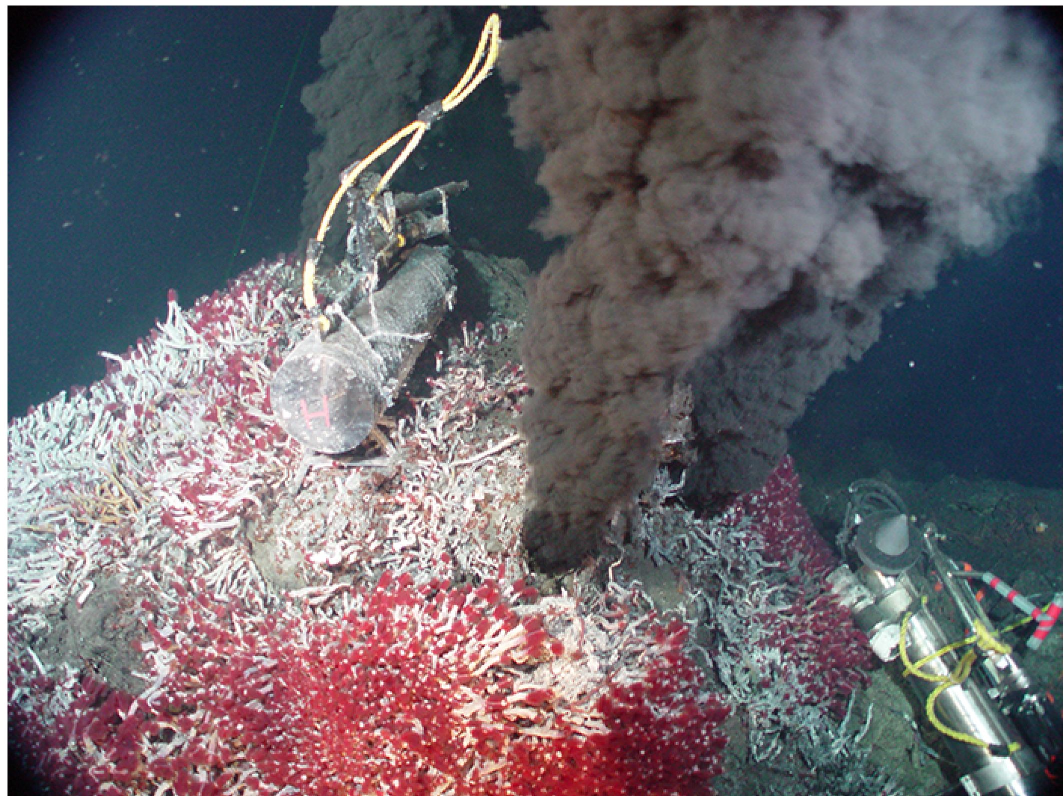


Figure 10.28 A black smoker at the bottom of the ocean belches hot, chemical-rich water, and heats the surrounding waters. Sea vents provide an extreme environment that is nonetheless teeming with macroscopic life (the red tubeworms) supported by an abundant microbial ecosystem. (credit: NOAA)

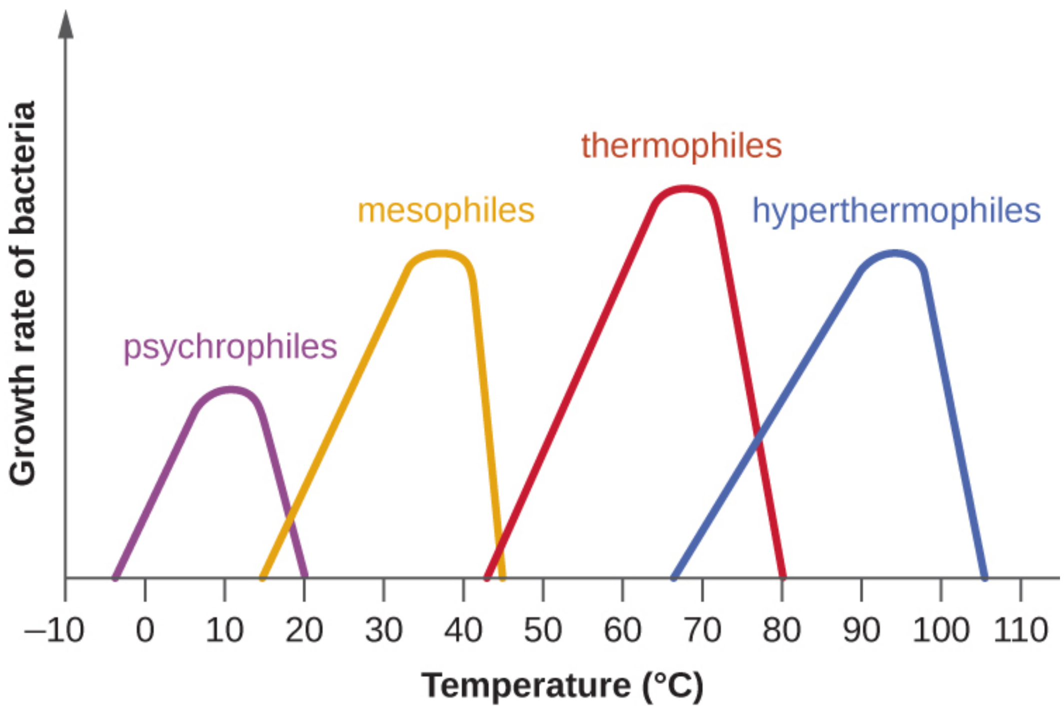


Figure 10.29 The graph shows growth rate of bacteria as a function of temperature. Notice that the curves are skewed toward the optimum temperature. The skewing of the growth curve is thought to reflect the rapid denaturation of proteins as the temperature rises past the optimum for growth of the microorganism.

Life in extreme environments raises fascinating questions about the adaptation of macromolecules and metabolic processes. Very low temperatures affect cells in many ways. Membranes lose their fluidity and are damaged by ice crystal formation. Chemical reactions and diffusion slow considerably. Proteins become too rigid to catalyze reactions and may undergo denaturation. At the opposite end of the temperature spectrum, heat denatures proteins and nucleic acids. Increased fluidity impairs metabolic processes in membranes. Some of the practical applications of the destructive effects of heat on microbes are sterilization by steam, pasteurization, and incineration of inoculating loops. Proteins in psychrophiles are, in general, rich in hydrophobic residues, display an increase in flexibility, and have a lower number of secondary stabilizing bonds when compared with homologous proteins from mesophiles. Antifreeze proteins and solutes that decrease the freezing temperature of the cytoplasm are common. The lipids in the membranes tend to be unsaturated to increase fluidity. Growth rates are much slower than those encountered at moderate temperatures. Under appropriate conditions, mesophiles and even thermophiles can survive freezing. Liquid cultures of bacteria are mixed with sterile glycerol solutions and frozen to −80 °C for long-term storage as stocks. Cultures can withstand freeze drying (lyophilization) and then be stored as powders in sealed ampules to be reconstituted with broth when needed.

Macromolecules in thermophiles and hyperthermophiles show some notable structural differences from what is observed in the mesophiles. The ratio of saturated to polyunsaturated lipids increases to limit the fluidity of the cell membranes. Their DNA sequences show a higher proportion of guanine–cytosine nitrogenous bases, which are held together by three hydrogen bonds in contrast to adenine and thymine, which are connected in the double helix by two hydrogen bonds. Additional secondary structures, ionic and covalent bonds, as well as the replacement of key amino acids to stabilize folding, contribute to the resistance of proteins to denaturation. The so-called thermoenzymes purified from thermophiles have important practical applications. For example, amplification of nucleic acids in the polymerase chain reaction (PCR) depends on the thermal stability of *Taq* polymerase, an enzyme isolated from *T. aquaticus*. Degradation enzymes from thermophiles are added as ingredients in hot-water detergents, increasing their effectiveness.

- What temperature requirements do most bacterial human pathogens have?
- What DNA adaptation do thermophiles exhibit?

Feeding the World…and the World's Algae

Artificial fertilizers have become an important tool in food production around the world. They are responsible for many of the gains of the so-called green revolution of the 20th century, which has allowed the planet to feed many of its more than 7 billion people. Artificial fertilizers provide nitrogen and phosphorus, key limiting nutrients, to crop plants, removing the normal barriers that would otherwise limit the rate of growth. Thus, fertilized crops grow much faster, and farms that use fertilizer produce higher crop yields.

However, careless use and overuse of artificial fertilizers have been demonstrated to have significant negative impacts on aquatic ecosystems, both freshwater and marine. Fertilizers that are applied at inappropriate times or in too-large quantities allow nitrogen and phosphorus compounds to escape use by crop plants and enter drainage systems. Inappropriate use of fertilizers in residential settings can also contribute to nutrient loads, which find their way to lakes and coastal marine ecosystems. As water warms and nutrients are plentiful, microscopic algae bloom, often changing the color of the water because of the high cell density.

Most algal blooms are not directly harmful to humans or wildlife; however, they can cause harm indirectly. As the algal population expands and then dies, it provides a large increase in organic matter to the bacteria that live in deep water. With this large supply of nutrients, the population of nonphotosynthetic microorganisms explodes, consuming available oxygen and creating " dead zones" where animal life has virtually disappeared.

Depletion of oxygen in the water is not the only damaging consequence of some algal blooms. The algae that produce red tides in the Gulf of Mexico, *Karenia brevis*, secrete potent toxins that can kill fish and other organisms and also accumulate in shellfish. Consumption of contaminated shellfish can cause severe neurological and gastrointestinal symptoms in humans. Shellfish beds must be regularly monitored for the presence of the toxins, and harvests are often shut down when it is present, incurring economic costs to the fishery. Cyanobacteria, which can form blooms in marine and freshwater ecosystems, produce toxins called microcystins, which can cause allergic reactions and liver damage when ingested in drinking water or during swimming. Recurring cyanobacterial algal blooms in Lake Erie (**Figure 10.30**) have forced municipalities to issue drinking water bans for days at a time because of unacceptable toxin levels.

This is just a small sampling of the negative consequences of algal blooms, red tides, and dead zones. Yet the benefits of crop fertilizer—the main cause of such blooms—are difficult to dispute. There is no easy solution to this dilemma, as a ban on fertilizers is not politically or economically feasible. In lieu of this, we must advocate for responsible use and regulation in agricultural and residential contexts, as well as the restoration of wetlands, which can absorb excess fertilizers before they reach lakes and oceans.

Figure 10.30 Heavy rains cause runoff of fertilizers into Lake Erie, triggering extensive algal blooms, which can be observed along the shoreline. Notice the brown unplanted and green planted agricultural land on the shore. (credit: NASA)

This **video (https://openstax.org/l/22algaebloomvid)** discusses algal blooms and dead zones in more depth.

10.5 | Other Environmental Conditions that Affect Growth

Learning Objectives

By the end of this section, you will be able to:

- Identify and describe different categories of microbes with specific growth requirements other than oxygen, pH, and temperature, such as altered barometric pressure, osmotic pressure, humidity, and light
- Give at least one example microorganism for each category of growth requirement

Microorganisms interact with their environment along more dimensions than pH, temperature, and free oxygen levels, although these factors require significant adaptations. We also find microorganisms adapted to varying levels of salinity, barometric pressure, humidity, and light.

Osmotic and Barometric Pressure

Most natural environments tend to have lower solute concentrations than the cytoplasm of most microorganisms. Rigid cell walls protect the cells from bursting in a dilute environment. Not much protection is available against high osmotic pressure. In this case, water, following its concentration gradient, flows out of the cell. This results in plasmolysis (the shrinking of the protoplasm away from the intact cell wall) and cell death. This fact explains why brines and layering meat and fish in salt are time-honored methods of preserving food. Microorganisms called **halophiles** ("salt loving") actually require high salt concentrations for growth. These organisms are found

in marine environments where salt concentrations hover at 3.5%. Extreme halophilic microorganisms, such as the red alga *Dunaliella salina* and the archaeal species *Halobacterium* in **Figure 10.31**, grow in hypersaline lakes such as the Great Salt Lake, which is 3.5–8 times saltier than the ocean, and the Dead Sea, which is 10 times saltier than the ocean.

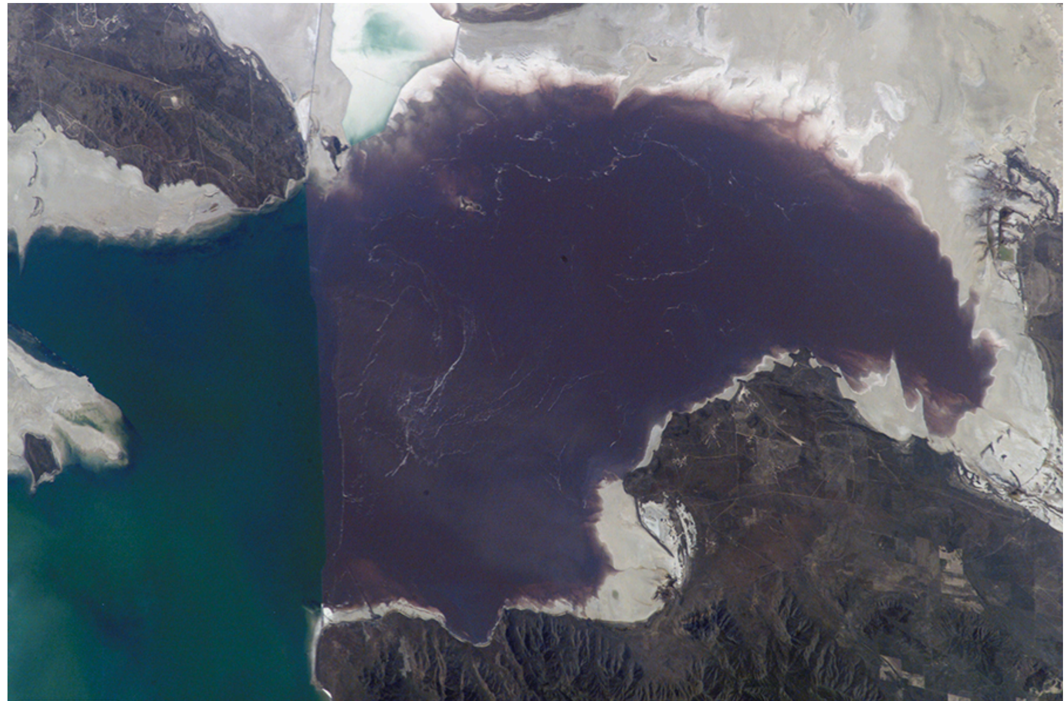


Figure 10.31 Photograph taken from space of the Great Salt Lake in Utah. The purple color is caused by high density of the alga *Dunaliella* and the archaean *Halobacterium* spp. (credit: NASA)

Dunaliella spp. counters the tremendous osmotic pressure of the environment with a high cytoplasmic concentration of glycerol and by actively pumping out salt ions. *Halobacterium* spp. accumulates large concentrations of K^+ and other ions in its cytoplasm. Its proteins are designed for high salt concentrations and lose activity at salt concentrations below 1–2 M. Although most **halotolerant** organisms, for example *Halomonas* spp. in salt marshes, do not need high concentrations of salt for growth, they will survive and divide in the presence of high salt. Not surprisingly, the staphylococci, micrococci, and corynebacteria that colonize our skin tolerate salt in their environment. Halotolerant pathogens are an important cause of food-borne illnesses because they survive and multiply in salty food. For example, the halotolerant bacteria *S. aureus, Bacillus cereus*, and *V. cholerae* produce dangerous enterotoxins and are major causes of food poisoning.

Microorganisms depend on available water to grow. Available moisture is measured as water activity (a_W), which is the ratio of the vapor pressure of the medium of interest to the vapor pressure of pure distilled water; therefore, the a_W of water is equal to 1.0. Bacteria require high a_W (0.97–0.99), whereas fungi can tolerate drier environments; for example, the range of a_W for growth of *Aspergillus* spp. is 0.8–0.75. Decreasing the water content of foods by drying, as in jerky, or through freeze-drying or by increasing osmotic pressure, as in brine and jams, are common methods of preventing spoilage.

Microorganisms that require high atmospheric pressure for growth are called **barophiles**. The bacteria that live at the bottom of the ocean must be able to withstand great pressures. Because it is difficult to retrieve intact specimens and reproduce such growth conditions in the laboratory, the characteristics of these microorganisms are largely unknown.

Light

Photoautotrophs, such as cyanobacteria or green sulfur bacteria, and photoheterotrophs, such as purple nonsulfur bacteria, depend on sufficient light intensity at the wavelengths absorbed by their pigments to grow and multiply. Energy from light is captured by pigments and converted into chemical energy that drives carbon fixation and other metabolic processes. The portion of the electromagnetic spectrum that is absorbed by these organisms is defined as photosynthetically active radiation (PAR). It lies within the visible light spectrum ranging

from 400 to 700 nanometers (nm) and extends in the near infrared for some photosynthetic bacteria. A number of accessory pigments, such as fucoxanthin in brown algae and phycobilins in cyanobacteria, widen the useful range of wavelengths for photosynthesis and compensate for the low light levels available at greater depths of water. Other microorganisms, such as the archaea of the class Halobacteria, use light energy to drive their proton and sodium pumps. The light is absorbed by a pigment protein complex called bacteriorhodopsin, which is similar to the eye pigment rhodopsin. Photosynthetic bacteria are present not only in aquatic environments but also in soil and in symbiosis with fungi in lichens. The peculiar watermelon snow is caused by a microalga *Chlamydomonas nivalis*, a green alga rich in a secondary red carotenoid pigment (astaxanthin) which gives the pink hue to the snow where the alga grows.

- Which photosynthetic pigments were described in this section?
- What is the fundamental stress of a hypersaline environment for a cell?

10.6 | Media Used for Bacterial Growth

Learning Objectives

By the end of this section, you will be able to:

- Identify and describe culture media for the growth of bacteria, including examples of all-purpose media, enriched, selective, differential, defined, and enrichment media

The study of microorganisms is greatly facilitated if we are able to culture them, that is, to keep reproducing populations alive under laboratory conditions. Culturing many microorganisms is challenging because of highly specific nutritional and environmental requirements and the diversity of these requirements among different species.

Nutritional Requirements

The number of available media to grow bacteria is considerable. Some media are considered general all-purpose media and support growth of a large variety of organisms. A prime example of an all-purpose medium is tryptic soy broth (TSB). Specialized media are used in the identification of bacteria and are supplemented with dyes, pH indicators, or antibiotics. One type, **enriched media**, contains growth factors, vitamins, and other essential nutrients to promote the growth of **fastidious organisms**, organisms that cannot make certain nutrients and require them to be added to the medium. When the complete chemical composition of a medium is known, it is called a **chemically defined medium**. For example, in EZ medium, all individual chemical components are identified and the exact amounts of each is known. In **complex media**, which contain extracts and digests of yeasts, meat, or plants, the precise chemical composition of the medium is not known. Amounts of individual components are undetermined and variable. Nutrient broth, tryptic soy broth, and brain heart infusion, are all examples of complex media.

Media that inhibit the growth of unwanted microorganisms and support the growth of the organism of interest by supplying nutrients and reducing competition are called **selective media**. An example of a selective medium is MacConkey agar. It contains bile salts and crystal violet, which interfere with the growth of many gram-positive bacteria and favor the growth of gram-negative bacteria, particularly the Enterobacteriaceae. These species are commonly named enterics, reside in the intestine, and are adapted to the presence of bile salts. The **enrichment culture**s foster the preferential growth of a desired microorganism that represents a fraction of the organisms present in an inoculum. For example, if we want to isolate bacteria that break down crude oil, hydrocarbonoclastic bacteria, sequential subculturing in a medium that supplies carbon only in the form of crude oil will enrich the cultures with oil-eating bacteria. The **differential media** make it easy to distinguish colonies of different bacteria by a change in the color of the colonies or the color of the medium. Color changes are the result of end products created by interaction of bacterial enzymes with differential substrates in the medium or, in the case of hemolytic reactions, the lysis of red blood cells in the medium. In Figure 10.32, the differential fermentation of lactose can be observed on MacConkey agar. The lactose fermenters produce acid, which turns the medium and the colonies of strong fermenters hot pink. The medium is supplemented with the pH indicator neutral red, which turns to hot pink at low pH. Selective and differential media can be combined and play an important role in the identification of bacteria by biochemical methods.

Figure 10.32 On this MacConkey agar plate, the lactose-fermenter *E. coli* colonies are bright pink. *Serratia marcescens*, which does not ferment lactose, forms a cream-colored streak on the tan medium. (credit: American Society for Microbiology)

- Distinguish complex and chemically defined media.
- Distinguish selective and enrichment media.

Compare the compositions of **EZ medium (https://openstax.org/l/22EZMedium)** and **sheep blood (https://openstax.org/l/22bloodagar)** agar.

The End-of-Year Picnic

The microbiology department is celebrating the end of the school year in May by holding its traditional picnic on the green. The speeches drag on for a couple of hours, but finally all the faculty and students can dig into the food: chicken salad, tomatoes, onions, salad, and custard pie. By evening, the whole department, except for two vegetarian students who did not eat the chicken salad, is stricken with nausea, vomiting, retching, and abdominal cramping. Several individuals complain of diarrhea. One patient shows signs of shock (low blood pressure). Blood and stool samples are collected from patients, and an analysis of all foods served at the meal is conducted.

Bacteria can cause gastroenteritis (inflammation of the stomach and intestinal tract) either by colonizing and replicating in the host, which is considered an infection, or by secreting toxins, which is considered intoxication. Signs and symptoms of infections are typically delayed, whereas intoxication manifests within hours, as happened after the picnic.

Blood samples from the patients showed no signs of bacterial infection, which further suggests that this was

a case of intoxication. Since intoxication is due to secreted toxins, bacteria are not usually detected in blood or stool samples. MacConkey agar and sorbitol-MacConkey agar plates and xylose-lysine-deoxycholate (XLD) plates were inoculated with stool samples and did not reveal any unusually colored colonies, and no black colonies or white colonies were observed on XLD. All lactose fermenters on MacConkey agar also ferment sorbitol. These results ruled out common agents of food-borne illnesses: *E. coli*, *Salmonella* spp., and *Shigella* spp.

Analysis of the chicken salad revealed an abnormal number of gram-positive cocci arranged in clusters (**Figure 10.33**). A culture of the gram-positive cocci releases bubbles when mixed with hydrogen peroxide. The culture turned mannitol salt agar yellow after a 24-hour incubation.

All the tests point to *Staphylococcus aureus* as the organism that secreted the toxin. Samples from the salad showed the presence of gram-positive cocci bacteria in clusters. The colonies were positive for catalase. The bacteria grew on mannitol salt agar fermenting mannitol, as shown by the change to yellow of the medium. The pH indicator in mannitol salt agar is phenol red, which turns to yellow when the medium is acidified by the products of fermentation.

The toxin secreted by *S. aureus* is known to cause severe gastroenteritis. The organism was probably introduced into the salad during preparation by the food handler and multiplied while the salad was kept in the warm ambient temperature during the speeches.

- What are some other factors that might have contributed to rapid growth of *S. aureus* in the chicken salad?
- Why would *S. aureus* not be inhibited by the presence of salt in the chicken salad?

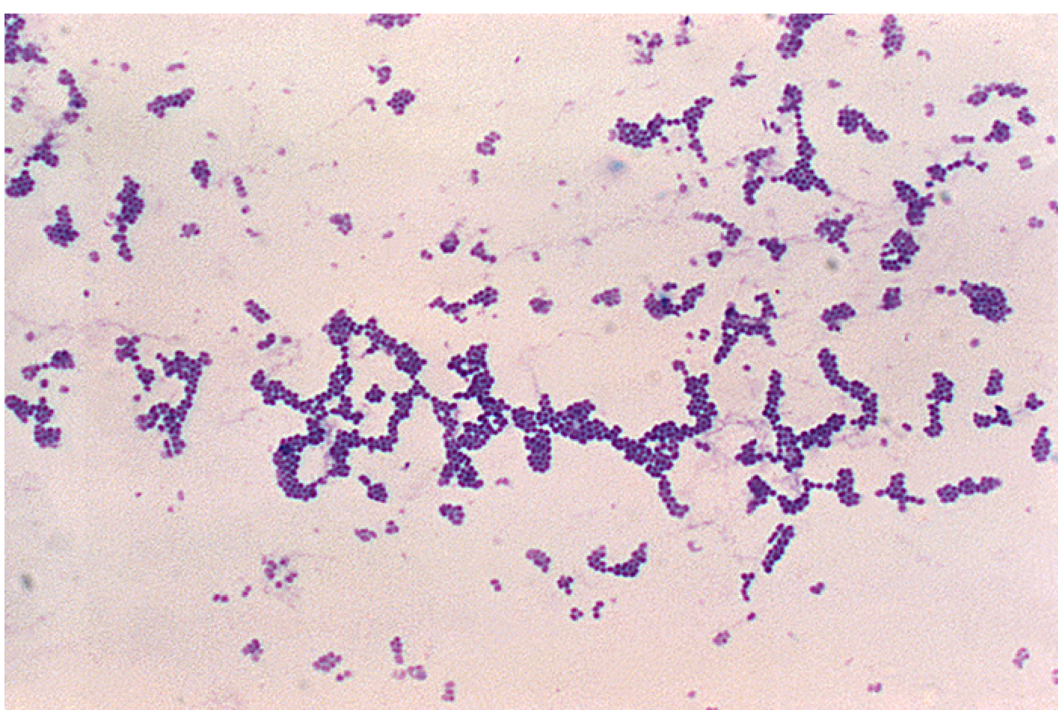


Figure 10.33 Gram-positive cocci in clusters. (credit: Centers for Disease Control and Prevention)

CHAPTER SUMMARY

10.1 How Microbes Grow

- Most bacterial cells divide by **binary fission**. **Generation time** in bacterial growth is defined as the **doubling time** of the population.
- Cells in a closed system follow a pattern of growth with four phases: **lag**, **logarithmic (exponential)**, **stationary**, and **death**.

- Cells can be counted by **direct viable cell count**. The **pour plate** and **spread plate** methods are used to plate **serial dilutions** into or onto, respectively, agar to allow counting of viable cells that give rise to **colony-forming units**. **Membrane filtration** is used to count live cells in dilute solutions. The **most probable cell number (MPN)** method allows estimation of cell numbers in cultures without using solid media.
- Indirect methods can be used to estimate **culture density** by measuring **turbidity** of a culture or live cell density by measuring metabolic activity.
- Other patterns of cell division include multiple nucleoid formation in cells; asymmetric division, as in **budding**; and the formation of hyphae and terminal spores.
- **Biofilms** are communities of microorganisms enmeshed in a matrix of **extracellular polymeric substance**. The formation of a biofilm occurs when **planktonic** cells attach to a substrate and become **sessile**. Cells in biofilms coordinate their activity by communicating through **quorum sensing**.
- Biofilms are commonly found on surfaces in nature and in the human body, where they may be beneficial or cause severe infections. Pathogens associated with biofilms are often more resistant to antibiotics and disinfectants.

10.2 Oxygen Requirements for Microbial Growth

- Aerobic and anaerobic environments can be found in diverse niches throughout nature, including different sites within and on the human body.
- Microorganisms vary in their requirements for molecular oxygen. **Obligate aerobes** depend on aerobic respiration and use oxygen as a terminal electron acceptor. They cannot grow without oxygen.
- **Obligate anaerobes** cannot grow in the presence of oxygen. They depend on fermentation and anaerobic respiration using a final electron acceptor other than oxygen.
- **Facultative anaerobes** show better growth in the presence of oxygen but will also grow without it.
- Although **aerotolerant anaerobes** do not perform aerobic respiration, they can grow in the presence of oxygen. Most aerotolerant anaerobes test negative for the enzyme **catalase**.
- **Microaerophiles** need oxygen to grow, albeit at a lower concentration than 21% oxygen in air.
- **Optimum oxygen concentration** for an organism is the oxygen level that promotes the fastest growth rate. The **minimum permissive oxygen concentration** and the **maximum permissive oxygen concentration** are, respectively, the lowest and the highest oxygen levels that the organism will tolerate.
- **Peroxidase**, **superoxide dismutase**, and **catalase** are the main enzymes involved in the detoxification of the **reactive oxygen species**. Superoxide dismutase is usually present in a cell that can tolerate oxygen. All three enzymes are usually detectable in cells that perform aerobic respiration and produce more ROS.
- A **capnophile** is an organism that requires a higher than atmospheric concentration of CO_2 to grow.

10.3 The Effects of pH on Microbial Growth

- Bacteria are generally **neutrophiles**. They grow best at neutral pH close to 7.0.
- **Acidophiles** grow optimally at a pH near 3.0. **Alkaliphiles** are organisms that grow optimally between a pH of 8 and 10.5. Extreme acidophiles and alkaliphiles grow slowly or not at all near neutral pH.
- Microorganisms grow best at their **optimum growth pH**. Growth occurs slowly or not at all below the **minimum growth pH** and above the **maximum growth pH**.

10.4 Temperature and Microbial Growth

- Microorganisms thrive at a wide range of temperatures; they have colonized different natural environments and have adapted to extreme temperatures. Both extreme cold and hot temperatures require evolutionary

adjustments to macromolecules and biological processes.

- **Psychrophiles** grow best in the temperature range of 0–15 °C whereas **psychrotrophs** thrive between 4°C and 25 °C.
- **Mesophiles** grow best at moderate temperatures in the range of 20 °C to about 45 °C. Pathogens are usually mesophiles.
- **Thermophiles** and **hyperthemophiles** are adapted to life at temperatures above 50 °C.
- Adaptations to cold and hot temperatures require changes in the composition of membrane lipids and proteins.

10.5 Other Environmental Conditions that Affect Growth

- **Halophiles** require high salt concentration in the medium, whereas **halotolerant** organisms can grow and multiply in the presence of high salt but do not require it for growth.
- Halotolerant pathogens are an important source of foodborne illnesses because they contaminate foods preserved in salt.
- Photosynthetic bacteria depend on visible light for energy.
- Most bacteria, with few exceptions, require high moisture to grow.

10.6 Media Used for Bacterial Growth

- **Chemically defined media** contain only chemically known components.
- **Selective media** favor the growth of some microorganisms while inhibiting others.
- **Enriched media** contain added essential nutrients a specific organism needs to grow
- **Differential media** help distinguish bacteria by the color of the colonies or the change in the medium.

REVIEW QUESTIONS

1. Which of the following methods would be used to measure the concentration of bacterial contamination in processed peanut butter?

A. turbidity measurement
B. total plate count
C. dry weight measurement
D. direct counting of bacteria on a calibrated slide under the microscope

2. In which phase would you expect to observe the most endospores in a *Bacillus* cell culture?

A. death phase
B. lag phase
C. log phase
D. log, lag, and death phases would all have roughly the same number of endospores.

3. During which phase would penicillin, an antibiotic that inhibits cell-wall synthesis, be most effective?

A. death phase
B. lag phase
C. log phase
D. stationary phase

4. Which of the following is the best definition of generation time in a bacterium?

A. the length of time it takes to reach the log phase
B. the length of time it takes for a population of cells to double
C. the time it takes to reach stationary phase
D. the length of time of the exponential phase

5. What is the function of the Z ring in binary fission?

A. It controls the replication of DNA.
B. It forms a contractile ring at the septum.
C. It separates the newly synthesized DNA molecules.
D. It mediates the addition of new peptidoglycan subunits.

6. If a culture starts with 50 cells, how many cells will be present after five generations with no cell death?

A. 200
B. 400
C. 1600
D. 3200

7. Filamentous cyanobacteria often divide by which

of the following?

A. budding
B. mitosis
C. fragmentation
D. formation of endospores

8. Which is a reason for antimicrobial resistance being higher in a biofilm than in free-floating bacterial cells?

A. The EPS allows faster diffusion of chemicals in the biofilm.
B. Cells are more metabolically active at the base of a biofilm.
C. Cells are metabolically inactive at the base of a biofilm.
D. The structure of a biofilm favors the survival of antibiotic resistant cells.

9. Quorum sensing is used by bacterial cells to determine which of the following?

A. the size of the population
B. the availability of nutrients
C. the speed of water flow
D. the density of the population

10. Which of the following statements about autoinducers is incorrect?

A. They bind directly to DNA to activate transcription.
B. They can activate the cell that secreted them.
C. N-acylated homoserine lactones are autoinducers in gram-negative cells.
D. Autoinducers may stimulate the production of virulence factors.

11. An inoculated thioglycolate medium culture tube shows dense growth at the surface and turbidity throughout the rest of the tube. What is your conclusion?

A. The organisms die in the presence of oxygen
B. The organisms are facultative anaerobes.
C. The organisms should be grown in an anaerobic chamber.
D. The organisms are obligate aerobes.

12. An inoculated thioglycolate medium culture tube is clear throughout the tube except for dense growth at the bottom of the tube. What is your conclusion?

A. The organisms are obligate anaerobes.
B. The organisms are facultative anaerobes.
C. The organisms are aerotolerant.
D. The organisms are obligate aerobes.

13. *Pseudomonas aeruginosa* is a common pathogen that infects the airways of patients with cystic fibrosis. It does not grow in the absence of oxygen. The bacterium is probably which of the following?

A. an aerotolerant anaerobe
B. an obligate aerobe
C. an obligate anaerobe
D. a facultative anaerobe

14. *Streptococcus mutans* is a major cause of cavities. It resides in the gum pockets, does not have catalase activity, and can be grown outside of an anaerobic chamber. The bacterium is probably which of the following?

A. a facultative anaerobe
B. an obligate aerobe
C. an obligate anaerobe
D. an aerotolerant anaerobe

15. Why do the instructions for the growth of *Neisseria gonorrhoeae* recommend a CO_2-enriched atmosphere?

A. It uses CO_2 as a final electron acceptor in respiration.
B. It is an obligate anaerobe.
C. It is a capnophile.
D. It fixes CO_2 through photosynthesis.

16. Bacteria that grow in mine drainage at pH 1–2 are probably which of the following?

A. alkaliphiles
B. acidophiles
C. neutrophiles
D. obligate anaerobes

17. Bacteria isolated from Lake Natron, where the water pH is close to 10, are which of the following?

A. alkaliphiles
B. facultative anaerobes
C. neutrophiles
D. obligate anaerobes

18. In which environment are you most likely to encounter an acidophile?

A. human blood at pH 7.2
B. a hot vent at pH 1.5
C. human intestine at pH 8.5
D. milk at pH 6.5

19. A soup container was forgotten in the refrigerator and shows contamination. The contaminants are probably which of the following?

A. thermophiles
B. acidophiles
C. mesophiles
D. psychrotrophs

20. Bacteria isolated from a hot tub at 39 °C are probably which of the following?

A. thermophiles
B. psychrotrophs
C. mesophiles
D. hyperthermophiles

21. In which environment are you most likely to encounter a hyperthermophile?

A. hot tub
B. warm ocean water in Florida
C. hydrothermal vent at the bottom of the ocean
D. human body

22. Which of the following environments would harbor psychrophiles?
A. mountain lake with a water temperature of 12 °C
B. contaminated plates left in a 35 °C incubator
C. yogurt cultured at room temperature
D. salt pond in the desert with a daytime temperature of 34 °C

23. Which of the following is the reason jams and dried meats often do not require refrigeration to prevent spoilage?
A. low pH
B. toxic alkaline chemicals
C. naturally occurring antibiotics
D. low water activity

24. Bacteria living in salt marshes are most likely which of the following?
A. acidophiles
B. barophiles
C. halotolerant
D. thermophiles

25. EMB agar is a medium used in the identification and isolation of pathogenic bacteria. It contains digested meat proteins as a source of organic nutrients. Two indicator dyes, eosin and methylene blue, inhibit the growth of gram-positive bacteria and distinguish between lactose fermenting and nonlactose fermenting organisms. Lactose fermenters form metallic green or deep purple colonies, whereas the nonlactose fermenters form completely colorless colonies. EMB agar is an example of which of the following?
A. a selective medium only
B. a differential medium only
C. a selective medium and a chemically defined medium
D. a selective medium, a differential medium, and a complex medium

26. *Haemophilus influenzae* must be grown on chocolate agar, which is blood agar treated with heat to release growth factors in the medium. *H. influenzae* is described as ________.
A. an acidophile
B. a thermophile
C. an obligate anaerobe
D. fastidious

MATCHING

10.1 How Microbes Grow

27. Match the definition with the name of the growth phase in the growth curve.

___Number of dying cells is higher than the number of cells dividing	A. Lag phase
___Number of new cells equal to number of dying cells	B. Log phase
___New enzymes to use available nutrients are induced	C. Stationary phase
___Binary fission is occurring at maximum rate	D. Death phase

10.2 Oxygen Requirements for Microbial Growth

28. Four tubes are illustrated with cultures grown in a medium that slows oxygen diffusion. Match the culture tube with the correct type of bacteria from the following list: facultative anaerobe, obligate anaerobe, microaerophile, aerotolerant anaerobe, obligate aerobe.

10.4 Temperature and Microbial Growth

29. Match the type of bacterium with its environment. Each choice may be used once, more than once, or

not at all. Put the appropriate letter beside the environment.

___psychotroph	A. food spoiling in refrigerator
___mesophile	B. hydrothermal vent
___thermophile	C. deep ocean waters
___hyperthermophile	D. human pathogen
___psychrophile	E. garden compost

FILL IN THE BLANK

10.1 How Microbes Grow

30. Direct count of total cells can be performed using a ________ or a ________.

31. The ________ method allows direct count of total cells growing on solid medium.

32. A statistical estimate of the number of live cells in a liquid is usually done by ________.

33. For this indirect method of estimating the growth of a culture, you measure ________ using a spectrophotometer.

34. Active growth of a culture may be estimated indirectly by measuring the following products of cell metabolism: ________ or ________.

10.3 The Effects of pH on Microbial Growth

35. A bacterium that thrives in a soda lake where the average pH is 10.5 can be classified as a(n) ________.

36. *Lactobacillus acidophilus* grows best at pH 4.5. It is considered a(n) ________.

10.5 Other Environmental Conditions that Affect Growth

37. A bacterium that thrives in the Great Salt Lake but not in fresh water is probably a ________.

38. Bacteria isolated from the bottom of the ocean need high atmospheric pressures to survive. They are ________.

39. *Staphylococcus aureus* can be grown on multipurpose growth medium or on mannitol salt agar that contains 7.5% NaCl. The bacterium is ________.

10.6 Media Used for Bacterial Growth

40. Blood agar contains many unspecified nutrients, supports the growth of a large number of bacteria, and allows differentiation of bacteria according to hemolysis (breakdown of blood). The medium is ________ and ________.

41. Rogosa agar contains yeast extract. The pH is adjusted to 5.2 and discourages the growth of many microorganisms; however, all the colonies look similar. The medium is ________ and ________.

SHORT ANSWER

10.1 How Microbes Grow

42. Why is it important to measure the transmission of light through a control tube with only broth in it when making turbidity measures of bacterial cultures?

43. In terms of counting cells, what does a plating method accomplish that an electronic cell counting method does not?

44. Order the following stages of the development of a biofilm from the earliest to the last step.

1. secretion of EPS
2. reversible attachment
3. dispersal
4. formation of water channels
5. irreversible attachment

45. Infections among hospitalized patients are often related to the presence of a medical device in the patient. Which conditions favor the formation of biofilms on in-dwelling catheters and prostheses?

10.2 Oxygen Requirements for Microbial Growth

46. Why are some obligate anaerobes able to grow in tissues (e.g., gum pockets) that are not completely free of oxygen?

47. Why should *Haemophilus influenzae* be grown in a candle jar?

48. In terms of oxygen requirements, what type of organism would most likely be responsible for a foodborne illness associated with canned foods?

10.3 The Effects of pH on Microbial Growth

49. Which macromolecule in the cell is most sensitive to changes in pH?

50. Which metabolic process in the bacterial cell is particularly challenging at high pH?

10.4 Temperature and Microbial Growth

51. How are hyperthermophile's proteins adapted to the high temperatures of their environment?

52. Why would NASA be funding microbiology research in Antarctica?

10.5 Other Environmental Conditions that Affect Growth

53. Fish sauce is a salty condiment produced using fermentation. What type of organism is likely responsible for the fermentation of the fish sauce?

10.6 Media Used for Bacterial Growth

54. What is the major difference between an enrichment culture and a selective culture?

CRITICAL THINKING

55. A patient in the hospital has an intravenous catheter inserted to allow for the delivery of medications, fluids, and electrolytes. Four days after the catheter is inserted, the patient develops a fever and an infection in the skin around the catheter. Blood cultures reveal that the patient has a blood-borne infection. Tests in the clinical laboratory identify the blood-borne pathogen as *Staphylococcus epidermidis*, and antibiotic susceptibility tests are performed to provide doctors with essential information for selecting the best drug for treatment of the infection. Antibacterial chemotherapy is initiated and delivered through the intravenous catheter that was originally inserted into the patient. Within 7 days, the skin infection is gone, blood cultures are negative for *S. epidermidis*, and the antibacterial chemotherapy is discontinued. However, 2 days after discontinuing the antibacterial chemotherapy, the patient develops another fever and skin infection and the blood cultures are positive for the same strain of *S. epidermidis* that had been isolated the previous week. This time, doctors remove the intravenous catheter and administer oral antibiotics, which successfully treat both the skin and blood-borne infection caused by *S. epidermidis.* Furthermore, the infection does not return after discontinuing the oral antibacterial chemotherapy. What are some possible reasons why intravenous chemotherapy failed to completely cure the patient despite laboratory tests showing the bacterial strain was susceptible to the prescribed antibiotic? Why might the second round of antibiotic therapy have been more successful? Justify your answers.

56. Why are autoinducers small molecules?

57. Refer to **Figure B1** in **Appendix B**. If the results from a pond water sample were recorded as 3, 2, 1, what would be the MPN of bacteria in 100 mL of pond water?

58. Refer to **Figure 10.15**. Why does turbidity lose reliability at high cell concentrations when the culture reaches the stationary phase?

59. A microbiology instructor prepares cultures for a gram-staining practical laboratory by inoculating growth medium with a gram-positive coccus (nonmotile) and a gram-negative rod (motile). The goal is to demonstrate staining of a mixed culture. The flask is incubated at 35 °C for 24 hours without aeration. A sample is stained and reveals only gram-negative rods. Both cultures are known facultative anaerobes. Give a likely reason for success of the gram-negative rod. Assume that the cultures have comparable intrinsic growth rates.

60. People who use proton pumps inhibitors or antacids are more prone to infections of the gastrointestinal tract. Can you explain the observation in light of what you have learned?

61. The bacterium that causes Hansen's disease (leprosy), *Mycobacterium leprae*, infects mostly the extremities of the body: hands, feet, and nose. Can you make an educated guess as to its optimum temperature of growth?

62. Refer to **Figure 10.29**. Some hyperthermophiles can survive autoclaving temperatures. Are they a concern in health care?

63. *Haemophilus, influenzae* grows best at 35–37 °C with ~5% CO_2 (or in a candle-jar) and requires hemin (X factor) and nicotinamide-adenine-dinucleotide (NAD, also known as V factor) for growth. (Centers for Disease Control and Prevention, World Health Organization. "*CDC Laboratory Methods for the Diagnosis of Meningitis Caused by* Neisseria meningitidis, Streptococcus pneumoniae, *and* Haemophilus influenza. WHO Manual, 2nd edition." 2011. http://www.cdc.gov/meningitis/lab-manual/full-manual.pdf) Using the vocabulary learned in this chapter, describe *H. influenzae*.

11 | CONTROL OF MICROBIAL GROWTH

Location	Average number CFUs per 6.5 × 6.5 cm area
Door latch	256
Door lock	14
Door lock control	182
Door handle	29
Window control	4
Cruise control button	69
Steering wheel	239
Interior steering wheel	390
Radio volume knob	99
Gear shifter	115
Center console	506

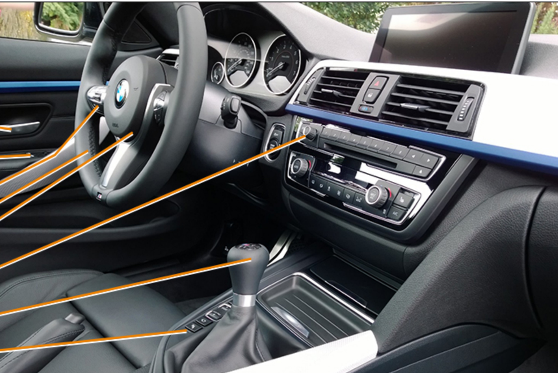


Figure 11.1 Most environments, including cars, are not sterile. A study[1] analyzed 11 locations within 18 different cars to determine the number of microbial colony-forming units (CFUs) present. The center console harbored by far the most microbes (506 CFUs), possibly because that is where drinks are placed (and often spilled). Frequently touched sites also had high concentrations. (credit "photo": modification of work by Jeff Wilcox)

Chapter Outline

11.1: Controlling Microbial Growth

11.2: Using Physical Methods to Control Microorganisms

11.3: Using Chemicals to Control Microorganisms

Introduction

How clean is clean? People wash their cars and vacuum the carpets, but most would not want to eat from these surfaces. Similarly, we might eat with silverware cleaned in a dishwasher, but we could not use the same dishwasher to clean surgical instruments. As these examples illustrate, "clean" is a relative term. Car washing, vacuuming, and dishwashing all reduce the microbial load on the items treated, thus making them "cleaner." But whether they are "clean enough" depends on their intended use. Because people do not normally eat from cars or carpets, these items do not require the same level of cleanliness that silverware does. Likewise, because silverware is not used for invasive surgery, these utensils do not require the same level of cleanliness as surgical equipment, which requires sterilization to prevent infection.

Why not play it safe and sterilize everything? Sterilizing everything we come in contact with is impractical, as well as potentially dangerous. As this chapter will demonstrate, sterilization protocols often require time- and labor-intensive treatments that may degrade the quality of the item being treated or have toxic effects on users. Therefore, the user must consider the item's intended application when choosing a cleaning method to ensure that it is "clean enough."

11.1 | Controlling Microbial Growth

1. R.E. Stephenson et al. "Elucidation of Bacteria Found in Car Interiors and Strategies to Reduce the Presence of Potential Pathogens." *Biofouling* 30 no. 3 (2014):337–346.

Learning Objectives

By the end of this section, you will be able to:

- Compare disinfectants, antiseptics, and sterilants
- Describe the principles of controlling the presence of microorganisms through sterilization and disinfection
- Differentiate between microorganisms of various biological safety levels and explain methods used for handling microbes at each level

Part 1

Roberta is a 46-year-old real estate agent who recently underwent a cholecystectomy (surgery to remove painful gallstones). The surgery was performed laparoscopically with the aid of a duodenoscope, a specialized endoscope that allows surgeons to see inside the body with the aid of a tiny camera. On returning home from the hospital, Roberta developed abdominal pain and a high fever. She also experienced a burning sensation during urination and noticed blood in her urine. She notified her surgeon of these symptoms, per her postoperative instructions.

- What are some possible causes of Roberta's symptoms?

*Jump to the **next** Clinical Focus box.*

To prevent the spread of human disease, it is necessary to control the growth and abundance of microbes in or on various items frequently used by humans. Inanimate items, such as doorknobs, toys, or towels, which may harbor microbes and aid in disease transmission, are called **fomites**. Two factors heavily influence the level of cleanliness required for a particular fomite and, hence, the protocol chosen to achieve this level. The first factor is the application for which the item will be used. For example, invasive applications that require insertion into the human body require a much higher level of cleanliness than applications that do not. The second factor is the level of resistance to antimicrobial treatment by potential pathogens. For example, foods preserved by canning often become contaminated with the bacterium *Clostridium botulinum*, which produces the neurotoxin that causes botulism. Because *C. botulinum* can produce endospores that can survive harsh conditions, extreme temperatures and pressures must be used to eliminate the endospores. Other organisms may not require such extreme measures and can be controlled by a procedure such as washing clothes in a laundry machine.

Laboratory Biological Safety Levels

For researchers or laboratory personnel working with pathogens, the risks associated with specific pathogens determine the levels of cleanliness and control required. The Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH) have established four classification levels, called " biological safety levels" (BSLs). Various organizations around the world, including the World Health Organization (WHO) and the European Union (EU), use a similar classification scheme. According to the CDC, the BSL is determined by the agent's infectivity, ease of transmission, and potential disease severity, as well as the type of work being done with the agent.[2]

Each BSL requires a different level of biocontainment to prevent contamination and spread of infectious agents to laboratory personnel and, ultimately, the community. For example, the lowest BSL, BSL-1, requires the fewest precautions because it applies to situations with the lowest risk for microbial infection.

BSL-1 agents are those that generally do not cause infection in healthy human adults. These include noninfectious bacteria, such as nonpathogenic strains of *Escherichia coli* and *Bacillus subtilis*, and viruses known to infect animals other than humans, such as baculoviruses (insect viruses). Because working with BSL-1 agents poses very little risk, few precautions are necessary. Laboratory workers use standard aseptic technique and may work with these agents at an open laboratory bench or table, wearing personal protective equipment (PPE) such as a laboratory coat, goggles, and gloves, as needed. Other than a sink for handwashing and doors to separate the laboratory from the rest of the building, no additional modifications are needed.

Agents classified as BSL-2 include those that pose moderate risk to laboratory workers and the community, and are typically "indigenous," meaning that they are commonly found in that geographical area. These include bacteria such as *Staphylococcus aureus* and *Salmonella* spp., and viruses like hepatitis, mumps, and measles viruses. BSL-2 laboratories require additional precautions beyond those of BSL-1, including restricted access;

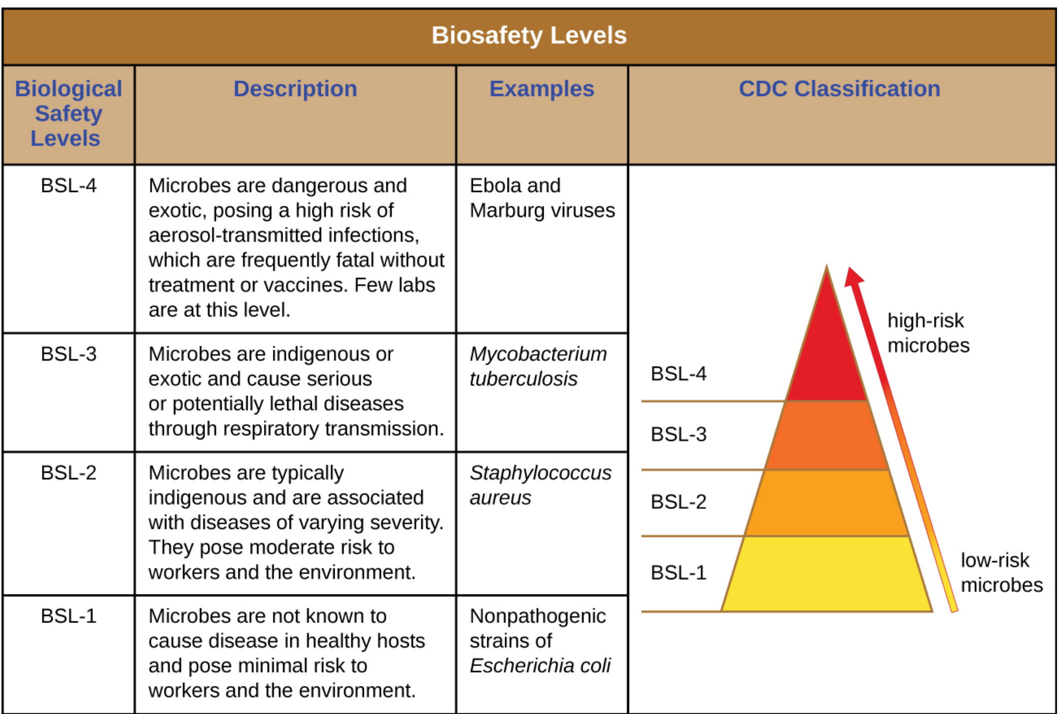
2. US Centers for Disease Control and Prevention. "Recognizing the Biosafety Levels." http://www.cdc.gov/training/quicklearns/biosafety/. Accessed June 7, 2016.

required PPE, including a face shield in some circumstances; and the use of biological safety cabinets for procedures that may disperse agents through the air (called “aerosolization”). BSL-2 laboratories are equipped with self-closing doors, an eyewash station, and an **autoclave**, which is a specialized device for sterilizing materials with pressurized steam before use or disposal. BSL-1 laboratories may also have an autoclave.

BSL-3 agents have the potential to cause lethal infections by inhalation. These may be either indigenous or “exotic,” meaning that they are derived from a foreign location, and include pathogens such as *Mycobacterium tuberculosis*, *Bacillus anthracis*, West Nile virus, and human immunodeficiency virus (HIV). Because of the serious nature of the infections caused by BSL-3 agents, laboratories working with them require restricted access. Laboratory workers are under medical surveillance, possibly receiving vaccinations for the microbes with which they work. In addition to the standard PPE already mentioned, laboratory personnel in BSL-3 laboratories must also wear a respirator and work with microbes and infectious agents in a biological safety cabinet at all times. BSL-3 laboratories require a hands-free sink, an eyewash station near the exit, and two sets of self-closing and locking doors at the entrance. These laboratories are equipped with directional airflow, meaning that clean air is pulled through the laboratory from clean areas to potentially contaminated areas. This air cannot be recirculated, so a constant supply of clean air is required.

BSL-4 agents are the most dangerous and often fatal. These microbes are typically exotic, are easily transmitted by inhalation, and cause infections for which there are no treatments or vaccinations. Examples include Ebola virus and Marburg virus, both of which cause hemorrhagic fevers, and smallpox virus. There are only a small number of laboratories in the United States and around the world appropriately equipped to work with these agents. In addition to BSL-3 precautions, laboratory workers in BSL-4 facilities must also change their clothing on entering the laboratory, shower on exiting, and decontaminate all material on exiting. While working in the laboratory, they must either wear a full-body protective suit with a designated air supply or conduct all work within a biological safety cabinet with a high-efficiency particulate air (HEPA)-filtered air supply and a doubly HEPA-filtered exhaust. If wearing a suit, the air pressure within the suit must be higher than that outside the suit, so that if a leak in the suit occurs, laboratory air that may be contaminated cannot be drawn into the suit (**Figure 11.2**). The laboratory itself must be located either in a separate building or in an isolated portion of a building and have its own air supply and exhaust system, as well as its own decontamination system. The BSLs are summarized in **Figure 11.3**.

Figure 11.2 A protective suit like this one is an additional precaution for those who work in BSL-4 laboratories. This suit has its own air supply and maintains a positive pressure relative to the outside, so that if a leak occurs, air will flow out of the suit, not into it from the laboratory. (Credit: James Gathany, CDC, public domain)



Biosafety Levels			
Biological Safety Levels	**Description**	**Examples**	**CDC Classification**
BSL-4	Microbes are dangerous and exotic, posing a high risk of aerosol-transmitted infections, which are frequently fatal without treatment or vaccines. Few labs are at this level.	Ebola and Marburg viruses	
BSL-3	Microbes are indigenous or exotic and cause serious or potentially lethal diseases through respiratory transmission.	*Mycobacterium tuberculosis*	
BSL-2	Microbes are typically indigenous and are associated with diseases of varying severity. They pose moderate risk to workers and the environment.	*Staphylococcus aureus*	
BSL-1	Microbes are not known to cause disease in healthy hosts and pose minimal risk to workers and the environment.	Nonpathogenic strains of *Escherichia coli*	

Figure 11.3 The CDC classifies infectious agents into four biosafety levels based on potential risk to laboratory personnel and the community. Each level requires a progressively greater level of precaution. (credit "pyramid": modification of work by Centers for Disease Control and Prevention)

To **learn more (https://openstax.org/l/22cdcfourbsls)** about the four BSLs, visit the CDC's website.

- What are some factors used to determine the BSL necessary for working with a specific pathogen?

Sterilization

The most extreme protocols for microbial control aim to achieve **sterilization**: the complete removal or killing of all vegetative cells, endospores, and viruses from the targeted item or environment. Sterilization protocols are generally reserved for laboratory, medical, manufacturing, and food industry settings, where it may be imperative for certain items to be completely free of potentially infectious agents. Sterilization can be accomplished through either physical means, such as exposure to high heat, pressure, or filtration through an appropriate filter, or by chemical means. Chemicals that can be used to achieve sterilization are called **sterilants**. Sterilants effectively kill all microbes and viruses, and, with appropriate exposure time, can also kill endospores.

For many clinical purposes, **aseptic technique** is necessary to prevent contamination of sterile surfaces. Aseptic technique involves a combination of protocols that collectively maintain sterility, or **asepsis**, thus preventing contamination of the patient with microbes and infectious agents. Failure to practice aseptic technique during many types of clinical procedures may introduce microbes to the patient's body and put the patient at risk for **sepsis**, a systemic inflammatory response to an infection that results in high fever, increased heart and respiratory rates, shock, and, possibly, death. Medical procedures that carry risk of contamination must be performed in a **sterile field**, a designated area that is kept free of all vegetative microbes, endospores, and viruses. Sterile fields are created according to protocols requiring the use of sterilized materials, such as packaging and drapings, and strict procedures for washing and application of sterilants. Other protocols are followed to maintain the sterile field while the medical procedure is being performed.

One food sterilization protocol, **commercial sterilization**, uses heat at a temperature low enough to preserve food quality but high enough to destroy common pathogens responsible for food poisoning, such as *C. botulinum*. Because *C. botulinum* and its endospores are commonly found in soil, they may easily contaminate crops during harvesting, and these endospores can later germinate within the anaerobic environment once foods are canned. Metal cans of food contaminated with *C. botulinum* will bulge due to the microbe's production

of gases; contaminated jars of food typically bulge at the metal lid. To eliminate the risk for *C. botulinum* contamination, commercial food-canning protocols are designed with a large margin of error. They assume an impossibly large population of endospores (10^{12} per can) and aim to reduce this population to 1 endospore per can to ensure the safety of canned foods. For example, low- and medium-acid foods are heated to 121 °C for a minimum of 2.52 minutes, which is the time it would take to reduce a population of 10^{12} endospores per can down to 1 endospore at this temperature. Even so, commercial sterilization does not eliminate the presence of all microbes; rather, it targets those pathogens that cause spoilage and foodborne diseases, while allowing many nonpathogenic organisms to survive. Therefore, "sterilization" is somewhat of a misnomer in this context, and commercial sterilization may be more accurately described as "quasi-sterilization."

- What is the difference between sterilization and aseptic technique?

The Association of Surgical Technologists publishes **standards (https://openstax.org/l/22ASTstanasepte)** for aseptic technique, including creating and maintaining a sterile field.

Other Methods of Control

Sterilization protocols require procedures that are not practical, or necessary, in many settings. Various other methods are used in clinical and nonclinical settings to reduce the microbial load on items. Although the terms for these methods are often used interchangeably, there are important distinctions (**Figure 11.4**).

The process of **disinfection** inactivates most microbes on the surface of a fomite by using antimicrobial chemicals or heat. Because some microbes remain, the disinfected item is not considered sterile. Ideally, **disinfectant**s should be fast acting, stable, easy to prepare, inexpensive, and easy to use. An example of a natural disinfectant is vinegar; its acidity kills most microbes. Chemical disinfectants, such as chlorine bleach or products containing chlorine, are used to clean nonliving surfaces such as laboratory benches, clinical surfaces, and bathroom sinks. Typical disinfection does not lead to sterilization because endospores tend to survive even when all vegetative cells have been killed.

Unlike disinfectants, **antiseptic**s are antimicrobial chemicals safe for use on living skin or tissues. Examples of antiseptics include hydrogen peroxide and isopropyl alcohol. The process of applying an antiseptic is called **antisepsis**. In addition to the characteristics of a good disinfectant, antiseptics must also be selectively effective against microorganisms and able to penetrate tissue deeply without causing tissue damage.

The type of protocol required to achieve the desired level of cleanliness depends on the particular item to be cleaned. For example, those used clinically are categorized as critical, semicritical, and noncritical. Critical items must be sterile because they will be used inside the body, often penetrating sterile tissues or the bloodstream; examples of **critical item**s include surgical instruments, catheters, and intravenous fluids. Gastrointestinal endoscopes and various types of equipment for respiratory therapies are examples of **semicritical item**s; they may contact mucous membranes or nonintact skin but do not penetrate tissues. Semicritical items do not typically need to be sterilized but do require a high level of disinfection. Items that may contact but not penetrate intact skin are **noncritical item**s; examples are bed linens, furniture, crutches, stethoscopes, and blood pressure cuffs. These articles need to be clean but not highly disinfected.

The act of handwashing is an example of **degerming**, in which microbial numbers are significantly reduced by gently scrubbing living tissue, most commonly skin, with a mild chemical (e.g., soap) to avoid the transmission of pathogenic microbes. Wiping the skin with an alcohol swab at an injection site is another example of degerming. These degerming methods remove most (but not all) microbes from the skin's surface.

The term **sanitization** refers to the cleansing of fomites to remove enough microbes to achieve levels deemed safe for public health. For example, commercial dishwashers used in the food service industry typically use very hot water and air for washing and drying; the high temperatures kill most microbes, sanitizing the dishes. Surfaces in hospital rooms are commonly sanitized using a chemical disinfectant to prevent disease transmission between patients. **Figure 11.4** summarizes common protocols, definitions, applications, and agents used to control microbial growth.

This OpenStax book is available for free at https://legacy.cnx.org/content/col26095/1.5

Common Protocols for Control of Microbial Growth			
Protocol	**Definition**	**Common Application**	**Common Agents**
For Use on Fomites			
Disinfection	Reduces or destroys microbial load of an inanimate item through application of heat or antimicrobial chemicals	Cleaning surfaces like laboratory benches, clinical surfaces, and bathrooms	Chlorine bleach, phenols (e.g., Lysol), glutaraldehyde
Sanitization	Reduces microbial load of an inanimate item to safe public health levels through application of heat or antimicrobial chemicals	Commercial dishwashing of eating utensils, cleaning public restrooms	Detergents containing phosphates (e.g., Finish), industrial-strength cleaners containing quaternary ammonium compounds
Sterilization	Completely eliminates all vegetative cells, endospores, and viruses from an inanimate item	Preparation of surgical equipment and of needles used for injection	Pressurized steam (autoclave), chemicals, radiation
For Use on Living Tissue			
Antisepsis	Reduces microbial load on skin or tissue through application of an antimicrobial chemical	Cleaning skin broken due to injury; cleaning skin before surgery	Boric acid, isopropyl alcohol, hydrogen peroxide, iodine (betadine)
Degerming	Reduces microbial load on skin or tissue through gentle to firm scrubbing and the use of mild chemicals	Handwashing	Soap, alcohol swab

Figure 11.4

- What is the difference between a disinfectant and an antiseptic?
- Which is most effective at removing microbes from a product: sanitization, degerming, or sterilization? Explain.

Part 2

Roberta's physician suspected that a bacterial infection was responsible for her sudden-onset high fever, abdominal pain, and bloody urine. Based on these symptoms, the physician diagnosed a urinary tract infection (UTI). A wide variety of bacteria may cause UTIs, which typically occur when bacteria from the lower gastrointestinal tract are introduced to the urinary tract. However, Roberta's recent gallstone surgery caused the physician to suspect that she had contracted a nosocomial (hospital-acquired) infection during her surgery. The physician took a urine sample and ordered a urine culture to check for the presence of white blood cells, red blood cells, and bacteria. The results of this test would help determine the cause of the infection. The physician also prescribed a course of the antibiotic ciprofloxacin, confident that it would clear Roberta's infection.

- What are some possible ways that bacteria could have been introduced to Roberta's urinary tract during her surgery?

Jump to the ***next (https://legacy.cnx.org/content/m58856/latest/#fs-id1167583850472)*** *Clinical Focus box. Go back to the* ***previous*** *Clinical Focus box.*

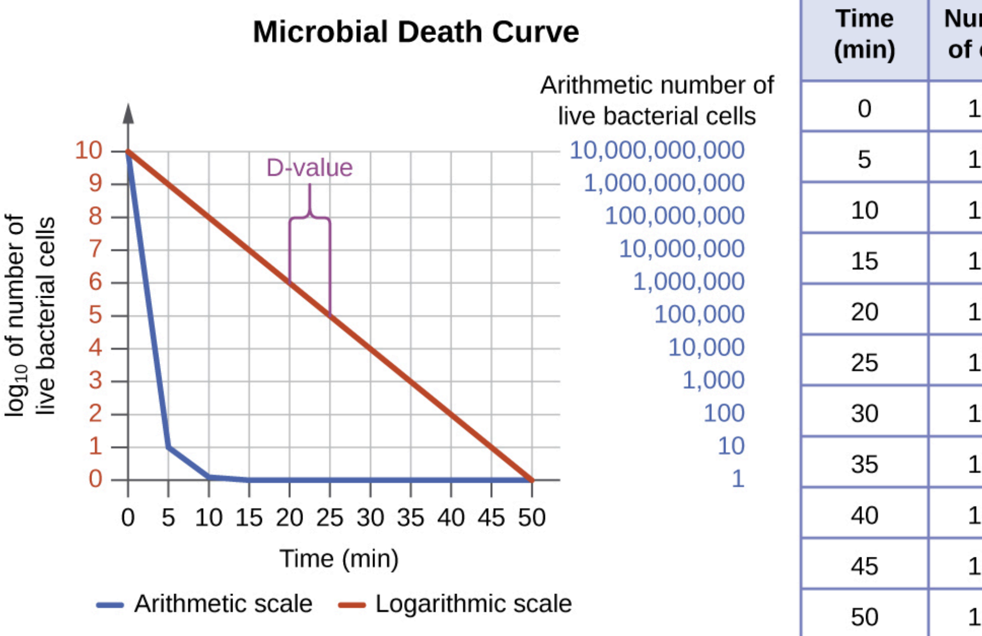
Measuring Microbial Control

Physical and chemical methods of microbial control that kill the targeted microorganism are identified by the suffix *-cide* (or *-cidal*). The prefix indicates the type of microbe or infectious agent killed by the treatment method: **bactericides** kill bacteria, **viricides** kill or inactivate viruses, and **fungicides** kill fungi. Other methods do not kill organisms but, instead, stop their growth, making their population static; such methods are identified by the suffix *-stat* (or *-static*). For example, **bacteriostatic** treatments inhibit the growth of bacteria, whereas **fungistatic** treatments inhibit the growth of fungi. Factors that determine whether a particular treatment is *-cidal* or *-static* include the types of microorganisms targeted, the concentration of the chemical used, and the nature of the

treatment applied.

Although *-static* treatments do not actually kill infectious agents, they are often less toxic to humans and other animals, and may also better preserve the integrity of the item treated. Such treatments are typically sufficient to keep the microbial population of an item in check. The reduced toxicity of some of these *-static* chemicals also allows them to be impregnated safely into plastics to prevent the growth of microbes on these surfaces. Such plastics are used in products such as toys for children and cutting boards for food preparation. When used to treat an infection, *-static* treatments are typically sufficient in an otherwise healthy individual, preventing the pathogen from multiplying, thus allowing the individual's immune system to clear the infection.

The degree of microbial control can be evaluated using a **microbial death curve** to describe the progress and effectiveness of a particular protocol. When exposed to a particular microbial control protocol, a fixed percentage of the microbes within the population will die. Because the rate of killing remains constant even when the population size varies, the percentage killed is more useful information than the absolute number of microbes killed. Death curves are often plotted as semilog plots just like microbial growth curves because the reduction in microorganisms is typically logarithmic (**Figure 11.5**). The amount of time it takes for a specific protocol to produce a one order-of-magnitude decrease in the number of organisms, or the death of 90% of the population, is called the **decimal reduction time (DRT)** or **D-value**.



Time (min)	Number of cells
0	10^{10}
5	10^{9}
10	10^{8}
15	10^{7}
20	10^{6}
25	10^{5}
30	10^{4}
35	10^{3}
40	10^{2}
45	10^{1}
50	10^{0}

Figure 11.5 Microbial death is logarithmic and easily observed using a semilog plot instead of an arithmetic one. The decimal reduction time (D-value) is the time it takes to kill 90% of the population (a 1-log decrease in the total population) when exposed to a specific microbial control protocol, as indicated by the purple bracket.

Several factors contribute to the effectiveness of a disinfecting agent or microbial control protocol. First, as demonstrated in **Figure 11.5**, the length of time of exposure is important. Longer exposure times kill more microbes. Because microbial death of a population exposed to a specific protocol is logarithmic, it takes longer to kill a high-population load than a low-population load exposed to the same protocol. A shorter treatment time (measured in multiples of the D-value) is needed when starting with a smaller number of organisms. Effectiveness also depends on the susceptibility of the agent to that disinfecting agent or protocol. The concentration of disinfecting agent or intensity of exposure is also important. For example, higher temperatures and higher concentrations of disinfectants kill microbes more quickly and effectively. Conditions that limit contact between the agent and the targeted cells cells—for example, the presence of bodily fluids, tissue, organic debris (e.g., mud or feces), or biofilms on surfaces—increase the cleaning time or intensity of the microbial control protocol required to reach the desired level of cleanliness. All these factors must be considered when choosing the appropriate protocol to control microbial growth in a given situation.

- What are two possible reasons for choosing a bacteriostatic treatment over a bactericidal one?
- Name at least two factors that can compromise the effectiveness of a disinfecting agent.

11.2 | Using Physical Methods to Control Microorganisms

Learning Objectives

By the end of this section, you will be able to:

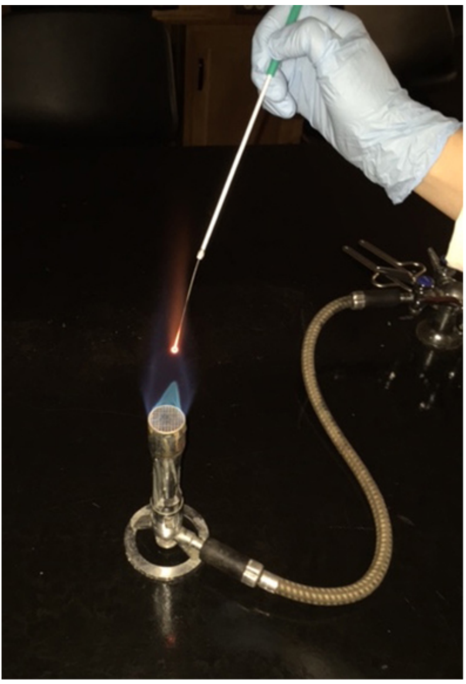
- Understand and compare various physical methods of controlling microbial growth, including heating, refrigeration, freezing, high-pressure treatment, desiccation, lyophilization, irradiation, and filtration

For thousands of years, humans have used various physical methods of microbial control for food preservation. Common control methods include the application of high temperatures, radiation, filtration, and desiccation (drying), among others. Many of these methods nonspecifically kill cells by disrupting membranes, changing membrane permeability, or damaging proteins and nucleic acids by denaturation, degradation, or chemical modification. Various physical methods used for microbial control are described in this section.

Heat

Heating is one of the most common—and oldest—forms of microbial control. It is used in simple techniques like cooking and canning. Heat can kill microbes by altering their membranes and denaturing proteins. The **thermal death point (TDP)** of a microorganism is the lowest temperature at which all microbes are killed in a 10-minute exposure. Different microorganisms will respond differently to high temperatures, with some (e.g., endospore-formers such as *C. botulinum*) being more heat tolerant. A similar parameter, the **thermal death time (TDT)**, is the length of time needed to kill all microorganisms in a sample at a given temperature. These parameters are often used to describe sterilization procedures that use high heat, such as autoclaving. Boiling is one of the oldest methods of moist-heat control of microbes, and it is typically quite effective at killing vegetative cells and some viruses. However, boiling is less effective at killing endospores; some endospores are able to survive up to 20 hours of boiling. Additionally, boiling may be less effective at higher altitudes, where the boiling point of water is lower and the boiling time needed to kill microbes is therefore longer. For these reasons, boiling is not considered a useful sterilization technique in the laboratory or clinical setting.

Many different heating protocols can be used for sterilization in the laboratory or clinic, and these protocols can be broken down into two main categories: **dry-heat sterilization** and **moist-heat sterilization**. Aseptic technique in the laboratory typically involves some dry-heat sterilization protocols using direct application of high heat, such as sterilizing inoculating loops (Figure 11.6). Incineration at very high temperatures destroys all microorganisms. Dry heat can also be applied for relatively long periods of time (at least 2 hours) at temperatures up to 170 °C by using a dry-heat sterilizer, such as an oven. However, moist-heat sterilization is typically the more effective protocol because it penetrates cells better than dry heat does.



(a) (b)

Figure 11.6 (a) Sterilizing a loop, often referred to as “flaming a loop,” is a common component of aseptic technique in the microbiology laboratory and is used to incinerate any microorganisms on the loop. (b) Alternatively, a bactericinerator may be used to reduce aerosolization of microbes and remove the presence of an open flame in the laboratory. These are examples of dry-heat sterilization by the direct application of high heat capable of incineration. (credit a: modification of work by Anh-Hue Tu; credit b: modification of work by Brian Forster)

Autoclaves

Autoclaves rely on moist-heat sterilization. They are used to raise temperatures above the boiling point of water to sterilize items such as surgical equipment from vegetative cells, viruses, and especially endospores, which are known to survive boiling temperatures, without damaging the items. Charles Chamberland (1851–1908) designed the modern autoclave in 1879 while working in the laboratory of Louis Pasteur. The autoclave is still considered the most effective method of sterilization (Figure 11.7). Outside laboratory and clinical settings, large industrial autoclaves called **retorts** allow for moist-heat sterilization on a large scale.

In general, the air in the chamber of an autoclave is removed and replaced with increasing amounts of steam trapped within the enclosed chamber, resulting in increased interior pressure and temperatures above the boiling point of water. The two main types of autoclaves differ in the way that air is removed from the chamber. In gravity displacement autoclaves, steam is introduced into the chamber from the top or sides. Air, which is heavier than steam, sinks to the bottom of the chamber, where it is forced out through a vent. Complete displacement of air is difficult, especially in larger loads, so longer cycles may be required for such loads. In prevacuum sterilizers, air is removed completely using a high-speed vacuum before introducing steam into the chamber. Because air is more completely eliminated, the steam can more easily penetrate wrapped items. Many autoclaves are capable of both gravity and prevacuum cycles, using the former for the decontamination of waste and sterilization of media and unwrapped glassware, and the latter for sterilization of packaged instruments.

Figure 11.7 A technician sterilizes a sample using an autoclave. (Credit: Martha Cooper / Picryl; Public Domain.

Standard operating temperatures for autoclaves are 121 °C or, in some cases, 132 °C, typically at a pressure of 15 to 20 pounds per square inch (psi). The length of exposure depends on the volume and nature of material being sterilized, but it is typically 20 minutes or more, with larger volumes requiring longer exposure times to ensure sufficient heat transfer to the materials being sterilized. The steam must directly contact the liquids or dry materials being sterilized, so containers are left loosely closed and instruments are loosely wrapped in paper or foil. The key to autoclaving is that the temperature must be high enough to kill endospores to achieve complete sterilization.

Because sterilization is so important to safe medical and laboratory protocols, quality control is essential. Autoclaves may be equipped with recorders to document the pressures and temperatures achieved during each run. Additionally, internal indicators of various types should be autoclaved along with the materials to be sterilized to ensure that the proper sterilization temperature has been reached (**Figure 11.8**). One common type of indicator is the use of heat-sensitive autoclave tape, which has white stripes that turn black when the appropriate temperature is achieved during a successful autoclave run. This type of indicator is relatively inexpensive and can be used during every run. However, autoclave tape provides no indication of length of exposure, so it cannot be used as an indicator of sterility. Another type of indicator, a biological indicator spore test, uses either a strip of paper or a liquid suspension of the endospores of *Geobacillus stearothermophilus* to determine whether the endospores are killed by the process. The endospores of the obligate thermophilic bacterium *G. stearothermophilus* are the gold standard used for this purpose because of their extreme heat resistance. Biological spore indicators can also be used to test the effectiveness of other sterilization protocols, including ethylene oxide, dry heat, formaldehyde, gamma radiation, and hydrogen peroxide plasma sterilization using either *G*. *stearothermophilus*, *Bacillus atrophaeus, B. subtilis,* or *B. pumilus* spores. In the case of validating autoclave function, the endospores are incubated after autoclaving to ensure no viable endospores remain. Bacterial growth subsequent to endospore germination can be monitored by biological indicator spore tests that detect acid metabolites or fluorescence produced by enzymes derived from viable *G. stearothermophilus*. A third type of autoclave indicator is the Diack tube, a glass ampule containing a temperature-sensitive pellet that melts at the proper sterilization temperature. Spore strips or Diack tubes are used periodically to ensure the autoclave is functioning properly.

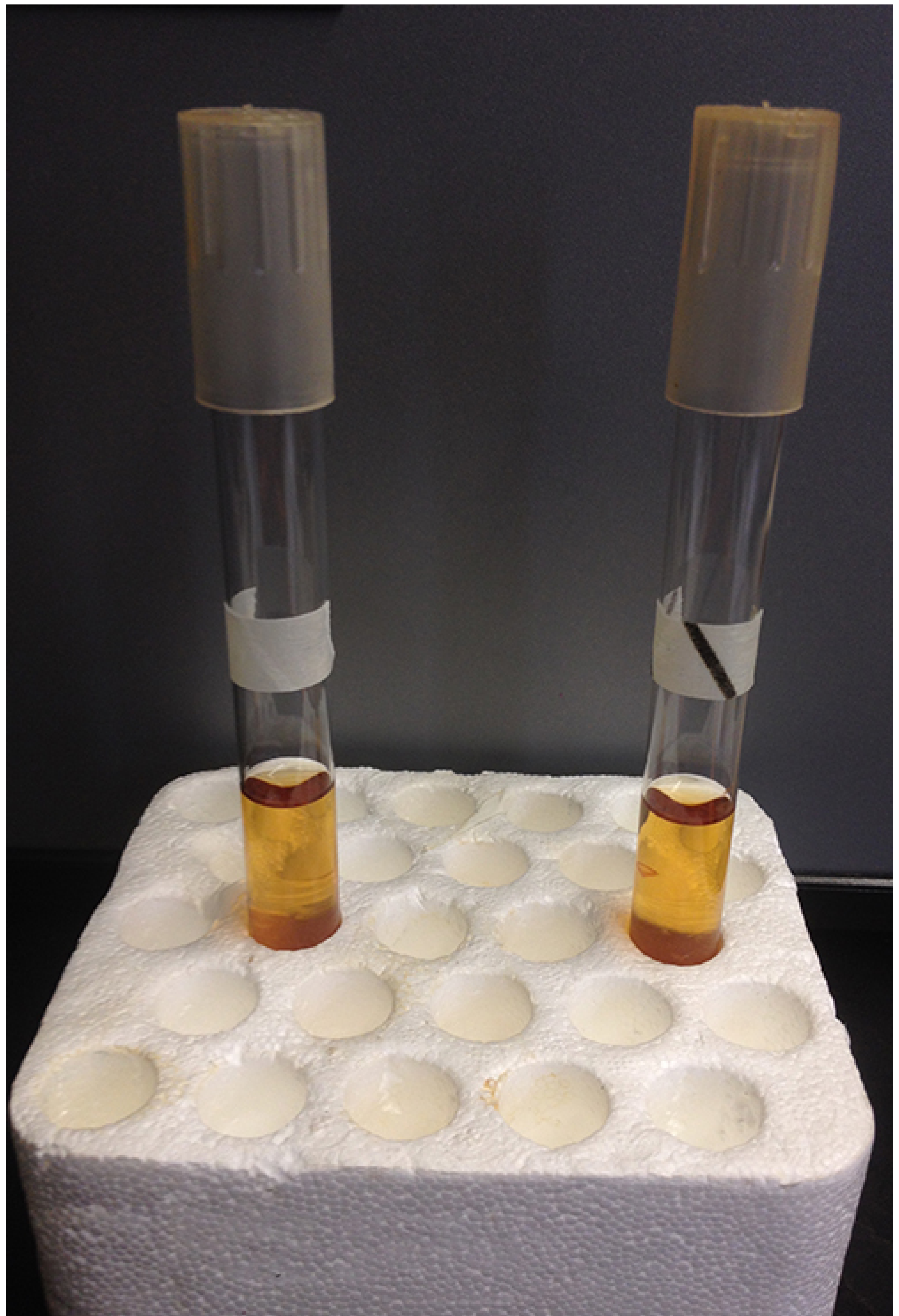


Figure 11.8 The white strips on autoclave tape (left tube) turn dark during a successful autoclave run (right tube). (credit: modification of work by Brian Forster)

Pasteurization

Although complete sterilization is ideal for many medical applications, it is not always practical for other applications and may also alter the quality of the product. Boiling and autoclaving are not ideal ways to control microbial growth in many foods because these methods may ruin the consistency and other organoleptic (sensory) qualities of the food. Pasteurization is a form of microbial control for food that uses heat but does not render the food sterile. Traditional **pasteurization** kills pathogens and reduces the number of spoilage-causing microbes while maintaining food quality. The process of pasteurization was first developed by Louis Pasteur in

the 1860s as a method for preventing the spoilage of beer and wine. Today, pasteurization is most commonly used to kill heat-sensitive pathogens in milk and other food products (e.g., apple juice and honey) (**Figure 11.9**). However, because pasteurized food products are not sterile, they will eventually spoil.

The methods used for milk pasteurization balance the temperature and the length of time of treatment. One method, **high-temperature short-time (HTST) pasteurization**, exposes milk to a temperature of 72 °C for 15 seconds, which lowers bacterial numbers while preserving the quality of the milk. An alternative is **ultra-high-temperature (UHT) pasteurization**, in which the milk is exposed to a temperature of 138 °C for 2 or more seconds. UHT pasteurized milk can be stored for a long time in sealed containers without being refrigerated; however, the very high temperatures alter the proteins in the milk, causing slight changes in the taste and smell. Still, this method of pasteurization is advantageous in regions where access to refrigeration is limited.

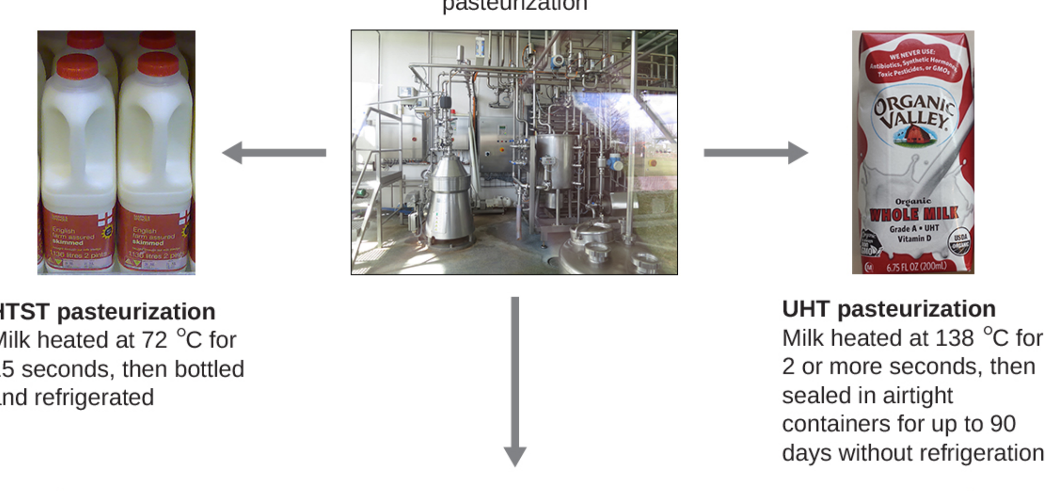


Figure 11.9 Two different methods of pasteurization, HTST and UHT, are commonly used to kill pathogens associated with milk spoilage. (credit left: modification of work by Mark Hillary; credit right: modification of work by Kerry Ceszyk)

- In an autoclave, how are temperatures above boiling achieved?
- How would the onset of spoilage compare between HTST-pasteurized and UHT-pasteurized milk?
- Why is boiling not used as a sterilization method in a clinical setting?

Refrigeration and Freezing

Just as high temperatures are effective for controlling microbial growth, exposing microbes to low temperatures can also be an easy and effective method of microbial control, with the exception of psychrophiles, which prefer cold temperatures (see **Temperature and Microbial Growth**). Refrigerators used in home kitchens or in the laboratory maintain temperatures between 0 °C and 7 °C. This temperature range inhibits microbial metabolism, slowing the growth of microorganisms significantly and helping preserve refrigerated products such as foods or medical supplies. Certain types of laboratory cultures can be preserved by refrigeration for later use.

Freezing below −2 °C may stop microbial growth and even kill susceptible organisms. According to the US Department of Agriculture (USDA), the only safe ways that frozen foods can be thawed are in the refrigerator, immersed in cold water changed every 30 minutes, or in the microwave, keeping the food at temperatures not conducive for bacterial growth.[3] In addition, halted bacterial growth can restart in thawed foods, so thawed foods should be treated like fresh perishables.

Bacterial cultures and medical specimens requiring long-term storage or transport are often frozen at ultra-low temperatures of −70 °C or lower. These ultra-low temperatures can be achieved by storing specimens on dry ice in an ultra-low freezer or in special liquid nitrogen tanks, which maintain temperatures lower than −196 °C

3. US Department of Agriculture. "Freezing and Food Safety." 2013. http://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/safe-food-handling/freezing-and-food-safety/CT_Index. Accessed June 8, 2016.

(**Figure 11.10**).

Figure 11.10 Cultures and other medical specimens can be stored for long periods at ultra-low temperatures. (a) An ultra-low freezer maintains temperatures at or below −70 °C. (b) Two people stand in a room with a large nitrogen storage unit emitting a large amount of fog. (credit a: modification of work by “Expert Infantry”/Flickr; credit b: Credit: US Navy; Public Domain.)

- Does placing food in a refrigerator kill bacteria on the food?

Pressure

Exposure to high pressure kills many microbes. In the food industry, high-pressure processing (also called pascalization) is used to kill bacteria, yeast, molds, parasites, and viruses in foods while maintaining food quality and extending shelf life. The application of high pressure between 100 and 800 MPa (sea level atmospheric pressure is about 0.1 MPa) is sufficient to kill vegetative cells by protein denaturation, but endospores may survive these pressures.[4][5]

In clinical settings, hyperbaric oxygen therapy is sometimes used to treat infections. In this form of therapy, a patient breathes pure oxygen at a pressure higher than normal atmospheric pressure, typically between 1 and 3 atmospheres (atm). This is achieved by placing the patient in a hyperbaric chamber or by supplying the pressurized oxygen through a breathing tube. Hyperbaric oxygen therapy helps increase oxygen saturation in tissues that become hypoxic due to infection and inflammation. This increased oxygen concentration enhances the body’s immune response by increasing the activities of neutrophils and macrophages, white blood cells that fight infections. Increased oxygen levels also contribute to the formation of toxic free radicals that inhibit the growth of oxygen-sensitive or anaerobic bacteria like as *Clostridium perfringens*, a common cause of gas gangrene. In *C. perfringens* infections, hyperbaric oxygen therapy can also reduce secretion of a bacterial toxin that causes tissue destruction. Hyperbaric oxygen therapy also seems to enhance the effectiveness of antibiotic treatments. Unfortunately, some rare risks include oxygen toxicity and effects on delicate tissues, such as the eyes, middle ear, and lungs, which may be damaged by the increased air pressure.

High pressure processing is not commonly used for disinfection or sterilization of fomites. Although the application of pressure and steam in an autoclave is effective for killing endospores, it is the high temperature achieved, and not the pressure directly, that results in endospore death.

A Streak of Bad Potluck

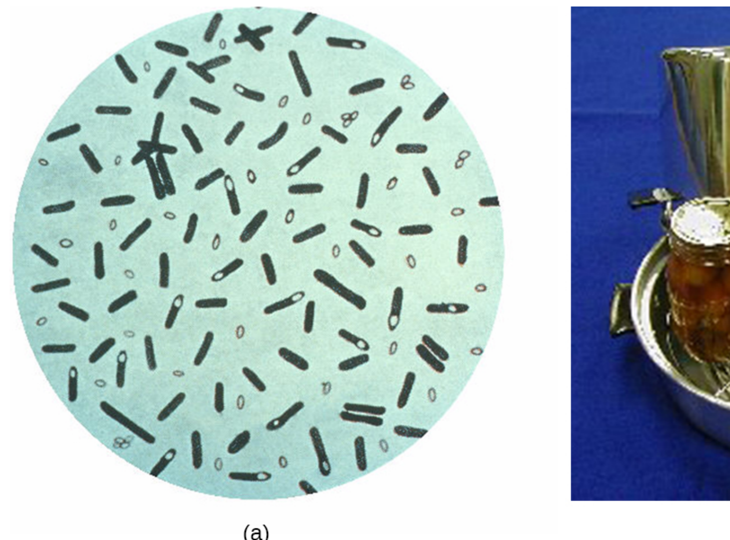
One Monday in spring 2015, an Ohio woman began to experience blurred, double vision; difficulty swallowing; and drooping eyelids. She was rushed to the emergency department of her local hospital. During the examination, she began to experience abdominal cramping, nausea, paralysis, dry mouth, weakness of facial muscles, and difficulty speaking and breathing. Based on these symptoms, the hospital’s incident command center was activated, and Ohio public health officials were notified of a possible case

4. C. Ferstl. “High Pressure Processing: Insights on Technology and Regulatory Requirements.” Food for Thought/White Paper. Series Volume 10. Livermore, CA: The National Food Lab; July 2013.
5. US Food and Drug Administration. “Kinetics of Microbial Inactivation for Alternative Food Processing Technologies: High Pressure Processing.” 2000. http://www.fda.gov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm101456.htm. Accessed July 19, 2106.

of botulism. Meanwhile, other patients with similar symptoms began showing up at other local hospitals. Because of the suspicion of botulism, antitoxin was shipped overnight from the CDC to these medical facilities, to be administered to the affected patients. The first patient died of respiratory failure as a result of paralysis, and about half of the remaining victims required additional hospitalization following antitoxin administration, with at least two requiring ventilators for breathing.

Public health officials investigated each of the cases and determined that all of the patients had attended the same church potluck the day before. Moreover, they traced the source of the outbreak to a potato salad made with home-canned potatoes. More than likely, the potatoes were canned using boiling water, a method that allows endospores of *Clostridium botulinum* to survive. *C. botulinum* produces botulinum toxin, a neurotoxin that is often deadly once ingested. According to the CDC, the Ohio case was the largest botulism outbreak in the United States in nearly 40 years.[6]

Killing *C. botulinum* endospores requires a minimum temperature of 116 °C (240 °F), well above the boiling point of water. This temperature can only be reached in a pressure canner, which is recommended for home canning of low-acid foods such as meat, fish, poultry, and vegetables (**Figure 11.11**). Additionally, the CDC recommends boiling home-canned foods for about 10 minutes before consumption. Since the botulinum toxin is heat labile (meaning that it is denatured by heat), 10 minutes of boiling will render nonfunctional any botulinum toxin that the food may contain.



(a)

(b)

Figure 11.11 (a) *Clostridium botulinum* is the causative agent of botulism. (b) A pressure canner is recommended for home canning because endospores of *C. botulinum* can survive temperatures above the boiling point of water. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by National Center for Home Food Preservation)

To **learn more (https://openstax.org/l/22cdccanathome)** about proper home-canning techniques, visit the CDC's website.

Desiccation

Drying, also known as **desiccation** or dehydration, is a method that has been used for millennia to preserve foods such as raisins, prunes, and jerky. It works because all cells, including microbes, require water for their metabolism and survival. Although drying controls microbial growth, it might not kill all microbes or their endospores, which may start to regrow when conditions are more favorable and water content is restored.

In some cases, foods are dried in the sun, relying on evaporation to achieve desiccation. Freeze-drying, or **lyophilization**, is another method of dessication in which an item is rapidly frozen ("snap-frozen") and placed under vacuum so that water is lost by sublimation. Lyophilization combines both exposure to cold

6. CL McCarty et al. "Large Outbreak of Botulism Associated with a Church Potluck Meal-Ohio, 2015." *Morbidity and Mortality Weekly Report* 64, no. 29 (2015):802–803.

temperatures and desiccation, making it quite effective for controlling microbial growth. In addition, lyophilization causes less damage to an item than conventional desiccation and better preserves the item's original qualities. Lyophilized items may be stored at room temperature if packaged appropriately to prevent moisture acquisition. Lyophilization is used for preservation in the food industry and is also used in the laboratory for the long-term storage and transportation of microbial cultures.

The water content of foods and materials, called the **water activity**, can be lowered without physical drying by the addition of solutes such as salts or sugars. At very high concentrations of salts or sugars, the amount of available water in microbial cells is reduced dramatically because water will be drawn from an area of low solute concentration (inside the cell) to an area of high solute concentration (outside the cell) (Figure 11.12). Many microorganisms do not survive these conditions of high osmotic pressure. Honey, for example, is 80% sucrose, an environment in which very few microorganisms are capable of growing, thereby eliminating the need for refrigeration. Salted meats and fish, like ham and cod, respectively, were critically important foods before the age of refrigeration. Fruits were preserved by adding sugar, making jams and jellies. However, certain microbes, such as molds and yeasts, tend to be more tolerant of desiccation and high osmotic pressures, and, thus, may still contaminate these types of foods.

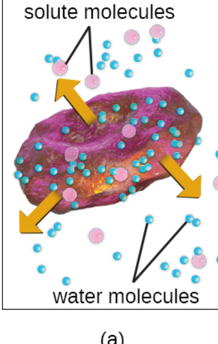


Figure 11.12 (a) The addition of a solute creates a hypertonic environment, drawing water out of cells. (b) Some foods can be dried directly, like raisins and jerky. Other foods are dried with the addition of salt, as in the case of salted fish, or sugar, as in the case of jam. (credit a: modification of work by "Bruce Blaus"/Wikimedia Commons; credit raisins: modification of work by Christian Schnettelker; credit jerky: modification of work by Larry Jacobsen; credit salted fish: modification of work by "The Photographer"/Wikimedia Commons; credit jam: modification of work by Kim Becker)

- How does the addition of salt or sugar to food affect its water activity?

Radiation

Radiation in various forms, from high-energy radiation to sunlight, can be used to kill microbes or inhibit their growth. **Ionizing radiation** includes X-rays, gamma rays, and high-energy electron beams. Ionizing radiation is strong enough to pass into the cell, where it alters molecular structures and damages cell components. For example, ionizing radiation introduces double-strand breaks in DNA molecules. This may directly cause DNA mutations to occur, or mutations may be introduced when the cell attempts to repair the DNA damage. As these mutations accumulate, they eventually lead to cell death.

Both X-rays and gamma rays easily penetrate paper and plastic and can therefore be used to sterilize many packaged materials. In the laboratory, ionizing radiation is commonly used to sterilize materials that cannot be autoclaved, such as plastic Petri dishes and disposable plastic inoculating loops. For clinical use, ionizing radiation is used to sterilize gloves, intravenous tubing, and other latex and plastic items used for patient care. Ionizing radiation is also used for the sterilization of other types of delicate, heat-sensitive materials used clinically, including tissues for transplantation, pharmaceutical drugs, and medical equipment.

In Europe, gamma irradiation for food preservation is widely used, although it has been slow to catch on in the United States (see the **Micro Connections** box on this topic). Packaged dried spices are also often gamma-irradiated. Because of their ability to penetrate paper, plastic, thin sheets of wood and metal, and tissue, great care must be taken when using X-rays and gamma irradiation. These types of ionizing irradiation cannot penetrate thick layers of iron or lead, so these metals are commonly used to protect humans who may be potentially exposed.

Another type of radiation, **nonionizing radiation**, is commonly used for disinfection and uses less energy than

ionizing radiation. It does not penetrate cells or packaging. Ultraviolet (UV) light is one example; it causes thymine dimers to form between adjacent thymines within a single strand of DNA (**Figure 11.13**). When DNA polymerase encounters the thymine dimer, it does not always incorporate the appropriate complementary nucleotides (two adenines), and this leads to formation of mutations that can ultimately kill microorganisms.

UV light can be used effectively by both consumers and laboratory personnel to control microbial growth. UV lamps are now commonly incorporated into water purification systems for use in homes. In addition, small portable UV lights are commonly used by campers to purify water from natural environments before drinking. Germicidal lamps are also used in surgical suites, biological safety cabinets, and transfer hoods, typically emitting UV light at a wavelength of 260 nm. Because UV light does not penetrate surfaces and will not pass through plastics or glass, cells must be exposed directly to the light source.

Sunlight has a very broad spectrum that includes UV and visible light. In some cases, sunlight can be effective against certain bacteria because of both the formation of thymine dimers by UV light and by the production of reactive oxygen products induced in low amounts by exposure to visible light.

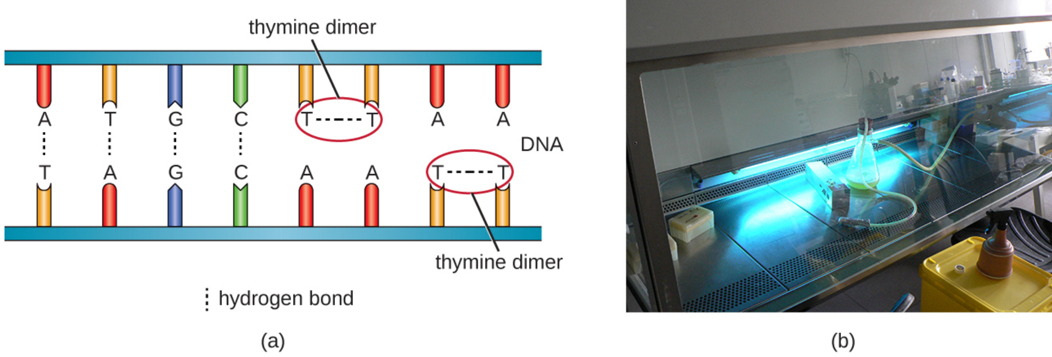


Figure 11.13 (a) UV radiation causes the formation of thymine dimers in DNA, leading to lethal mutations in the exposed microbes. (b) Germicidal lamps that emit UV light are commonly used in the laboratory to disinfect equipment.

- What are two advantages of ionizing radiation as a sterilization method?
- How does the effectiveness of ionizing radiation compare with that of nonionizing radiation?

Irradiated Food: Would You Eat That?

Of all the ways to prevent food spoilage and foodborne illness, gamma irradiation may be the most unappetizing. Although gamma irradiation is a proven method of eliminating potentially harmful microbes from food, the public has yet to buy in. Most of their concerns, however, stem from misinformation and a poor understanding of the basic principles of radiation.

The most common method of irradiation is to expose food to cobalt-60 or cesium-137 by passing it through a radiation chamber on a conveyor belt. The food does not directly contact the radioactive material and does not become radioactive itself. Thus, there is no risk for exposure to radioactive material through eating gamma-irradiated foods. Additionally, irradiated foods are not significantly altered in terms of nutritional quality, aside from the loss of certain vitamins, which is also exacerbated by extended storage. Alterations in taste or smell may occur in irradiated foods with high fat content, such as fatty meats and dairy products, but this effect can be minimized by using lower doses of radiation at colder temperatures.

In the United States, the CDC, Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA) have deemed irradiation safe and effective for various types of meats, poultry, shellfish, fresh fruits and vegetables, eggs with shells, and spices and seasonings. Gamma irradiation of foods has also been approved for use in many other countries, including France, the Netherlands, Portugal, Israel, Russia, China, Thailand, Belgium, Australia, and South Africa. To help ameliorate consumer concern and assist with education efforts, irradiated foods are now clearly labeled and marked with the international irradiation symbol, called the “radura” (**Figure 11.14**). Consumer acceptance seems to be rising, as indicated by several recent studies.[7]

7. AM Johnson et al. “Consumer Acceptance of Electron-Beam Irradiated Ready-to-Eat Poultry Meats.” *Food Processing Preservation*, 28 no. 4 (2004):302–319.

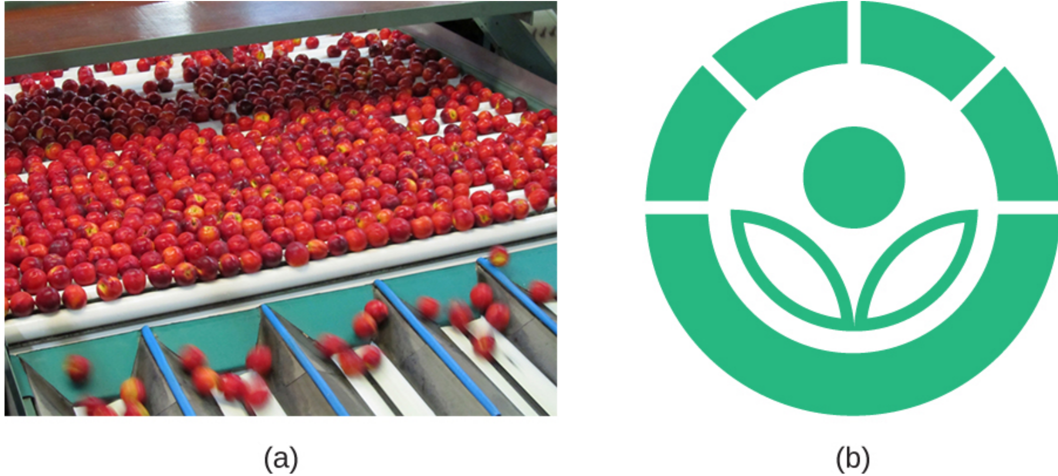


Figure 11.14 (a) Foods are exposed to gamma radiation by passage on a conveyor belt through a radiation chamber. (b) Gamma-irradiated foods must be clearly labeled and display the irradiation symbol, known as the "radura." (credit a, b: modification of work by U.S. Department of Agriculture)

Sonication

The use of high-frequency ultrasound waves to disrupt cell structures is called **sonication**. Application of ultrasound waves causes rapid changes in pressure within the intracellular liquid; this leads to cavitation, the formation of bubbles inside the cell, which can disrupt cell structures and eventually cause the cell to lyse or collapse. Sonication is useful in the laboratory for efficiently lysing cells to release their contents for further research; outside the laboratory, sonication is used for cleaning surgical instruments, lenses, and a variety of other objects such as coins, tools, and musical instruments.

Filtration

Filtration is a method of physically separating microbes from samples. Air is commonly filtered through **high-efficiency particulate air (HEPA) filter**s (Figure 11.15). HEPA filters have effective pore sizes of 0.3 µm, small enough to capture bacterial cells, endospores, and many viruses, as air passes through these filters, nearly sterilizing the air on the other side of the filter. HEPA filters have a variety of applications and are used widely in clinical settings, in cars and airplanes, and even in the home. For example, they may be found in vacuum cleaners, heating and air-conditioning systems, and air purifiers.

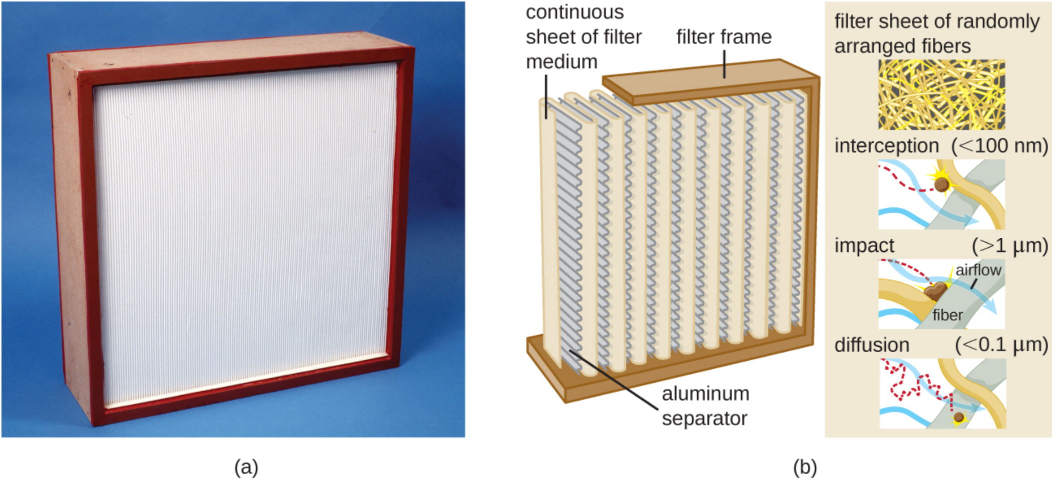


Figure 11.15 (a) HEPA filters like this one remove microbes, endospores, and viruses as air flows through them. (b) A schematic of a HEPA filter. (credit a: modification of work by CSIRO; credit b: modification of work by "LadyofHats"/Mariana Ruiz Villareal)

Biological Safety Cabinets

Biological safety cabinets are a good example of the use of HEPA filters. HEPA filters in biological safety cabinets (BSCs) are used to remove particulates in the air either entering the cabinet (air intake), leaving the cabinet (air exhaust), or treating both the intake and exhaust. Use of an air-intake HEPA filter prevents environmental contaminants from entering the BSC, creating a clean area for handling biological materials. Use of an air-exhaust HEPA filter prevents laboratory pathogens from contaminating the laboratory, thus maintaining a safe work area for laboratory personnel.

There are three classes of BSCs: I, II, and III. Each class is designed to provide a different level of protection for laboratory personnel and the environment; BSC II and III are also designed to protect the materials or devices in the cabinet. Table 11.1 summarizes the level of safety provided by each class of BSC for each BSL.

Biological Risks and BSCs

Biological Risk Assessed	BSC Class	Protection of Personnel	Protection of Environment	Protection of Product
BSL-1, BSL-2, BSL-3	I	Yes	Yes	No
BSL-1, BSL-2, BSL-3	II	Yes	Yes	Yes
BSL-4	III; II when used in suit room with suit	Yes	Yes	Yes

Table 11.1

Class I BSCs protect laboratory workers and the environment from a low to moderate risk for exposure to biological agents used in the laboratory. Air is drawn into the cabinet and then filtered before exiting through the building's exhaust system. Class II BSCs use directional air flow and partial barrier systems to contain infectious agents. Class III BSCs are designed for working with highly infectious agents like those used in BSL-4 laboratories. They are gas tight, and materials entering or exiting the cabinet must be passed through a double-door system, allowing the intervening space to be decontaminated between uses. All air is passed through one or two HEPA filters and an air incineration system before being exhausted directly to the outdoors (not through the building's exhaust system). Personnel can manipulate materials inside the Class III cabinet by using long rubber gloves sealed to the cabinet.

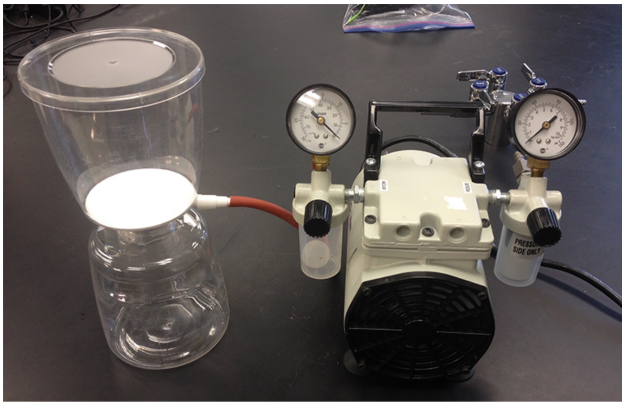
This **video (https://openstax.org/l/22BSCsdesvideo)** shows how BSCs are designed and explains how they protect personnel, the environment, and the product.

Filtration in Hospitals

HEPA filters are also commonly used in hospitals and surgical suites to prevent contamination and the spread of airborne microbes through ventilation systems. HEPA filtration systems may be designed for entire buildings or for individual rooms. For example, burn units, operating rooms, or isolation units may require special HEPA-filtration systems to remove opportunistic pathogens from the environment because patients in these rooms are particularly vulnerable to infection.

Membrane Filters

Filtration can also be used to remove microbes from liquid samples using **membrane filtration**. Membrane filters for liquids function similarly to HEPA filters for air. Typically, membrane filters that are used to remove bacteria have an effective pore size of 0.2 µm, smaller than the average size of a bacterium (1 µm), but filters with smaller pore sizes are available for more specific needs. Membrane filtration is useful for removing bacteria from various types of heat-sensitive solutions used in the laboratory, such as antibiotic solutions and vitamin solutions. Large volumes of culture media may also be filter sterilized rather than autoclaved to protect heat-sensitive components. Often when filtering small volumes, syringe filters are used, but vacuum filters are typically used for filtering larger volumes (Figure 11.16).



(a) (b)

Figure 11.16 Membrane filters come in a variety of sizes, depending on the volume of solution being filtered. (a) Larger volumes are filtered in units like these. The solution is drawn through the filter by connecting the unit to a vacuum. (b) Smaller volumes are often filtered using syringe filters, which are units that fit on the end of a syringe. In this case, the solution is pushed through by depressing the syringe's plunger. (credit a, b: modification of work by Brian Forster)

- Would membrane filtration with a 0.2-µm filter likely remove viruses from a solution? Explain.
- Name at least two common uses of HEPA filtration in clinical or laboratory settings.

Figure 11.17 and **Figure 11.18** summarize the physical methods of control discussed in this section.

Physical Methods of Control			
Method	**Conditions**	**Mode of Action**	**Example Uses**
Heat			
Boiling	100 °C at sea level	Denatures proteins and alters membranes	Cooking, personal use, preparing certain laboratory media
Dry-heat oven	170 °C for 2 hours	Denatures proteins and alters membranes, dehydration, desiccation	Sterilization of heat-stable medical and laboratory equipment and glassware
Incineration	Exposure to flame	Destroy by burning	Flaming loop, microincinerator
Autoclave	Typical settings: 121 °C for 15 minutes at 15 pounds per square inch (psi)	Denatures proteins and alters membranes	Sterilization of microbiological media, heat-stable medical and laboratory equipment, and other heat-stable items
Pasteurization	Can vary. One type is 72 °C for 15 seconds (HTST)	Denatures proteins and alters membranes	Prevents spoilage of milk, apple juice, honey, and other ingestible liquids
Cold			
Refrigeration	0 °C to 7 °C	Inhibits metabolism (slows or arrests cell division)	Preservation of food or laboratory materials (solutions, cultures)
Freezing	Below −2 °C	Stops metabolism, may kill microbes	Long-term storage of food, laboratory cultures, or medical specimens
Pressure			
High-pressure processing	100–800 MPa	Denatures proteins and can cause cell lysis	Preservation of food
Hyberbaric oxygen therapy	Air pressure three times higher than normal	Inhibits metabolism and growth of anaerobic microbes	Treatment of certain infections (e.g., gas gangrene)
Desiccation			
Simple desiccation	Drying	Inhibits metabolism	Dried fruits, jerky
Reduce water activity	Addition of salt or water	Inhibits metabolism and can cause lysis	Salted meats and fish, honey, jams and jellies
Lyophilization	Rapid freezing under vacuum	Inhibits metabolism	Preservation of food, laboratory cultures, or reagents
Radiation			
Ionizing radiation	Exposure to X-rays or gamma rays	Alters molecular structures, introduces double-strand breaks into DNA	Sterilization of spices and heat-sensitive laboratory and medical items; used for food sterilization in Europe but not widely accepted in US
Nonionizing radiation	Exposure to ultraviolet light	Introduces thymine dimers, leading to mutations	Disinfection of surfaces in laboratories and rooms in health-care environment, and disinfection of water and air

Figure 11.17

Physical Methods of Control (continued)			
Method	**Conditions**	**Mode of Action**	**Example Uses**
Sonication			
Sonication	Exposure to ultrasonic waves	Cavitation (formation of empty space) disrupts cells, lysing them	Laboratory research to lyse cells; cleaning jewelry, lenses, and equipment
Filtration			
HEPA filtration	Use of high-efficiency particulate air (HEPA) filter with 0.3 μm pore size	Physically removes microbes from air	Laboratory biological safety cabinets, operating rooms, isolation units, heating and air conditioning systems, vacuum cleaners
Membrane filtration	Use of membrane filter with 0.2-μm or smaller pore size	Physically removes microbes from liquid solutions	Removal of bacteria from heat-sensitive solutions like vitamins, antibiotics, and media with heat-sensitive components

Figure 11.18

11.3 | Using Chemicals to Control Microorganisms

Learning Objectives

By the end of this section, you will be able to:

- Understand and compare various chemicals used to control microbial growth, including their uses, advantages and disadvantages, chemical structure, and mode of action

In addition to physical methods of microbial control, chemicals are also used to control microbial growth. A wide variety of chemicals can be used as disinfectants or antiseptics. When choosing which to use, it is important to consider the type of microbe targeted; how clean the item needs to be; the disinfectant's effect on the item's integrity; its safety to animals, humans, and the environment; its expense; and its ease of use. This section describes the variety of chemicals used as disinfectants and antiseptics, including their mechanisms of action and common uses.

Phenolics

In the 1800s, scientists began experimenting with a variety of chemicals for disinfection. In the 1860s, British surgeon Joseph Lister (1827–1912) began using carbolic acid, known as phenol, as a disinfectant for the treatment of surgical wounds (see **Foundations of Modern Cell Theory (https://legacy.cnx.org/content/m58791/latest/)**). In 1879, Lister's work inspired the American chemist Joseph Lawrence (1836–1909) to develop Listerine, an alcohol-based mixture of several related compounds that is still used today as an oral antiseptic. Today, carbolic acid is no longer used as a surgical disinfectant because it is a skin irritant, but the chemical compounds found in antiseptic mouthwashes and throat lozenges are called **phenolics**.

Chemically, phenol consists of a benzene ring with an –OH group, and phenolics are compounds that have this group as part of their chemical structure (**Figure 11.19**). Phenolics such as thymol and eucalyptol occur naturally in plants. Other phenolics can be derived from creosote, a component of coal tar. Phenolics tend to be stable, persistent on surfaces, and less toxic than phenol. They inhibit microbial growth by denaturing proteins and disrupting membranes.

OH
OH
HO OH
Cl Cl
Cl Cl
Cl Cl
(a) (b) (c)

Figure 11.19 Phenol and phenolic compounds have been used to control microbial growth. (a) Chemical structure of phenol, also known as carbolic acid. (b) o-Phenylphenol, a type of phenolic, has been used as a disinfectant as well as to control bacterial and fungal growth on harvested citrus fruits. (c) Hexachlorophene, another phenol, known as a bisphenol (two rings), is the active ingredient in pHisoHex.

Since Lister's time, several phenolic compounds have been used to control microbial growth. Phenolics like cresols (methylated phenols) and o-phenylphenol were active ingredients in various formulations of Lysol since its invention in 1889. o-Phenylphenol was also commonly used in agriculture to control bacterial and fungal growth on harvested crops, especially citrus fruits, but its use in the United States is now far more limited. The bisphenol hexachlorophene, a disinfectant, is the active ingredient in pHisoHex, a topical cleansing detergent widely used for handwashing in hospital settings. pHisoHex is particularly effective against gram-positive bacteria, including those causing staphylococcal and streptococcal skin infections. pHisoHex was formerly used for bathing infants, but this practice has been discontinued because it has been shown that exposure to hexachlorophene can lead to neurological problems.

Triclosan is another bisphenol compound that has seen widespread application in antibacterial products over the last several decades. Initially used in toothpastes, triclosan has also been used in hand soaps and impregnated into a wide variety of other products, including cutting boards, knives, shower curtains, clothing, and concrete, to make them antimicrobial. However, in 2016 the FDA banned the marketing of over-the-counter antiseptic products containing triclosan and 18 other chemicals. This ruling was based on the lack of evidence of safety or efficacy, as well as concerns about the health risks of long-term exposure (See Micro Connections below). In 2019 the FDA issued an updated ban ruling to included 28 chemicals. Rulings on benzalkonium chloride, ethyl alcohol, and isopropyl alcohol have been deferred to allow for the submission of additional safety and efficacy data.[8]

Triclosan: Antibacterial Overkill?

Hand soaps and other cleaning products are often marketed as "antibacterial," suggesting that they provide a level of cleanliness superior to that of conventional soaps and cleansers. But are the antibacterial ingredients in these products really safe and effective?

About 75% of antibacterial liquid hand soaps and 30% of bar soaps contain the chemical triclosan, a phenolic, (**Figure 11.20**).[9] Triclosan blocks an enzyme in the bacterial fatty acid-biosynthesis pathway that is not found in the comparable human pathway. Although the use of triclosan in the home increased dramatically during the 1990s, more than 40 years of research by the FDA have turned up no conclusive evidence that washing with triclosan-containing products provides increased health benefits compared with washing with traditional soap. Although some studies indicate that fewer bacteria may remain on a person's hands after washing with triclosan-based soap, compared with traditional soap, no evidence points to any reduction in the transmission of bacteria that cause respiratory and gastrointestinal illness. In short, soaps with triclosan may remove or kill a few more germs but not enough to reduce the spread of disease.

Perhaps more disturbing, some clear risks associated with triclosan-based soaps have come to light. The widespread use of triclosan has led to an increase in triclosan-resistant bacterial strains, including those of clinical importance, such as *Salmonella enterica*; this resistance may render triclosan useless as an antibacterial in the long run.[10][11] Bacteria can easily gain resistance to triclosan through a change to a

8. US Food and Drug Administration. "FDA Issues Final Rule on Safety and Effectiveness of Antibacterial Soaps." 2016. https://www.fda.gov/news-events/press-announcements/fda-issues-final-rule-safety-and-effectiveness-antibacterial-soaps. Accessed October 29, 2020.
9. J. Stromberg. "Five Reasons Why You Should Probably Stop Using Antibacterial Soap." *Smithsonian.com* January 3, 2014. http://www.smithsonianmag.com/science-nature/five-reasons-why-you-should-probably-stop-using-antibacterial-soap-180948078/?no-ist. Accessed June 9, 2016.

single gene encoding the targeted enzyme in the bacterial fatty acid-synthesis pathway. Other disinfectants with a less specific mode of action are much less prone to engendering resistance because it would take much more than a single genetic change.

Use of triclosan over the last several decades has also led to a buildup of the chemical in the environment. Triclosan in hand soap is directly introduced into wastewater and sewage systems as a result of the handwashing process. There, its antibacterial properties can inhibit or kill bacteria responsible for the decomposition of sewage, causing septic systems to clog and back up. Eventually, triclosan in wastewater finds its way into surface waters, streams, lakes, sediments, and soils, disrupting natural populations of bacteria that carry out important environmental functions, such as inhibiting algae. Triclosan also finds its way into the bodies of amphibians and fish, where it can act as an endocrine disruptor. Detectable levels of triclosan have also been found in various human bodily fluids, including breast milk, plasma, and urine.[12] In fact, a study conducted by the CDC found detectable levels of triclosan in the urine of 75% of 2,517 people tested in 2003–2004.[13] This finding is even more troubling given the evidence that triclosan may affect immune function in humans.[14]

In December 2013, the FDA gave soap manufacturers until 2016 to prove that antibacterial soaps provide a significant benefit over traditional soaps; if unable to do so, manufacturers will be forced to remove these products from the market.

(a) (b) (c)

Figure 11.20 Triclosan is a common ingredient in antibacterial soaps despite evidence that it poses environmental and health risks and offers no significant health benefit compared to conventional soaps. (credit b: modification of work by FDA; c: Michelle Gigante/US Air Force; Public Domain.)

- Why is triclosan more like an antibiotic than a traditional disinfectant?

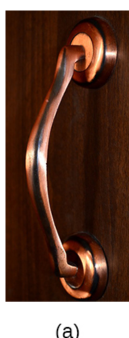
Heavy Metals

Some of the first chemical disinfectants and antiseptics to be used were heavy metals. Heavy metals kill microbes by binding to proteins, thus inhibiting enzymatic activity (**Figure 11.21**). Heavy metals are oligodynamic, meaning that very small concentrations show significant antimicrobial activity. Ions of heavy metals bind to sulfur-containing amino acids strongly and bioaccumulate within cells, allowing these metals to

10. SP Yazdankhah et al. “Triclosan and Antimicrobial Resistance in Bacteria: An Overview.” *Microbial Drug Resistance* 12 no. 2 (2006):83–90.
11. L. Birošová, M. Mikulášová. “Development of Triclosan and Antibiotic Resistance in *Salmonella enterica* serovar Typhimurium.” *Journal of Medical Microbiology* 58 no. 4 (2009):436–441.
12. AB Dann, A. Hontela. “Triclosan: Environmental Exposure, Toxicity and Mechanisms of Action.” *Journal of Applied Toxicology* 31 no. 4 (2011):285–311.
13. US Centers for Disease Control and Prevention. “Triclosan Fact Sheet.” 2013. http://www.cdc.gov/biomonitoring/Triclosan_FactSheet.html. Accessed June 9, 2016.
14. EM Clayton et al. “The Impact of Bisphenol A and Triclosan on Immune Parameters in the US Population, NHANES 2003-2006.” *Environmental Health Perspectives* 119 no. 3 (2011):390.

reach high localized concentrations. This causes proteins to denature.

Heavy metals are not selectively toxic to microbial cells. They may bioaccumulate in human or animal cells, as well, and excessive concentrations can have toxic effects on humans. If too much silver accumulates in the body, for example, it can result in a condition called argyria, in which the skin turns irreversibly blue-gray. One way to reduce the potential toxicity of heavy metals is by carefully controlling the duration of exposure and concentration of the heavy metal.

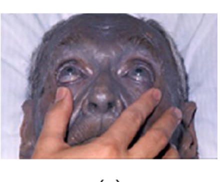


(a)

(b)

(c)

(d)



(e)

Figure 11.21 Heavy metals denature proteins, impairing cell function and, thus, giving them strong antimicrobial properties. (a) Copper in fixtures like this door handle kills microbes that otherwise might accumulate on frequently touched surfaces. (b) Eating utensils contain small amounts of silver to inhibit microbial growth. (c) Copper commonly lines incubators to minimize contamination of cell cultures stored inside. (d) Antiseptic mouthwashes commonly contain zinc chloride. (e) This patient is suffering from argyria, an irreversible condition caused by bioaccumulation of silver in the body. (credit b: modification of work by "Shoshanah"/Flickr; credit e: modification of work by Herbert L. Fred and Hendrik A. van Dijk)

Mercury

Mercury is an example of a heavy metal that has been used for many years to control microbial growth. It was used for many centuries to treat syphilis. Mercury compounds like mercuric chloride are mainly bacteriostatic and have a very broad spectrum of activity. Various forms of mercury bind to sulfur-containing amino acids within proteins, inhibiting their functions.

In recent decades, the use of such compounds has diminished because of mercury's toxicity. It is toxic to the central nervous, digestive, and renal systems at high concentrations, and has negative environmental effects, including bioaccumulation in fish. Topical antiseptics such as mercurochrome, which contains mercury in low concentrations, and merthiolate, a **tincture** (a solution of mercury dissolved in alcohol) were once commonly used. However, because of concerns about using mercury compounds, these antiseptics are no longer sold in the United States.

Silver

Silver has long been used as an antiseptic. In ancient times, drinking water was stored in silver jugs.[15] Silvadene cream is commonly used to treat topical wounds and is particularly helpful in preventing infection in burn wounds. Silver nitrate drops were once routinely applied to the eyes of newborns to protect against ophthalmia neonatorum, eye infections that can occur due to exposure to pathogens in the birth canal, but antibiotic creams are more now commonly used. Silver is often combined with antibiotics, making the antibiotics thousands of times more effective.[16] Silver is also commonly incorporated into catheters and bandages, rendering them antimicrobial; however, there is evidence that heavy metals may also enhance selection for antibiotic resistance.[17]

Copper, Nickel, and Zinc

Several other heavy metals also exhibit antimicrobial activity. Copper sulfate is a common algicide used to control algal growth in swimming pools and fish tanks. The use of metallic copper to minimize microbial growth is also becoming more widespread. Copper linings in incubators help reduce contamination of cell cultures. The use of copper pots for water storage in underdeveloped countries is being investigated as a way to combat diarrheal diseases. Copper coatings are also becoming popular for frequently handled objects such as

15. N. Silvestry-Rodriguez et al. "Silver as a Disinfectant." In *Reviews of Environmental Contamination and Toxicology*, pp. 23-45. Edited by GW Ware and DM Whitacre. New York: Springer, 2007.
16. B. Owens. "Silver Makes Antibiotics Thousands of Times More Effective." *Nature* June 19 2013. http://www.nature.com/news/silver-makes-antibiotics-thousands-of-times-more-effective-1.13232
17. C. Seiler, TU Berendonk. "Heavy Metal Driven Co-Selection of Antibiotic Resistance in Soil and Water Bodies Impacted by Agriculture and Aquaculture." *Frontiers in Microbiology* 3 (2012):399.

doorknobs, cabinet hardware, and other fixtures in health-care facilities in an attempt to reduce the spread of microbes.

Nickel and zinc coatings are now being used in a similar way. Other forms of zinc, including zinc chloride and zinc oxide, are also used commercially. Zinc chloride is quite safe for humans and is commonly found in mouthwashes, substantially increasing their length of effectiveness. Zinc oxide is found in a variety of products, including topical antiseptic creams such as calamine lotion, diaper ointments, baby powder, and dandruff shampoos.

- Why are many heavy metals both antimicrobial and toxic to humans?

Halogens

Other chemicals commonly used for disinfection are the halogens iodine, chlorine, and fluorine. Iodine works by oxidizing cellular components, including sulfur-containing amino acids, nucleotides, and fatty acids, and destabilizing the macromolecules that contain these molecules. It is often used as a topical tincture, but it may cause staining or skin irritation. An **iodophor** is a compound of iodine complexed with an organic molecule, thereby increasing iodine's stability and, in turn, its efficacy. One common iodophor is povidone-iodine, which includes a wetting agent that releases iodine relatively slowly. Betadine is a brand of povidone-iodine commonly used as a hand scrub by medical personnel before surgery and for topical antisepsis of a patient's skin before incision (**Figure 11.22**).

Figure 11.22 (a) Betadine is a solution of the iodophor povidone-iodine. (b) It is commonly used as a topical antiseptic on a patient's skin before incision during surgery. (credit b: modification of work by Andrew Ratto)

Chlorine is another halogen commonly used for disinfection. When chlorine gas is mixed with water, it produces a strong oxidant called hypochlorous acid, which is uncharged and enters cells easily. Chlorine gas is commonly used in municipal drinking water and wastewater treatment plants, with the resulting hypochlorous acid producing the actual antimicrobial effect. Those working at water treatment facilities need to take great care to minimize personal exposure to chlorine gas. Sodium hypochlorite is the chemical component of common household bleach, and it is also used for a wide variety of disinfecting purposes. Hypochlorite salts, including sodium and calcium hypochlorites, are used to disinfect swimming pools. Chlorine gas, sodium hypochlorite, and calcium hypochlorite are also commonly used disinfectants in the food processing and restaurant industries to reduce the spread of foodborne diseases. Workers in these industries also need to take care to use these products correctly to ensure their own safety as well as the safety of consumers. A recent joint statement published by the Food and Agriculture Organization (FAO) of the United Nations and WHO indicated that none of the many beneficial uses of chlorine products in food processing to reduce the spread of foodborne illness posed risks to consumers.[18]

Another class of chlorinated compounds called chloramines are widely used as disinfectants. Chloramines are relatively stable, releasing chlorine over long periods time. Chloramines are derivatives of ammonia by

18. World Health Organization. "Benefits and Risks of the Use of Chlorine-Containing Disinfectants in Food Production and Food Processing: Report of a Joint FAO/WHO Expert Meeting." Geneva, Switzerland: World Health Organization, 2009.

substitution of one, two, or all three hydrogen atoms with chlorine atoms (Figure 11.23).

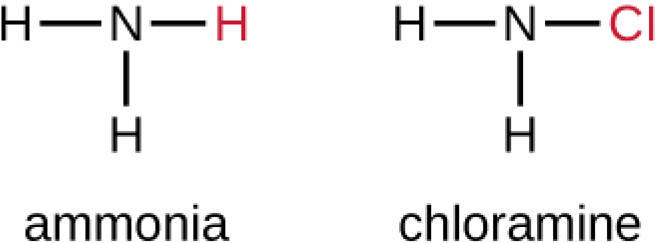


Figure 11.23 Monochloroamine, one of the chloramines, is derived from ammonia by the replacement of one hydrogen atom with a chlorine atom.

Chloramines and other cholorine compounds may be used for disinfection of drinking water, and chloramine tablets are frequently used by the military for this purpose. After a natural disaster or other event that compromises the public water supply, the CDC recommends disinfecting tap water by adding small amounts of regular household bleach. Recent research suggests that sodium dichloroisocyanurate (NaDCC) may also be a good alternative for drinking water disinfection. Currently, NaDCC tablets are available for general use and for use by the military, campers, or those with emergency needs; for these uses, NaDCC is preferable to chloramine tablets. Chlorine dioxide, a gaseous agent used for fumigation and sterilization of enclosed areas, is also commonly used for the disinfection of water.

Although chlorinated compounds are relatively effective disinfectants, they have their disadvantages. Some may irritate the skin, nose, or eyes of some individuals, and they may not completely eliminate certain hardy organisms from contaminated drinking water. The protozoan parasite *Cryptosporidium*, for example, has a protective outer shell that makes it resistant to chlorinated disinfectants. Thus, boiling of drinking water in emergency situations is recommended when possible.

The halogen fluorine is also known to have antimicrobial properties that contribute to the prevention of dental caries (cavities).[19] Fluoride is the main active ingredient of toothpaste and is also commonly added to tap water to help communities maintain oral health. Chemically, fluoride can become incorporated into the hydroxyapatite of tooth enamel, making it more resistant to corrosive acids produced by the fermentation of oral microbes. Fluoride also enhances the uptake of calcium and phosphate ions in tooth enamel, promoting remineralization. In addition to strengthening enamel, fluoride also seems to be bacteriostatic. It accumulates in plaque-forming bacteria, interfering with their metabolism and reducing their production of the acids that contribute to tooth decay.

- What is a benefit of a chloramine over hypochlorite for disinfecting?

Alcohols

Alcohols make up another group of chemicals commonly used as disinfectants and antiseptics. They work by rapidly denaturing proteins, which inhibits cell metabolism, and by disrupting membranes, which leads to cell lysis. Once denatured, the proteins may potentially refold if enough water is present in the solution. Alcohols are typically used at concentrations of about 70% aqueous solution and, in fact, work better in aqueous solutions than 100% alcohol solutions. This is because alcohols coagulate proteins. In higher alcohol concentrations, rapid coagulation of surface proteins prevents effective penetration of cells. The most commonly used alcohols for disinfection are ethyl alcohol (ethanol) and isopropyl alcohol (isopropanol, rubbing alcohol) (Figure 11.24).

Alcohols tend to be bactericidal and fungicidal, but may also be viricidal for enveloped viruses only. Although alcohols are not sporicidal, they do inhibit the processes of sporulation and germination. Alcohols are volatile and dry quickly, but they may also cause skin irritation because they dehydrate the skin at the site of application. One common clinical use of alcohols is swabbing the skin for degerming before needle injection. Alcohols also are the active ingredients in instant hand sanitizers, which have gained popularity in recent years. The alcohol in these hand sanitizers works both by denaturing proteins and by disrupting the microbial cell membrane, but will not work effectively in the presence of visible dirt.

Last, alcohols are used to make tinctures with other antiseptics, such as the iodine tinctures discussed previously in this chapter. All in all, alcohols are inexpensive and quite effective for the disinfection of a broad range of

19. RE Marquis. "Antimicrobial Actions of Fluoride for Oral Bacteria." *Canadian Journal of Microbiology* 41 no. 11 (1995):955–964.

vegetative microbes. However, one disadvantage of alcohols is their high volatility, limiting their effectiveness to immediately after application.

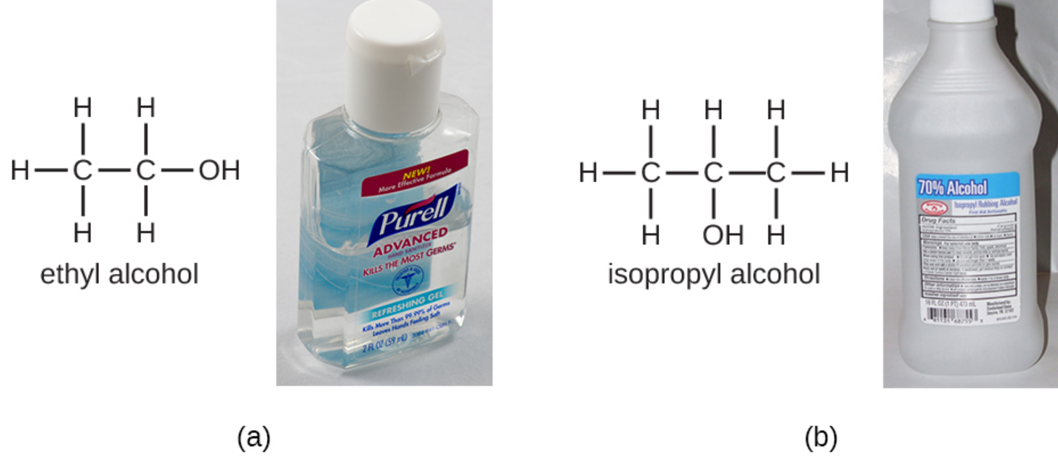


Figure 11.24 (a) Ethyl alcohol, the intoxicating ingredient found in alcoholic drinks, is also used commonly as a disinfectant. (b) Isopropyl alcohol, also called rubbing alcohol, has a related molecular structure and is another commonly used disinfectant. (credit a photo: modification of work by D Coetzee; credit b photo: modification of work by Craig Spurrier)

- Name at least three advantages of alcohols as disinfectants.
- Describe several specific applications of alcohols used in disinfectant products.

Surfactants

Surface-active agents, or **surfactants**, are a group of chemical compounds that lower the surface tension of water. Surfactants are the major ingredients in soaps and detergents. Soaps are salts of long-chain fatty acids and have both polar and nonpolar regions, allowing them to interact with polar and nonpolar regions in other molecules (**Figure 11.25**). They can interact with nonpolar oils and grease to create emulsions in water, loosening and lifting away dirt and microbes from surfaces and skin. Soaps do not kill or inhibit microbial growth and so are not considered antiseptics or disinfectants. However, proper use of soaps mechanically carries away microorganisms, effectively degerming a surface. Some soaps contain added bacteriostatic agents such as triclocarban or cloflucarban, compounds structurally related to triclosan, that introduce antiseptic or disinfectant properties to the soaps.

Na^+

$O^- - C(=O) - (CH_2)_{16} - CH_3$

hydrophilic head hydrophobic tail

Figure 11.25 Soaps are the salts (sodium salt in the illustration) of fatty acids and have the ability to emulsify lipids, fats, and oils by interacting with water through their hydrophilic heads and with the lipid at their hydrophobic tails.

Soaps, however, often form films that are difficult to rinse away, especially in hard water, which contains high concentrations of calcium and magnesium mineral salts. Detergents contain synthetic surfactant molecules with both polar and nonpolar regions that have strong cleansing activity but are more soluble, even in hard water, and, therefore, leave behind no soapy deposits. Anionic detergents, such as those used for laundry, have a negatively charged anion at one end attached to a long hydrophobic chain, whereas cationic detergents have a positively charged cation instead. Cationic detergents include an important class of disinfectants and antiseptics called the **quaternary ammonium salts (quats)**, named for the characteristic quaternary nitrogen atom that confers the positive charge (**Figure 11.26**). Overall, quats have properties similar to phospholipids, having hydrophilic and hydrophobic ends. As such, quats have the ability to insert into the bacterial phospholipid bilayer and disrupt

membrane integrity. The cationic charge of quats appears to confer their antimicrobial properties, which are diminished when neutralized. Quats have several useful properties. They are stable, nontoxic, inexpensive, colorless, odorless, and tasteless. They tend to be bactericidal by disrupting membranes. They are also active against fungi, protozoans, and enveloped viruses, but endospores are unaffected. In clinical settings, they may be used as antiseptics or to disinfect surfaces. Mixtures of quats are also commonly found in household cleaners and disinfectants, including many current formulations of Lysol brand products, which contain benzalkonium chlorides as the active ingredients. Benzalkonium chlorides, along with the quat cetylpyrimidine chloride, are also found in products such as skin antiseptics, oral rinses, and mouthwashes.

Figure 11.26 (a) Two common quats are benzylalkonium chloride and cetylpyrimidine chloride. Note the hydrophobic nonpolar carbon chain at one end and the nitrogen-containing cationic component at the other end. (b) Quats are able to infiltrate the phospholipid plasma membranes of bacterial cells and disrupt their integrity, leading to death of the cell.

- Why are soaps not considered disinfectants?

Handwashing the Right Way

Handwashing is critical for public health and should be emphasized in a clinical setting. For the general public, the CDC recommends handwashing before, during, and after food handling; before eating; before and after interacting with someone who is ill; before and after treating a wound; after using the toilet or changing diapers; after coughing, sneezing, or blowing the nose; after handling garbage; and after interacting with an animal, its feed, or its waste. **Figure 11.27** illustrates the five steps of proper handwashing recommended by the CDC.

Handwashing is even more important for health-care workers, who should wash their hands thoroughly between every patient contact, after the removal of gloves, after contact with bodily fluids and potentially infectious fomites, and before and after assisting a surgeon with invasive procedures. Even with the use of proper surgical attire, including gloves, scrubbing for surgery is more involved than routine handwashing. The goal of surgical scrubbing is to reduce the normal microbiota on the skin's surface to prevent the introduction of these microbes into a patient's surgical wounds.

There is no single widely accepted protocol for surgical scrubbing. Protocols for length of time spent scrubbing may depend on the antimicrobial used; health-care workers should always check the manufacturer's recommendations. According to the Association of Surgical Technologists (AST), surgical scrubs may be performed with or without the use of brushes (**Figure 11.27**).

(a)

(b)

Figure 11.27 (a) The CDC recommends five steps as part of typical handwashing for the general public. (b) Surgical scrubbing is more extensive, requiring scrubbing starting from the fingertips, extending to the hands and forearms, and then up beyond the elbows, as shown here. (credit a: modification of work by World Health Organization; credit b: b: Staff Sgt. Kevin Iinuma / US Air Force; Public Domain)

To **learn more (https://openstax.org/l/22CDChandwash)** about proper handwashing, visit the CDC's website.

Bisbiguanides

Bisbiguanides were first synthesized in the 20th century and are cationic (positively charged) molecules known for their antiseptic properties (**Figure 11.28**). One important **bisbiguanide** antiseptic is chlorhexidine. It has broad-spectrum activity against yeasts, gram-positive bacteria, and gram-negative bacteria, with the exception of *Pseudomonas aeruginosa*, which may develop resistance on repeated exposure.[20] Chlorhexidine disrupts cell membranes and is bacteriostatic at lower concentrations or bactericidal at higher concentrations, in which it actually causes the cells' cytoplasmic contents to congeal. It also has activity against enveloped viruses. However, chlorhexidine is poorly effective against *Mycobacterium tuberculosis* and nonenveloped viruses, and it is not sporicidal. Chlorhexidine is typically used in the clinical setting as a surgical scrub and for other handwashing needs for medical personnel, as well as for topical antisepsis for patients before surgery or needle injection. It is more persistent than iodophors, providing long-lasting antimicrobial activity. Chlorhexidine solutions may also be used as oral rinses after oral procedures or to treat gingivitis. Another bisbiguanide, alexidine, is gaining popularity as a surgical scrub and an oral rinse because it acts faster than chlorhexidine.

20. L. Thomas et al. "Development of Resistance to Chlorhexidine Diacetate in *Pseudomonas aeruginosa* and the Effect of a 'Residual' Concentration." *Journal of Hospital Infection* 46 no. 4 (2000):297–303.

chlorhexidine alexidine

Figure 11.28 The bisbiguanides chlorhexadine and alexidine are cationic antiseptic compounds commonly used as surgical scrubs.

- What two effects does chlorhexidine have on bacterial cells?

Alkylating Agents

The **alkylating agent**s are a group of strong disinfecting chemicals that act by replacing a hydrogen atom within a molecule with an alkyl group (C_nH_{2n+1}), thereby inactivating enzymes and nucleic acids (**Figure 11.29**). The alkylating agent formaldehyde (CH_2OH) is commonly used in solution at a concentration of 37% (known as formalin) or as a gaseous disinfectant and biocide. It is a strong, broad-spectrum disinfectant and biocide that has the ability to kill bacteria, viruses, fungi, and endospores, leading to sterilization at low temperatures, which is sometimes a convenient alternative to the more labor-intensive heat sterilization methods. It also cross-links proteins and has been widely used as a chemical fixative. Because of this, it is used for the storage of tissue specimens and as an embalming fluid. It also has been used to inactivate infectious agents in vaccine preparation. Formaldehyde is very irritating to living tissues and is also carcinogenic; therefore, it is not used as an antiseptic.

Glutaraldehyde is structurally similar to formaldehyde but has two reactive aldehyde groups, allowing it to act more quickly than formaldehyde. It is commonly used as a 2% solution for sterilization and is marketed under the brand name Cidex. It is used to disinfect a variety of surfaces and surgical and medical equipment. However, similar to formaldehyde, glutaraldehyde irritates the skin and is not used as an antiseptic.

A new type of disinfectant gaining popularity for the disinfection of medical equipment is o-phthalaldehyde (OPA), which is found in some newer formulations of Cidex and similar products, replacing glutaraldehyde. o-Phthalaldehyde also has two reactive aldehyde groups, but they are linked by an aromatic bridge. o-Phthalaldehyde is thought to work similarly to glutaraldehyde and formaldehyde, but is much less irritating to skin and nasal passages, produces a minimal odor, does not require processing before use, and is more effective against mycobacteria.

Ethylene oxide is a type of alkylating agent that is used for gaseous sterilization. It is highly penetrating and can sterilize items within plastic bags such as catheters, disposable items in laboratories and clinical settings (like packaged Petri dishes), and other pieces of equipment. Ethylene oxide exposure is a form of cold sterilization, making it useful for the sterilization of heat-sensitive items. Great care needs to be taken with the use of ethylene oxide, however; it is carcinogenic, like the other alkylating agents, and is also highly explosive. With careful use and proper aeration of the products after treatment, ethylene oxide is highly effective, and ethylene oxide sterilizers are commonly found in medical settings for sterilizing packaged materials.

β-Propionolactone is an alkylating agent with a different chemical structure than the others already discussed. Like other alkylating agents, β-propionolactone binds to DNA, thereby inactivating it (**Figure 11.29**). It is a clear liquid with a strong odor and has the ability to kill endospores. As such, it has been used in either liquid form or as a vapor for the sterilization of medical instruments and tissue grafts, and it is a common component of vaccines, used to maintain their sterility. It has also been used for the sterilization of nutrient broth, as well as blood plasma, milk, and water. It is quickly metabolized by animals and humans to lactic acid. It is also an irritant, however, and may lead to permanent damage of the eyes, kidneys, or liver. Additionally, it has been shown to be carcinogenic in animals; thus, precautions are necessary to minimize human exposure to β-propionolactone.[21]

21. Institute of Medicine. "Long-Term Health Effects of Participation in Project SHAD (Shipboard Hazard and Defense)." Washington, DC: The National Academies Press, 2007.

(a)

(b)

Figure 11.29 (a) Alkylating agents replace hydrogen atoms with alkyl groups. Here, guanine is alkylated, resulting in its hydrogen bonding with thymine, instead of cytosine. (b) The chemical structures of several alkylating agents.

- What chemical reaction do alkylating agents participate in?
- Why are alkylating agents not used as antiseptics?

Diehard Prions

Prions, the acellular, misfolded proteins responsible for incurable and fatal diseases such as kuru and Creutzfeldt-Jakob disease (see **Viroids, Virusoids, and Prions (https://legacy.cnx.org/content/m58811/latest/)**), are notoriously difficult to destroy. Prions are extremely resistant to heat, chemicals, and radiation. They are also extremely infectious and deadly; thus, handling and disposing of prion-infected items requires extensive training and extreme caution.

Typical methods of disinfection can reduce but not eliminate the infectivity of prions. Autoclaving is not completely effective, nor are chemicals such as phenol, alcohols, formalin, and β-propiolactone. Even when fixed in formalin, affected brain and spinal cord tissues remain infectious.

Personnel who handle contaminated specimens or equipment or work with infected patients must wear a protective coat, face protection, and cut-resistant gloves. Any contact with skin must be immediately washed with detergent and warm water without scrubbing. The skin should then be washed with 1 N NaOH or a 1:10 dilution of bleach for 1 minute. Contaminated waste must be incinerated or autoclaved in a strong basic solution, and instruments must be cleaned and soaked in a strong basic solution.

For more information on the handling of animals and prion-contaminated materials, visit the guidelines published on the **WHO (https://openstax.org/l/22WHOhandanipri)** website.

Peroxygens

Peroxygens are strong oxidizing agents that can be used as disinfectants or antiseptics. The most widely used **peroxygen** is hydrogen peroxide (H_2O_2), which is often used in solution to disinfect surfaces and may also be used as a gaseous agent. Hydrogen peroxide solutions are inexpensive skin antiseptics that break down into water and oxygen gas, both of which are environmentally safe. This decomposition is accelerated in the presence of light, so hydrogen peroxide solutions typically are sold in brown or opaque bottles. One disadvantage of using hydrogen peroxide as an antiseptic is that it also causes damage to skin that may delay

healing or lead to scarring. Contact lens cleaners often include hydrogen peroxide as a disinfectant.

Hydrogen peroxide works by producing free radicals that damage cellular macromolecules. Hydrogen peroxide has broad-spectrum activity, working against gram-positive and gram-negative bacteria (with slightly greater efficacy against gram-positive bacteria), fungi, viruses, and endospores. However, bacteria that produce the oxygen-detoxifying enzymes catalase or peroxidase may have inherent tolerance to low hydrogen peroxide concentrations (**Figure 11.30**). To kill endospores, the length of exposure or concentration of solutions of hydrogen peroxide must be increased. Gaseous hydrogen peroxide has greater efficacy and can be used as a sterilant for rooms or equipment.

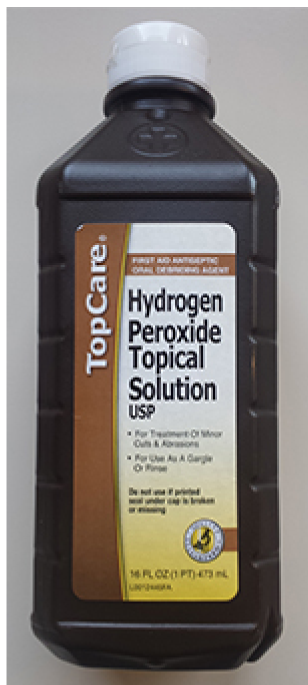


Figure 11.30 Catalase enzymatically converts highly reactive hydrogen peroxide (H_2O_2) into water and oxygen. Hydrogen peroxide can be used to clean wounds. Hydrogen peroxide is used to sterilize items such as contact lenses. (credit photos: modification of work by Kerry Ceszyk)

Plasma, a hot, ionized gas, described as the fourth state of matter, is useful for sterilizing equipment because it penetrates surfaces and kills vegetative cells and endospores. Hydrogen peroxide and peracetic acid, another commonly used peroxygen, each may be introduced as a plasma. Peracetic acid can be used as a liquid or plasma sterilant insofar as it readily kills endospores, is more effective than hydrogen peroxide even at rather low concentrations, and is immune to inactivation by catalases and peroxidases. It also breaks down to environmentally innocuous compounds; in this case, acetic acid and oxygen.

Other examples of peroxygens include benzoyl peroxide and carbamide peroxide. Benzoyl peroxide is a peroxygen that used in acne medication solutions. It kills the bacterium *Propionibacterium acnes*, which is associated with acne. Carbamide peroxide, an ingredient used in toothpaste, is a peroxygen that combats oral biofilms that cause tooth discoloration and halitosis (bad breath).[22] Last, ozone gas is a peroxygen with disinfectant qualities and is used to clean air or water supplies. Overall, peroxygens are highly effective and commonly used, with no associated environmental hazard.

- How do peroxides kill cells?

22. Yao, C.S. et al. "In vitro antibacterial effect of carbamide peroxide on oral biofilm." *Journal of Oral Microbiology* Jun 12, 2013. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3682087/. doi: 10.3402/jom.v5i0.20392.

Supercritical Fluids

Within the last 15 years, the use of **supercritical fluids**, especially supercritical carbon dioxide ($scCO_2$), has gained popularity for certain sterilizing applications. When carbon dioxide is brought to approximately 10 times atmospheric pressure, it reaches a supercritical state that has physical properties between those of liquids and gases. Materials put into a chamber in which carbon dioxide is pressurized in this way can be sterilized because of the ability of $scCO_2$ to penetrate surfaces.

Supercritical carbon dioxide works by penetrating cells and forming carbonic acid, thereby lowering the cell pH considerably. This technique is effective against vegetative cells and is also used in combination with peracetic acid to kill endospores. Its efficacy can also be augmented with increased temperature or by rapid cycles of pressurization and depressurization, which more likely produce cell lysis.

Benefits of $scCO_2$ include the nonreactive, nontoxic, and nonflammable properties of carbon dioxide, and this protocol is effective at low temperatures. Unlike other methods, such as heat and irradiation, that can degrade the object being sterilized, the use of $scCO_2$ preserves the object's integrity and is commonly used for treating foods (including spices and juices) and medical devices such as endoscopes. It is also gaining popularity for disinfecting tissues such as skin, bones, tendons, and ligaments prior to transplantation. $scCO_2$ can also be used for pest control because it can kill insect eggs and larvae within products.

- Why is the use of supercritical carbon dioxide gaining popularity for commercial and medical uses?

Chemical Food Preservatives

Chemical preservatives are used to inhibit microbial growth and minimize spoilage in some foods. Commonly used chemical preservatives include sorbic acid, benzoic acid, and propionic acid, and their more soluble salts potassium sorbate, sodium benzoate, and calcium propionate, all of which are used to control the growth of molds in acidic foods. Each of these preservatives is nontoxic and readily metabolized by humans. They are also flavorless, so they do not compromise the flavor of the foods they preserve.

Sorbic and benzoic acids exhibit increased efficacy as the pH decreases. Sorbic acid is thought to work by inhibiting various cellular enzymes, including those in the citric acid cycle, as well as catalases and peroxidases. It is added as a preservative in a wide variety of foods, including dairy, bread, fruit, and vegetable products. Benzoic acid is found naturally in many types of fruits and berries, spices, and fermented products. It is thought to work by decreasing intracellular pH, interfering with mechanisms such as oxidative phosphorylation and the uptake of molecules such as amino acids into cells. Foods preserved with benzoic acid or sodium benzoate include fruit juices, jams, ice creams, pastries, soft drinks, chewing gum, and pickles.

Propionic acid is thought to both inhibit enzymes and decrease intracellular pH, working similarly to benzoic acid. However, propionic acid is a more effective preservative at a higher pH than either sorbic acid or benzoic acid. Propionic acid is naturally produced by some cheeses during their ripening and is added to other types of cheese and baked goods to prevent mold contamination. It is also added to raw dough to prevent contamination by the bacterium *Bacillus mesentericus*, which causes bread to become ropy.

Other commonly used chemical preservatives include sulfur dioxide and nitrites. Sulfur dioxide prevents browning of foods and is used for the preservation of dried fruits; it has been used in winemaking since ancient times. Sulfur dioxide gas dissolves in water readily, forming sulfites. Although sulfites can be metabolized by the body, some people have sulfite allergies, including asthmatic reactions. Additionally, sulfites degrade thiamine, an important nutrient in some foods. The mode of action of sulfites is not entirely clear, but they may interfere with the disulfide bond (see **m58816 (https://legacy.cnx.org/content/m58816/latest/#OSC_Microbio_07_04_tertiary)**) formation in proteins, inhibiting enzymatic activity. Alternatively, they may reduce the intracellular pH of the cell, interfering with proton motive force-driven mechanisms.

Nitrites are added to processed meats to maintain color and stop the germination of *Clostridium botulinum* endospores. Nitrites are reduced to nitric oxide, which reacts with heme groups and iron-sulfur groups. When nitric oxide reacts with the heme group within the myoglobin of meats, a red product forms, giving meat its red color. Alternatively, it is thought that when nitric acid reacts with the iron-sulfur enzyme ferredoxin within bacteria, this electron transport-chain carrier is destroyed, preventing ATP synthesis. Nitrosamines, however, are carcinogenic and can be produced through exposure of nitrite-preserved meats (e.g., hot dogs, lunch meat, breakfast sausage, bacon, meat in canned soups) to heat during cooking.

Natural Chemical Food Preservatives

The discovery of natural antimicrobial substances produced by other microbes has added to the arsenal of preservatives used in food. Nisin is an antimicrobial peptide produced by the bacterium *Lactococcus lactis* and is particularly effective against gram-positive organisms. Nisin works by disrupting cell wall production, leaving cells more prone to lysis. It is used to preserve cheeses, meats, and beverages.

Natamycin is an antifungal macrolide antibiotic produced by the bacterium *Streptomyces natalensis*. It was approved by the FDA in 1982 and is used to prevent fungal growth in various types of dairy products, including cottage cheese, sliced cheese, and shredded cheese. Natamycin is also used for meat preservation in countries outside the United States.

- What are the advantages and drawbacks of using sulfites and nitrites as food preservatives?

Chemical Disinfectants

Chemical	Mode of Action	Example Uses
Phenolics		
Cresols o-Phenylphenol Hexachlorophene Triclosan	Denature proteins and disrupt membranes	Disinfectant in Lysol Prevent contamination of crops (citrus) Antibacterial soap pHisoHex for handwashing in hospitals
Metals		
Mercury Silver Copper Nickel Zinc	Bind to proteins and inhibit enzyme activity	Topical antiseptic Treatment of wounds and burns Prevention of eye infections in newborns Antibacterial in catheters and bandages Mouthwash Algicide for pools and fish tanks Containers for long-term water storage
Halogens		
Iodine Chlorine Fluorine	Oxidation and destabilization of cellular macromolecules	Topical antiseptic Hand scrub for medical personnel Water disinfectant Water treatment plants Household bleach Food processing Prevention of dental carries
Alcohols		
Ethanol Isopropanol	Denature proteins and disrupt membranes	Disinfectant Antiseptic
Surfactants		

Chemical Disinfectants

Chemical	Mode of Action	Example Uses
Quaternary ammonium salts	Lowers surface tension of water to help with washing away of microbes, and disruption of cell membranes	Soaps and detergent Disinfectant Antiseptic Mouthwash
Bisbiguanides		
Chlorhexidine Alexidine	Disruption of cell membranes	Oral rinse Hand scrub for medical personnel
Alkylating Agents		
Formaldehyde Glutaraldehyde o-Phthalaldehyde Ethylene oxide β-Propionolactone	Inactivation of enzymes and nucleic acid	Disinfectant Tissue specimen storage Embalming Sterilization of medical equipment Vaccine component for sterility
Peroxygens		
Hydrogen peroxide Peracetic acid Benzoyl peroxide Carbamide peroxide Ozone gas	Oxidation and destabilization of cellular macromolecules	Antiseptic Disinfectant Acne medication Toothpaste ingredient
Supercritical Gases		
Carbon dioxide	Penetrates cells, forms carbonic acid, lowers intracellular pH	Food preservation Disinfection of medical devices Disinfection of transplant tissues
Chemical Food Preservatives		
Sorbic acid Benzoic acid Propionic acid Potassium sorbate Sodium benzoate Calcium propionate Sulfur dioxide Nitrites	Decrease pH and inhibit enzymatic function	Preservation of food products
Natural Food Preservatives		
Nisin Natamycin	Inhibition of cell wall synthesis (Nisin)	Preservation of dairy products, meats, and beverages

This OpenStax book is available for free at https://legacy.cnx.org/content/col26095/1.5

CHAPTER SUMMARY

11.1 Controlling Microbial Growth

- Inanimate items that may harbor microbes and aid in their transmission are called **fomites**. The level of cleanliness required for a fomite depends both on the item's use and the infectious agent with which the item may be contaminated.
- The CDC and the NIH have established four **biological safety levels (BSLs)** for laboratories performing research on infectious agents. Each level is designed to protect laboratory personnel and the community. These BSLs are determined by the agent's infectivity, ease of transmission, and potential disease severity, as well as the type of work being performed with the agent.
- **Disinfection** removes potential pathogens from a fomite, whereas **antisepsis** uses antimicrobial chemicals safe enough for tissues; in both cases, microbial load is reduced, but microbes may remain unless the chemical used is strong enough to be a **sterilant**.
- The amount of cleanliness (**sterilization** versus high-level disinfection versus general cleanliness) required for items used clinically depends on whether the item will come into contact with sterile tissues (**critical item)**, mucous membranes (**semicritical item**), or intact skin (**noncritical item**).
- Medical procedures with a risk for contamination should be carried out in a **sterile field** maintained by proper **aseptic technique** to prevent **sepsis**.
- Sterilization is necessary for some medical applications as well as in the food industry, where endospores of *Clostridium botulinum* are killed through **commercial sterilization** protocols.
- Physical or chemical methods to control microbial growth that result in death of the microbe are indicated by the suffixes *-cide* or *-cidal* (e.g., as with **bactericides**, **viricides**, and **fungicides**), whereas those that inhibit microbial growth are indicated by the suffixes *-stat* or-*static* (e.g., **bacteriostatic**, **fungistatic**).
- **Microbial death curves** display the logarithmic decline of living microbes exposed to a method of microbial control. The time it takes for a protocol to yield a 1-log (90%) reduction in the microbial population is the **decimal reduction time**, or **D-value**.
- When choosing a microbial control protocol, factors to consider include the length of exposure time, the type of microbe targeted, its susceptibility to the protocol, the intensity of the treatment, the presence of organics that may interfere with the protocol, and the environmental conditions that may alter the effectiveness of the protocol.

11.2 Using Physical Methods to Control Microorganisms

- Heat is a widely used and highly effective method for controlling microbial growth.
- **Dry-heat sterilization** protocols are used commonly in aseptic techniques in the laboratory. However, **moist-heat sterilization** is typically the more effective protocol because it penetrates cells better than dry heat does.
- **Pasteurization** is used to kill pathogens and reduce the number of microbes that cause food spoilage. **High-temperature, short-time pasteurization** is commonly used to pasteurize milk that will be refrigerated; **ultra-high temperature pasteurization** can be used to pasteurize milk for long-term storage without refrigeration.
- Refrigeration slows microbial growth; freezing stops growth, killing some organisms. Laboratory and medical specimens may be frozen on dry ice or at ultra-low temperatures for storage and transport.
- High-pressure processing can be used to kill microbes in food. Hyperbaric oxygen therapy to increase oxygen saturation has also been used to treat certain infections.
- **Desiccation** has long been used to preserve foods and is accelerated through the addition of salt or sugar, which decrease water activity in foods.

- **Lyophilization** combines cold exposure and desiccation for the long-term storage of foods and laboratory materials, but microbes remain and can be rehydrated.
- **Ionizing radiation**, including gamma irradiation, is an effective way to sterilize heat-sensitive and packaged materials. **Nonionizing radiation**, like ultraviolet light, is unable to penetrate surfaces but is useful for surface sterilization.
- **HEPA** filtration is commonly used in hospital ventilation systems and biological safety cabinets in laboratories to prevent transmission of airborne microbes. **Membrane filtration** is commonly used to remove bacteria from heat-sensitive solutions.

11.3 Using Chemicals to Control Microorganisms

- **Heavy metals**, including mercury, silver, copper, and zinc, have long been used for disinfection and preservation, although some have toxicity and environmental risks associated with them.
- **Halogens**, including chlorine, fluorine, and iodine, are also commonly used for disinfection. Chlorine compounds, including **sodium hypochlorite**, **chloramines**, and **chlorine dioxide**, are commonly used for water disinfection. Iodine, in both **tincture** and **iodophor** forms, is an effective antiseptic.
- **Alcohols**, including ethyl alcohol and isopropyl alcohol, are commonly used antiseptics that act by denaturing proteins and disrupting membranes.
- **Phenolics** are stable, long-acting disinfectants that denature proteins and disrupt membranes. They are commonly found in household cleaners, mouthwashes, and hospital disinfectants, and are also used to preserve harvested crops.
- The phenolic compound **triclosan**, found in antibacterial soaps, plastics, and textiles is technically an antibiotic because of its specific mode of action of inhibiting bacterial fatty-acid synthesis..
- **Surfactants**, including soaps and detergents, lower the surface tension of water to create emulsions that mechanically carry away microbes. Soaps are long-chain fatty acids, whereas detergents are synthetic surfactants.
- **Quaternary ammonium compounds** (**quats**) are cationic detergents that disrupt membranes. They are used in household cleaners, skin disinfectants, oral rinses, and mouthwashes.
- **Bisbiguanides** disrupt cell membranes, causing cell contents to gel. **Chlorhexidine** and **alexidine** are commonly used for surgical scrubs, for handwashing in clinical settings, and in prescription oral rinses.
- **Alkylating agents** effectively sterilize materials at low temperatures but are carcinogenic and may also irritate tissue. **Glutaraldehyde** and **o-phthalaldehyde** are used as hospital disinfectants but not as antiseptics. **Formaldehyde** is used for the storage of tissue specimens, as an embalming fluid, and in vaccine preparation to inactivate infectious agents. **Ethylene oxide** is a gas sterilant that can permeate heat-sensitive packaged materials, but it is also explosive and carcinogenic.
- **Peroxygens**, including **hydrogen peroxide**, **peracetic acid**, **benzoyl peroxide**, and ozone gas, are strong oxidizing agents that produce free radicals in cells, damaging their macromolecules. They are environmentally safe and are highly effective disinfectants and antiseptics.
- Pressurized carbon dioxide in the form of a **supercritical fluid** easily permeates packaged materials and cells, forming carbonic acid and lowering intracellular pH. Supercritical carbon dioxide is nonreactive, nontoxic, nonflammable, and effective at low temperatures for sterilization of medical devices, implants, and transplanted tissues.
- Chemical preservatives are added to a variety of foods. **Sorbic acid**, **benzoic acid**, **propionic acid**, and their more soluble salts inhibit enzymes or reduce intracellular pH.
- **Sulfites** are used in winemaking and food processing to prevent browning of foods.
- **Nitrites** are used to preserve meats and maintain color, but cooking nitrite-preserved meats may produce carcinogenic nitrosamines.

- **Nisin** and **natamycin** are naturally produced preservatives used in cheeses and meats. Nisin is effective against gram-positive bacteria and natamycin against fungi.

REVIEW QUESTIONS

1. Which of the following types of medical items requires sterilization?
   A. needles
   B. bed linens
   C. respiratory masks
   D. blood pressure cuffs

2. Which of the following is suitable for use on tissues for microbial control to prevent infection?
   A. disinfectant
   B. antiseptic
   C. sterilant
   D. water

3. Which biosafety level is appropriate for research with microbes or infectious agents that pose moderate risk to laboratory workers and the community, and are typically indigenous?
   A. BSL-1
   B. BSL-2
   C. BSL-3
   D. BSL-4

4. Which of the following best describes a microbial control protocol that inhibits the growth of molds and yeast?
   A. bacteriostatic
   B. fungicidal
   C. bactericidal
   D. fungistatic

5. The decimal reduction time refers to the amount of time it takes to which of the following?
   A. reduce a microbial population by 10%
   B. reduce a microbial population by 0.1%
   C. reduce a microbial population by 90%
   D. completely eliminate a microbial population

6. Which of the following methods brings about cell lysis due to cavitation induced by rapid localized pressure changes?
   A. microwaving
   B. gamma irradiation
   C. ultraviolet radiation
   D. sonication

7. Which of the following terms is used to describe the time required to kill all of the microbes within a sample at a given temperature?
   A. D-value
   B. thermal death point
   C. thermal death time
   D. decimal reduction time

8. Which of the following microbial control methods does not actually kill microbes or inhibit their growth but instead removes them physically from samples?
   A. filtration
   B. desiccation
   C. lyophilization
   D. nonionizing radiation

9. Which of the following refers to a disinfecting chemical dissolved in alcohol?
   A. iodophor
   B. tincture
   C. phenolic
   D. peroxygen

10. Which of the following peroxygens is widely used as a household disinfectant, is inexpensive, and breaks down into water and oxygen gas?
   A. hydrogen peroxide
   B. peracetic acid
   C. benzoyl peroxide
   D. ozone

11. Which of the following chemical food preservatives is used in the wine industry but may cause asthmatic reactions in some individuals?
   A. nitrites
   B. sulfites
   C. propionic acid
   D. benzoic acid

12. Bleach is an example of which group of chemicals used for disinfection?
   A. heavy metals
   B. halogens
   C. quats
   D. bisbiguanides

13. Which chemical disinfectant works by methylating enzymes and nucleic acids and is known for being toxic and carcinogenic?
   A. sorbic acid
   B. triclosan
   C. formaldehyde
   D. hexaclorophene

TRUE/FALSE

11.1 Controlling Microbial Growth

14. Sanitization leaves an object free of microbes.

11.2 Using Physical Methods to Control Microorganisms

15. Ionizing radiation can penetrate surfaces, but nonionizing radiation cannot.

16. Moist-heat sterilization protocols require the use of higher temperatures for longer periods of time than do dry-heat sterilization protocols do.

11.3 Using Chemicals to Control Microorganisms

17. Soaps are classified as disinfectants.

18. Mercury-based compounds have fallen out of favor for use as preservatives and antiseptics.

FILL IN THE BLANK

11.1 Controlling Microbial Growth

19. A medical item that comes into contact with intact skin and does not penetrate sterile tissues or come into contact with mucous membranes is called a(n) ________ item.

20. The goal of ________ ________ protocols is to rid canned produce of *Clostridium botulinum* endospores.

11.2 Using Physical Methods to Control Microorganisms

21. In an autoclave, the application of pressure to ________ is increased to allow the steam to achieve temperatures above the boiling point of water.

11.3 Using Chemicals to Control Microorganisms

22. Doorknobs and other surfaces in clinical settings are often coated with ________, ________, or ________ to prevent the transmission of microbes.

SHORT ANSWER

11.1 Controlling Microbial Growth

23. What are some characteristics of microbes and infectious agents that would require handling in a BSL-3 laboratory?

24. What is the purpose of degerming? Does it completely eliminate microbes?

25. What are some factors that alter the effectiveness of a disinfectant?

11.2 Using Physical Methods to Control Microorganisms

26. What is the advantage of HTST pasteurization compared with sterilization? What is an advantage of UHT treatment?

27. How does the addition of salt or sugar help preserve food?

28. Which is more effective at killing microbes: autoclaving or freezing? Explain.

11.3 Using Chemicals to Control Microorganisms

29. Which solution of ethyl alcohol is more effective at inhibiting microbial growth: a 70% solution or a 100% solution? Why?

30. When might a gas treatment be used to control microbial growth instead of autoclaving? What are some examples?

31. What is the advantage of using an iodophor rather than iodine or an iodine tincture?

CRITICAL THINKING

32. When plotting microbial death curves, how might they look different for bactericidal versus bacteriostatic treatments?

33. What are the benefits of cleaning something to a level of cleanliness beyond what is required? What are some possible disadvantages of doing so?

34. In 2001, endospores of *Bacillus anthracis*, the causative agent of anthrax, were sent to government officials and news agencies via the mail. In response, the US Postal Service began to irradiate mail with UV light. Was this an effective strategy? Why or why not?

35. Looking at **Figure 11.29** and reviewing the functional groups in **m58813 (https://legacy.cnx.org/content/m58813/latest/#OSC_Microbio_07_01_TBLcomFun)** , which alkylating agent shown lacks an aldehyde group?

36. Do you think naturally produced antimicrobial products like nisin and natamycin should replace sorbic acid for food preservation? Why or why not?

37. Why is the use of skin disinfecting compounds required for surgical scrubbing and not for everyday handwashing?

12 | NUCLEIC ACID STRUCTURE & FUNCTION

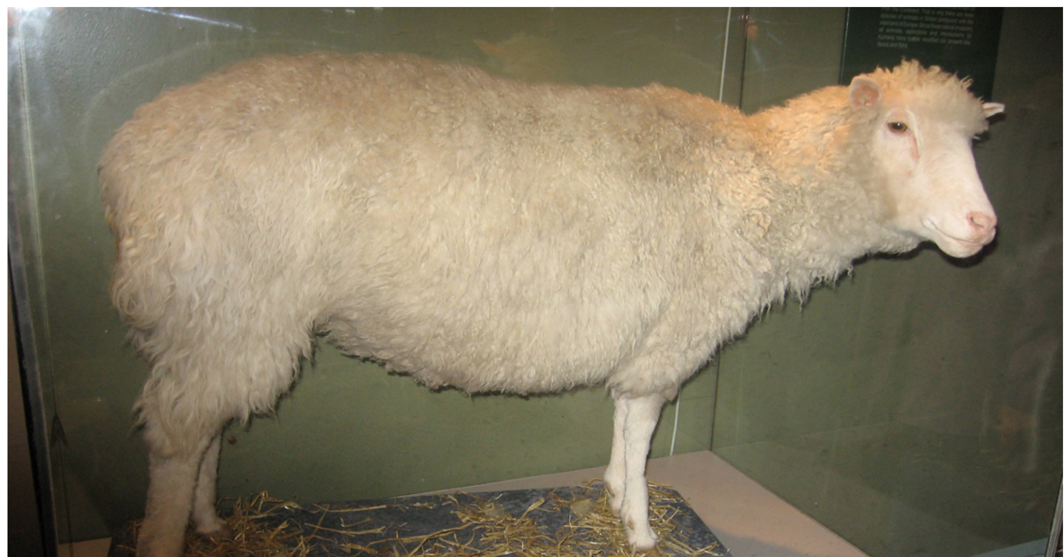


Figure 12.1 Dolly the sheep was the first cloned mammal.

Chapter Outline

12.1: Structure and Function of DNA
12.2: Rosalind Franklin
12.3: DNA Replication
12.4: Structure and Function of RNA
12.5: Transcription
12.6: Translation
12.7: How Genes Are Regulated
12.8: Structure and Function of Cellular Genomes
12.9: Mapping Genomes
12.10: Applying Genomics

Introduction

The three letters "DNA" have now become associated with crime solving, paternity testing, human identification, and genetic testing. DNA can be retrieved from hair, blood, or saliva. With the exception of identical twins, each person's DNA is unique and it is possible to detect differences between human beings on the basis of their unique DNA sequence.

DNA analysis has many practical applications beyond forensics and paternity testing. DNA testing is used for tracing genealogy and identifying pathogens. In the medical field, DNA is used in diagnostics, new vaccine development, and cancer therapy. It is now possible to determine predisposition to many diseases by analyzing genes.

DNA is the genetic material passed from parent to offspring for all life on Earth. The technology of molecular genetics developed in the last half century has enabled us to see deep into the history of life to deduce the

relationships between living things in ways never thought possible. It also allows us to understand the workings of evolution in populations of organisms. Over a thousand species have had their entire genome sequenced, and there have been thousands of individual human genome sequences completed. These sequences will allow us to understand human disease and the relationship of humans to the rest of the tree of life. Finally, molecular genetics techniques have revolutionized plant and animal breeding for human agricultural needs. All of these advances in biotechnology depended on basic research leading to the discovery of the structure of DNA in 1953, and the research since then that has uncovered the details of DNA replication and the complex process leading to the expression of DNA in the form of proteins in the cell.

12.1 | Structure and Function of DNA

Learning Objectives

By the end of this section, you will be able to:

- Describe the biochemical structure of deoxyribonucleotides
- Identify the base pairs used in the synthesis of deoxyribonucleotides
- Explain why the double helix of DNA is described as antiparallel

In **Microbial Metabolism (https://legacy.cnx.org/content/m58818/latest/)** , we discussed the microbial catabolism of three classes of macromolecules: proteins, lipids and carbohydrates. In this chapter, we will discuss the genetic role of a fourth class of molecules: nucleic acids. Like other macromolecules, **nucleic acid**s are composed of monomers, called **nucleotide**s, which are polymerized to form large strands. Each nucleic acid strand contains certain nucleotides that appear in a certain order within the strand, called its **base sequence**. The base sequence of **deoxyribonucleic acid (DNA)** is responsible for carrying and retaining the hereditary information in a cell. In **Mechanisms of Microbial Genetics (https://legacy.cnx.org/content/m58839/latest/)** , we will discuss in detail the ways in which DNA uses its own base sequence to direct its own synthesis, as well as the synthesis of RNA and proteins, which, in turn, gives rise to products with diverse structure and function. In this section, we will discuss the basic structure and function of DNA.

DNA Nucleotides

The building blocks of nucleic acids are nucleotides. Nucleotides that compose DNA are called **deoxyribonucleotides**. The three components of a deoxyribonucleotide are a five-carbon sugar called deoxyribose, a phosphate group, and a **nitrogenous base**, a nitrogen-containing ring structure that is responsible for complementary base pairing between nucleic acid strands (**Figure 12.2**). The carbon atoms of the five-carbon deoxyribose are numbered 1′, 2′, 3′, 4′, and 5′ (1′ is read as “one prime”). A nucleoside comprises the five-carbon sugar and nitrogenous base.

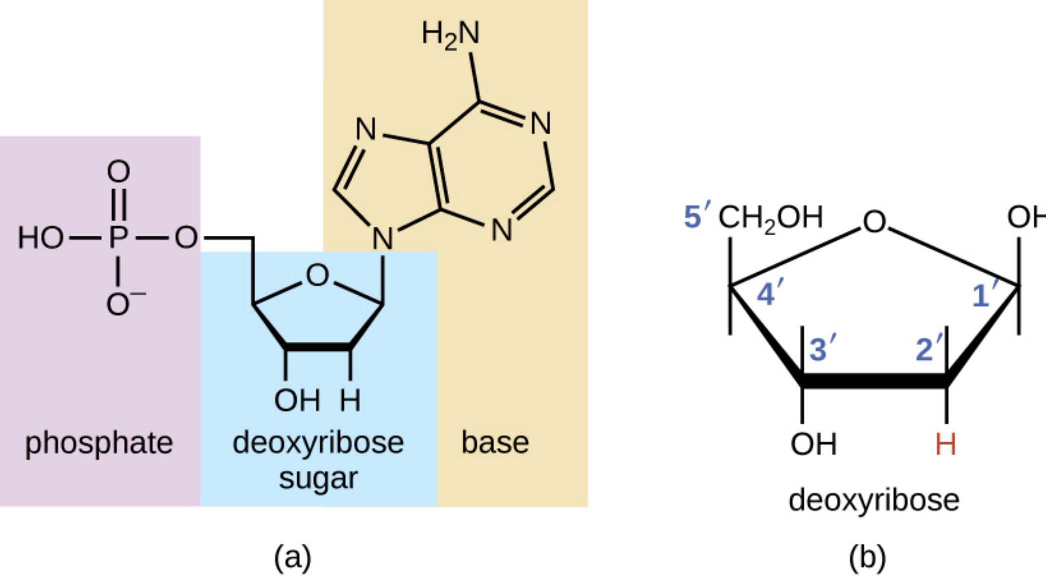


Figure 12.2 (a) Each deoxyribonucleotide is made up of a sugar called deoxyribose, a phosphate group, and a nitrogenous base—in this case, adenine. (b) The five carbons within deoxyribose are designated as 1′, 2′, 3′, 4′, and 5′.

The deoxyribonucleotide is named according to the nitrogenous bases (**Figure 12.3**). The nitrogenous bases **adenine** (A) and **guanine** (G) are the **purines**; they have a double-ring structure with a six-carbon ring fused to a five-carbon ring. The **pyrimidines**, **cytosine** (C) and **thymine** (T), are smaller nitrogenous bases that have only a six-carbon ring structure.

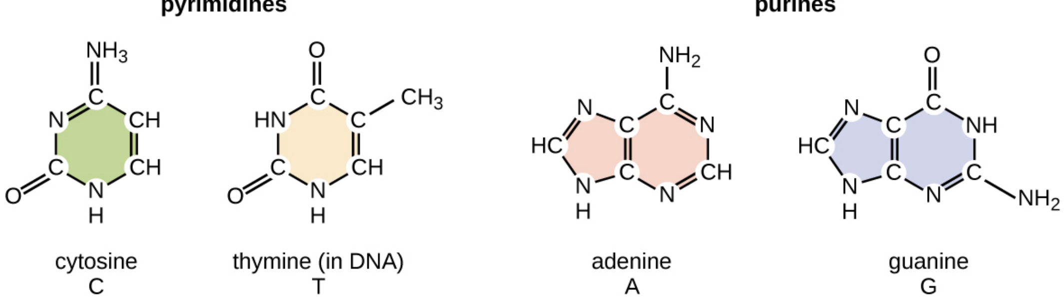


Figure 12.3 Nitrogenous bases within DNA are categorized into the two-ringed purines adenine and guanine and the single-ringed pyrimidines cytosine and thymine. Thymine is unique to DNA.

Individual nucleoside triphosphates combine with each other by covalent bonds known as 5′-3′ **phosphodiester bonds**, or linkages whereby the phosphate group attached to the 5′ carbon of the sugar of one nucleotide bonds to the hydroxyl group of the 3′ carbon of the sugar of the next nucleotide. Phosphodiester bonding between nucleotides forms the **sugar-phosphate backbone**, the alternating sugar-phosphate structure composing the framework of a nucleic acid strand (**Figure 12.4**). During the polymerization process, deoxynucleotide triphosphates (dNTP) are used. To construct the sugar-phosphate backbone, the two terminal phosphates are released from the dNTP as a pyrophosphate. The resulting strand of nucleic acid has a free phosphate group at the 5′ carbon end and a free hydroxyl group at the 3′ carbon end. The two unused phosphate groups from the nucleotide triphosphate are released as pyrophosphate during phosphodiester bond formation. Pyrophosphate is subsequently hydrolyzed, releasing the energy used to drive nucleotide polymerization.

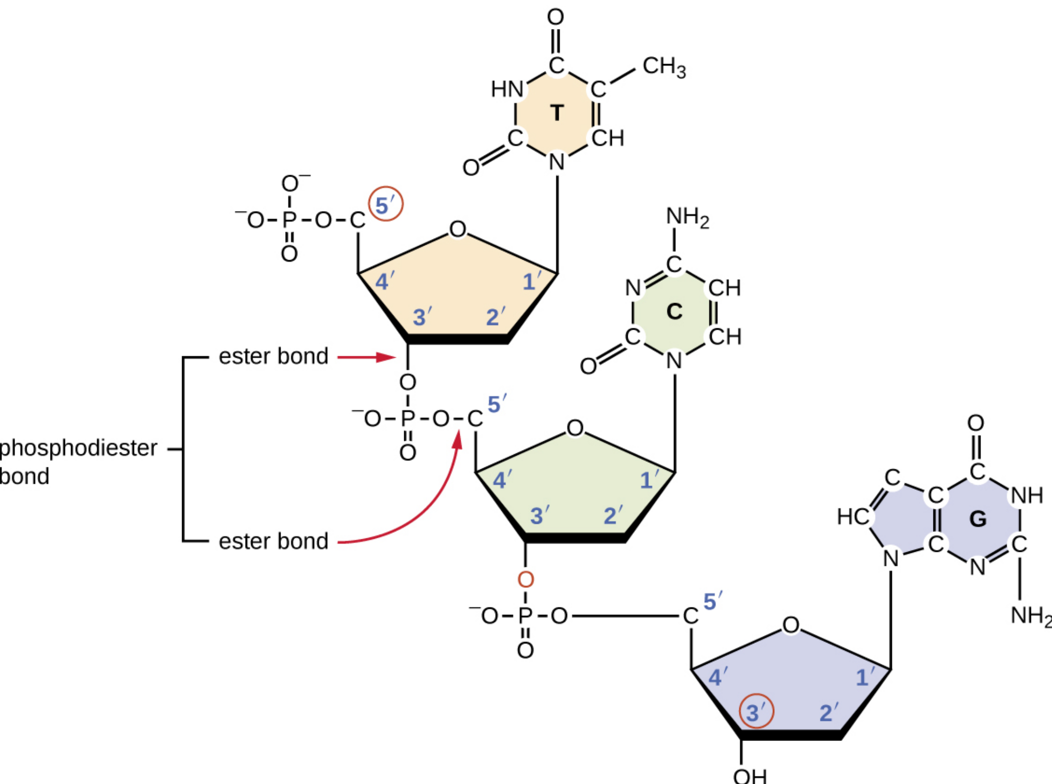


Figure 12.4 Phosphodiester bonds form between the phosphate group attached to the 5′ carbon of one nucleotide and the hydroxyl group of the 3′ carbon in the next nucleotide, bringing about polymerization of nucleotides in to nucleic acid strands. Note the 5′ and 3′ ends of this nucleic acid strand.

- What is meant by the 5′ and 3′ ends of a nucleic acid strand?

Discovering the Double Helix

By the early 1950s, considerable evidence had accumulated indicating that DNA was the genetic material of cells, and now the race was on to discover its three-dimensional structure. Around this time, Austrian biochemist Erwin Chargaff[1] (1905–2002) examined the content of DNA in different species and discovered that adenine, thymine, guanine, and cytosine were not found in equal quantities, and that it varied from species to species, but not between individuals of the same species. He found that the amount of adenine was very close to equaling the amount of thymine, and the amount of cytosine was very close to equaling the amount of guanine, or A = T and G = C. These relationships are also known as Chargaff's rules.

Other scientists were also actively exploring this field during the mid-20th century. In 1952, American scientist Linus Pauling (1901–1994) was the world's leading structural chemist and odds-on favorite to solve the structure of DNA. Pauling had earlier discovered the structure of protein α helices, using X-ray diffraction, and, based upon X-ray diffraction images of DNA made in his laboratory, he proposed a triple-stranded model of DNA.[2] At the same time, British researchers Rosalind Franklin (1920–1958) and her graduate student R.G. Gosling were also using X-ray diffraction to understand the structure of DNA (**Figure 12.5**). It was Franklin's scientific expertise that resulted in the production of more well-defined X-ray diffraction images of DNA that would clearly show the overall double-helix structure of DNA.

1. N. Kresge et al. "Chargaff's Rules: The Work of Erwin Chargaff." *Journal of Biological Chemistry* 280 (2005):e21.
2. L. Pauling, "A Proposed Structure for the Nucleic Acids." *Proceedings of the National Academy of Science of the United States of America* 39 no. 2 (1953):84–97.

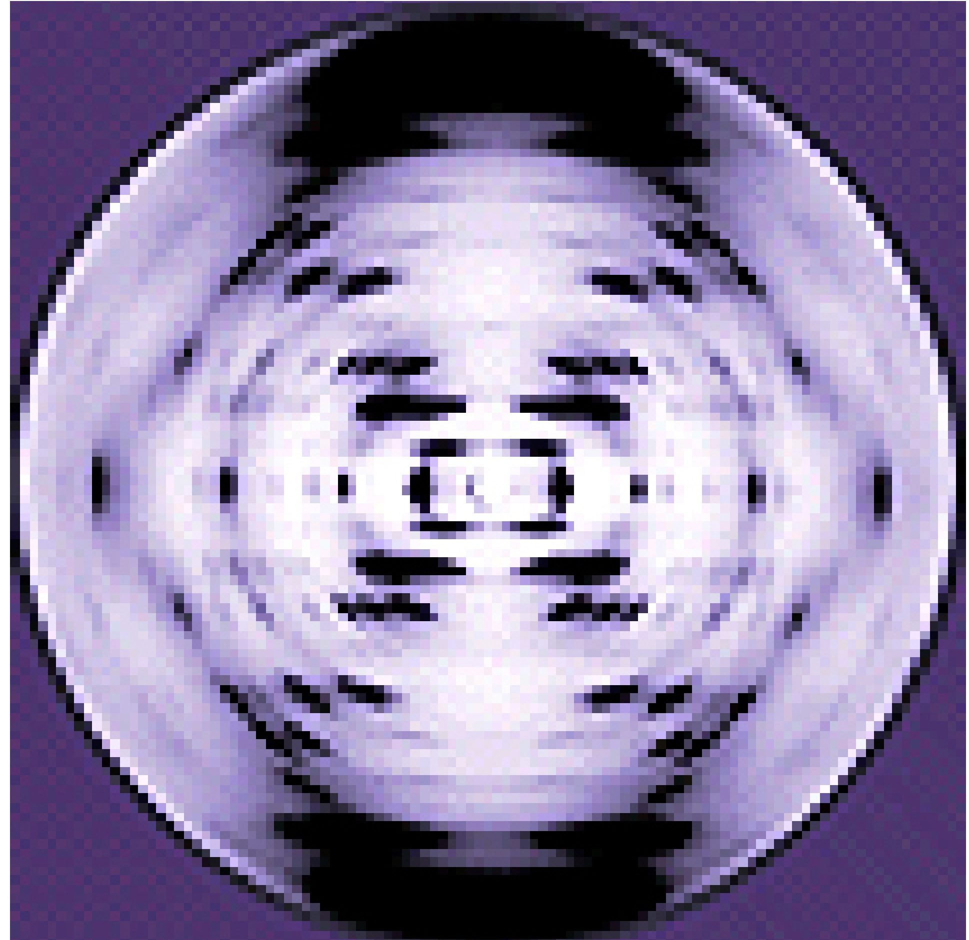


Figure 12.5 The X-ray diffraction pattern of DNA shows its helical nature. (credit: National Institutes of Health)

James Watson (1928–), an American scientist, and Francis Crick (1916–2004), a British scientist, were working together in the 1950s to discover DNA's structure. They used Chargaff's rules and Franklin and Wilkins' X-ray diffraction images of DNA fibers to piece together the purine-pyrimidine pairing of the double helical DNA molecule (**Figure 12.6**). In April 1953, Watson and Crick published their model of the DNA double helix in *Nature*.[3] The same issue additionally included papers by Wilkins and colleagues,[4] as well as by Franklin and Gosling,[5] each describing different aspects of the molecular structure of DNA. In 1962, James Watson, Francis Crick, and Maurice Wilkins were awarded the Nobel Prize in Physiology and Medicine. Unfortunately, by then Franklin had died, and Nobel prizes at the time were not awarded posthumously. Work continued, however, on learning about the structure of DNA. In 1973, Alexander Rich (1924–2015) and colleagues were able to analyze DNA crystals to confirm and further elucidate DNA structure.[6]

3. J.D. Watson, F.H.C. Crick. "A Structure for Deoxyribose Nucleic Acid." *Nature* 171 no. 4356 (1953):737–738.
4. M.H.F. Wilkins et al. "Molecular Structure of Deoxypentose Nucleic Acids." *Nature* 171 no. 4356 (1953):738–740.
5. R. Franklin, R.G. Gosling. "Molecular Configuration in Sodium Thymonucleate." *Nature* 171 no. 4356 (1953):740–741.
6. R.O. Day et al. "A Crystalline Fragment of the Double Helix: The Structure of the Dinucleoside Phosphate Guanylyl-3',5'-Cytidine." *Proceedings of the National Academy of Sciences of the United States of America* 70 no. 3 (1973):849–853.

Figure 12.6 In 1953, James Watson and Francis Crick built this model of the structure of DNA, shown here on display at the Science Museum in London.

- Which scientists are given most of the credit for describing the molecular structure of DNA?

DNA Structure

Watson and Crick proposed that DNA is made up of two strands that are twisted around each other to form a right-handed helix. The two DNA strands are **antiparallel**, such that the 3′ end of one strand faces the 5′ end of the other (**Figure 12.7**). The 3′ end of each strand has a free hydroxyl group, while the 5′ end of each strand has a free phosphate group. The sugar and phosphate of the polymerized nucleotides form the backbone of the structure, whereas the nitrogenous bases are stacked inside. These nitrogenous bases on the interior of the molecule interact with each other, base pairing.

Analysis of the diffraction patterns of DNA has determined that there are approximately 10 bases per turn in DNA. The asymmetrical spacing of the sugar-phosphate backbones generates major grooves (where the backbone is far apart) and minor grooves (where the backbone is close together) (**Figure 12.7**). These grooves are locations where proteins can bind to DNA. The binding of these proteins can alter the structure of DNA, regulate replication, or regulate transcription of DNA into RNA.

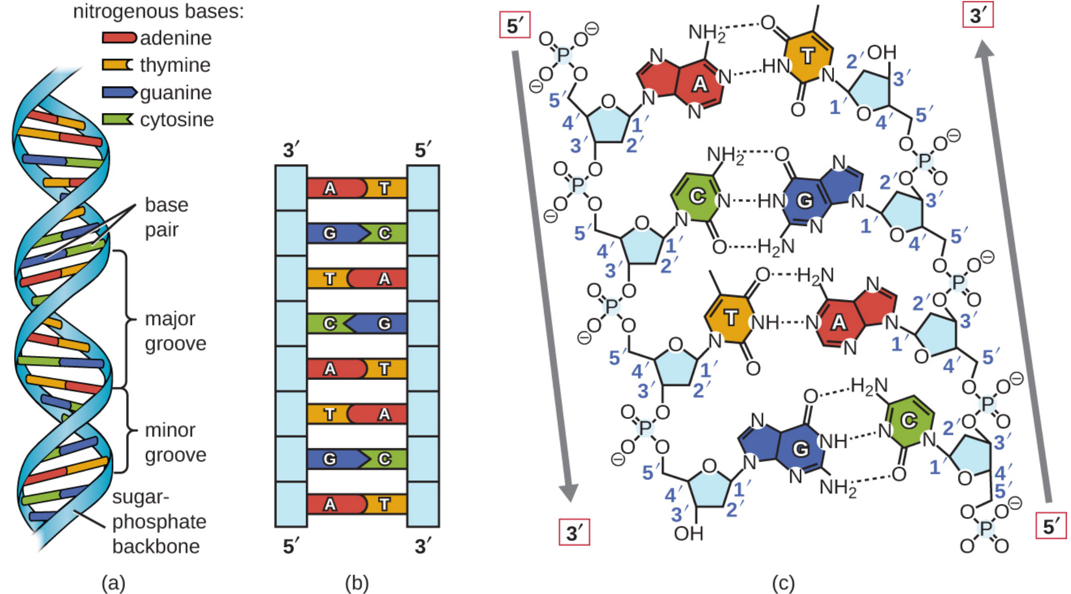


Figure 12.7 Watson and Crick proposed the double helix model for DNA. (a) The sugar-phosphate backbones are on the outside of the double helix and purines and pyrimidines form the “rungs” of the DNA helix ladder. (b) The two DNA strands are antiparallel to each other. (c) The direction of each strand is identified by numbering the carbons (1 through 5) in each sugar molecule. The 5′ end is the one where carbon #5 is not bound to another nucleotide; the 3′ end is the one where carbon #3 is not bound to another nucleotide.

Base pairing takes place between a purine and pyrimidine. In DNA, adenine (A) and thymine (T) are **complementary base pairs**, and cytosine (C) and guanine (G) are also complementary base pairs, explaining Chargaff’s rules (**Figure 12.8**). The base pairs are stabilized by hydrogen bonds; adenine and thymine form two hydrogen bonds between them, whereas cytosine and guanine form three hydrogen bonds between them.

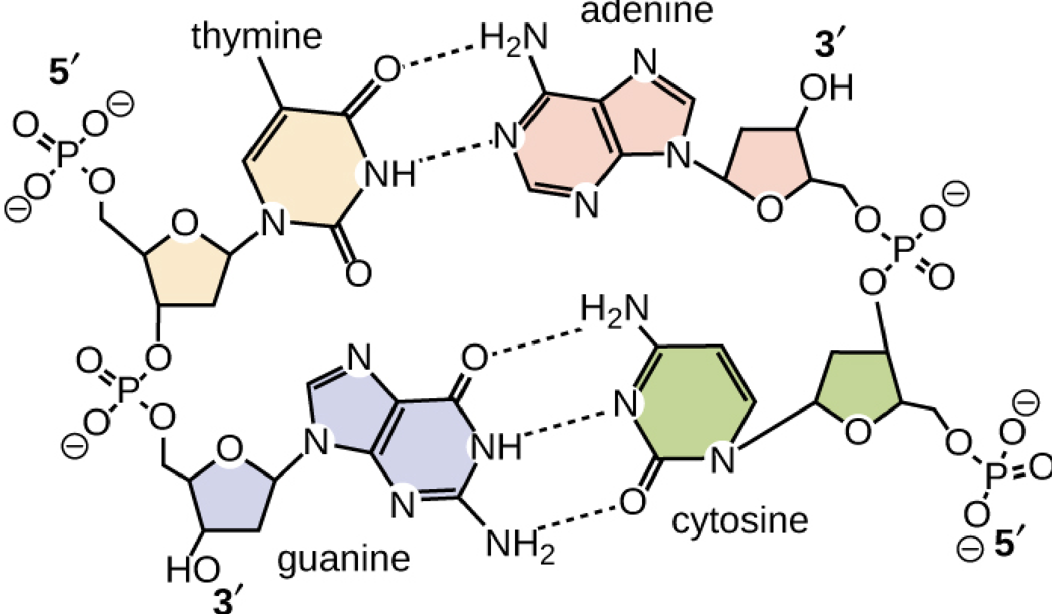


Figure 12.8 Hydrogen bonds form between complementary nitrogenous bases on the interior of DNA.

In the laboratory, exposing the two DNA strands of the double helix to high temperatures or to certain chemicals can break the hydrogen bonds between complementary bases, thus separating the strands into two separate single strands of DNA (single-stranded DNA [ssDNA]). This process is called DNA denaturation and is analogous to protein denaturation, as described in **Proteins (https://legacy.cnx.org/content/m58816/latest/)** . The ssDNA strands can also be put back together as double-stranded DNA (dsDNA), through reannealing

or renaturing by cooling or removing the chemical denaturants, allowing these hydrogen bonds to reform. The ability to artificially manipulate DNA in this way is the basis for several important techniques in biotechnology (**Figure 12.9**). Because of the additional hydrogen bonding between the C = G base pair, DNA with a high GC content is more difficult to denature than DNA with a lower GC content.

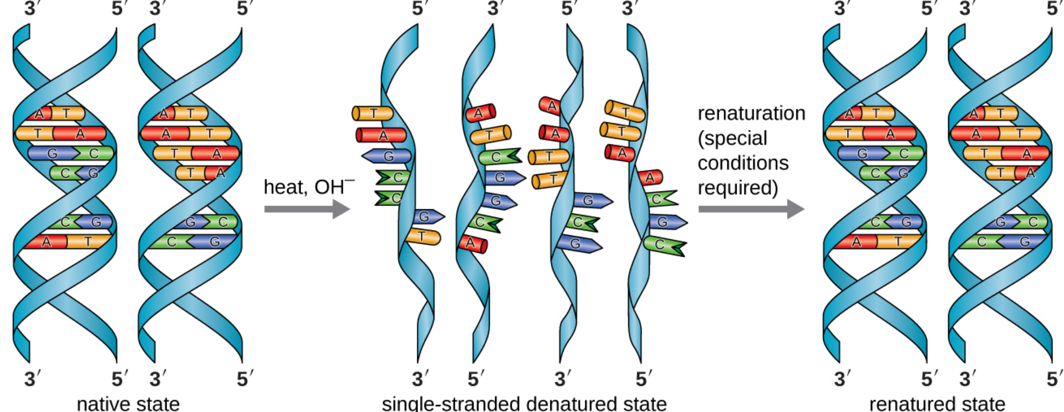


Figure 12.9 In the laboratory, the double helix can be denatured to single-stranded DNA through exposure to heat or chemicals, and then renatured through cooling or removal of chemical denaturants to allow the DNA strands to reanneal. (credit: modification of work by Hernández-Lemus E, Nicasio-Collazo LA, Castañeda-Priego R)

View an **animation (https://www.openstax.org/l/22dnastruanim)** on DNA structure from the DNA Learning Center to learn more.

- What are the two complementary base pairs of DNA and how are they bonded together?

DNA Function

DNA stores the information needed to build and control the cell. The transmission of this information from mother to daughter cells is called **vertical gene transfer** and it occurs through the process of DNA replication. DNA is replicated when a cell makes a duplicate copy of its DNA, then the cell divides, resulting in the correct distribution of one DNA copy to each resulting cell. DNA can also be enzymatically degraded and used as a source of nucleosides and nucleotides for the cell. Unlike other macromolecules, DNA does not serve a structural role in cells.

- How does DNA transmit genetic information to offspring?

Paving the Way for Women in Science and Health Professions

Historically, women have been underrepresented in the sciences and in medicine, and often their pioneering contributions have gone relatively unnoticed. For example, although Rosalind Franklin performed the X-ray diffraction studies demonstrating the double helical structure of DNA, it is Watson and Crick who became famous for this discovery, building on her data. There still remains great controversy over whether their acquisition of her data was appropriate and whether personality conflicts and gender bias contributed to the delayed recognition of her significant contributions. Similarly, Barbara McClintock did pioneering work in maize (corn) genetics from the 1930s through 1950s, discovering transposons (jumping genes), but she was not recognized until much later, receiving a Nobel Prize in Physiology or Medicine in 1983 (**Figure 12.10**).

Today, women still remain underrepresented in many fields of science and medicine. While more than half of the undergraduate degrees in science are awarded to women, only 46% of doctoral degrees in science are awarded to women. In academia, the number of women at each level of career advancement continues to decrease, with women holding less than one-third of the positions of Ph.D.-level scientists in tenure-track positions, and less than one-quarter of the full professorships at 4-year colleges and universities.[7] Even

7. N.H. Wolfinger "For Female Scientists, There's No Good Time to Have Children." *The Atlantic* July 29, 2013. http://www.theatlantic.com/sexes/archive/2013/07/for-female-scientists-theres-no-good-time-to-have-children/278165/.

in the health professions, like nearly all other fields, women are often underrepresented in many medical careers and earn significantly less than their male counterparts, as shown in a 2013 study published by the *Journal of the American Medical Association*.[8]

Why do such disparities continue to exist and how do we break these cycles? The situation is complex and likely results from the combination of various factors, including how society conditions the behaviors of girls from a young age and supports their interests, both professionally and personally. Some have suggested that women do not belong in the laboratory, including Nobel Prize winner Tim Hunt, whose 2015 public comments suggesting that women are too emotional for science[9] were met with widespread condemnation.

Perhaps girls should be supported more from a young age in the areas of science and math (**Figure 12.10**). Science, technology, engineering, and mathematics (STEM) programs sponsored by the American Association of University Women (AAUW)[10] and National Aeronautics and Space Administration (NASA)[11] are excellent examples of programs that offer such support. Contributions by women in science should be made known more widely to the public, and marketing targeted to young girls should include more images of historically and professionally successful female scientists and medical professionals, encouraging all bright young minds, including girls and women, to pursue careers in science and medicine.

(a)

(b)

Figure 12.10 (a) Barbara McClintock's work on maize genetics in the 1930s through 1950s resulted in the discovery of transposons, but its significance was not recognized at the time. (b) Efforts to appropriately mentor and to provide continued societal support for women in science and medicine may someday help alleviate some of the issues preventing gender equality at all levels in science and medicine. (credit a: modification of work by Smithsonian Institution; credit b: modification of work by Haynie SL, Hinkle AS, Jones NL, Martin CA, Olsiewski PJ, Roberts MF)

Part 2

Based upon his symptoms, Alex's physician suspects that he is suffering from a foodborne illness that he acquired during his travels. Possibilities include bacterial infection (e.g., enterotoxigenic *E. coli*, *Vibrio cholerae*, *Campylobacter jejuni*, *Salmonella*), viral infection (rotavirus or norovirus), or protozoan infection (*Giardia lamblia*, *Cryptosporidium parvum*, or *Entamoeba histolytica*).

His physician orders a stool sample to identify possible causative agents (e.g., bacteria, cysts) and to look for the presence of blood because certain types of infectious agents (like *C. jejuni*, *Salmonella*, and *E. histolytica*) are associated with the production of bloody stools.

Alex's stool sample showed neither blood nor cysts. Following analysis of his stool sample and based upon

8. S.A. Seabury et al. "Trends in the Earnings of Male and Female Health Care Professionals in the United States, 1987 to 2010." *Journal of the American Medical Association Internal Medicine* 173 no. 18 (2013):1748–1750.

9. E. Chung. "Tim Hunt, Sexism and Science: The Real 'Trouble With Girls' in Labs." *CBC News Technology and Science*, June 12, 2015. http://www.cbc.ca/news/technology/tim-hunt-sexism-and-science-the-real-trouble-with-girls-in-labs-1.3110133. Accessed 8/4/2016.

10. American Association of University Women. "Building a STEM Pipeline for Girls and Women." http://www.aauw.org/what-we-do/stem-education/. Accessed June 10, 2016.

11. National Aeronautics and Space Administration. "Outreach Programs: Women and Girls Initiative." http://women.nasa.gov/outreach-programs/. Accessed June 10, 2016.

his recent travel history, the hospital physician suspected that Alex was suffering from traveler's diarrhea caused by enterotoxigenic *E. coli* (ETEC), the causative agent of most traveler's diarrhea. To verify the diagnosis and rule out other possibilities, Alex's physician ordered a diagnostic lab test of his stool sample to look for DNA sequences encoding specific virulence factors of ETEC. The physician instructed Alex to drink lots of fluids to replace what he was losing and discharged him from the hospital.

ETEC produces several plasmid-encoded virulence factors that make it pathogenic compared with typical *E. coli*. These include the secreted toxins heat-labile enterotoxin (LT) and heat-stabile enterotoxin (ST), as well as colonization factor (CF). Both LT and ST cause the excretion of chloride ions from intestinal cells to the intestinal lumen, causing a consequent loss of water from intestinal cells, resulting in diarrhea. CF encodes a bacterial protein that aids in allowing the bacterium to adhere to the lining of the small intestine.

- Why did Alex's physician use genetic analysis instead of either isolation of bacteria from the stool sample or direct Gram stain of the stool sample alone?

Jump to the ***next*** *Clinical Focus box. Go back to the* ***previous (https://legacy.cnx.org/content/m58835/latest/#fs-id1172098310660)*** *Clinical Focus box.*

Fill in the Blank

Exercise 12.1

The end of a nucleic acid strand with a free phosphate group is called the ________.

Solution
5′ end

True/False

Exercise 12.2

The work of Rosalind Franklin and R.G. Gosling was important in demonstrating the helical nature of DNA.

Solution
True

Exercise 12.3

The A-T base pair has more hydrogen bonding than the C-G base pair.

Solution
False

Short Answer

Exercise 12.4

What is the role of phosphodiester bonds within the sugar-phosphate backbone of DNA?

Exercise 12.5

What is meant by the term “antiparallel?”

Exercise 12.6

Why is DNA with a high GC content more difficult to denature than that with a low GC content?

12.2 | Rosalind Franklin

A short biography of Rosalind Franklin and her contributions to the discovery of the structure of DNA

Rosalind Franklin and Photo 51

Rosalind Elsie Franklin (25 July 1920 – 16 April 1958) was an English chemist and X-ray crystallographer who made contributions to the understanding of the ***molecular structures of DNA*** (deoxyribonucleic acid), RNA (ribonucleic acid), viruses, coal, and graphite. Although her works on coal and viruses were appreciated in her lifetime, her contributions to the discovery of the structure of DNA were largely recognized posthumously.

Figure 12.11 Rosalind Elsie Franklin (25 July 1920 – 16 April 1958). Image Credit (left): By Jewish Chronicle Archive/Heritage-Imageshttp://www.britannica.com/EBchecked/topic-art/217394/99712/Rosalind-Franklin, Fair use, **https://en.wikipedia.org/w/index.php?curid=24959067 (https://en.wikipedia.org/w/index.php?curid=24959067)**

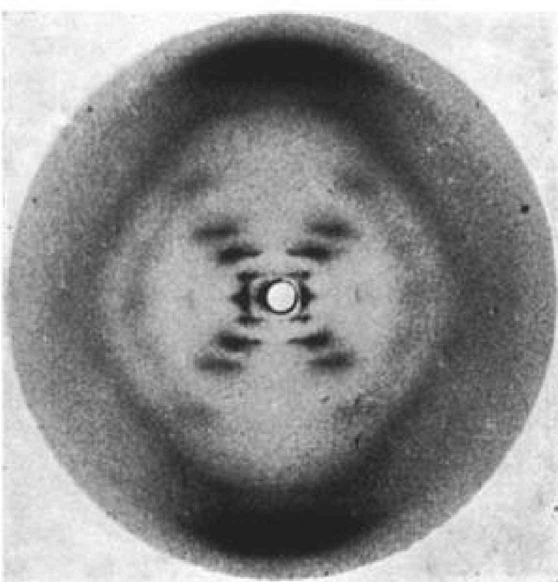


Figure 12.12 Rosalind Franklin's Photo 51, credited for the elucidation of the alpha helix structure theory of DNA. By Source (WP:NFCC#4), Fair use, **https://en.wikipedia.org/w/index.php?curid=38068629 (https://en.wikipedia.org/w/index.php?curid=38068629)**

Born to a prominent British Jewish family, Franklin studied the Natural Sciences Tripos at Newnham College, Cambridge, from which she graduated in 1941. Earning a research fellowship, she joined the University of Cambridge physical chemistry laboratory under Ronald George Wreyford Norrish, where she earned a Ph.D. in 1945. She went to Paris in 1947 as a chercheur (post-doctoral researcher) under **Jacques Mering (https://en.wikipedia.org/wiki/Jacques_Mering)** at the Laboratoire Central des Services Chimiques de l'Etat, where she became an accomplished X-ray crystallographer. She became a research associate at King's College London in 1951 and worked on X-ray diffraction studies, which would ***facilitate the*** **double helix (https://en.wikipedia.org/wiki/Double_helix)** ***theory of the DNA***. She died in 1958 at the age of 37 of ovarian cancer.

Franklin is best known for her work on the ***X-ray diffraction images of DNA, particularly*** **Photo 51 (https://en.wikipedia.org/wiki/Photo_51)** , while at King's College London, which led to the discovery of the DNA double helix for which **James Watson (https://en.wikipedia.org/wiki/James_Watson)** , **Francis Crick (https://en.wikipedia.org/wiki/Francis_Crick)** and **Maurice Wilkins (https://en.wikipedia.org/wiki/Maurice_Wilkins)** shared the Nobel Prize in Physiology or Medicine in 1962. Watson suggested that Franklin would have ideally been awarded a Nobel Prize in Chemistry, along with Wilkins, but, although there was not yet a rule against posthumous awards, the Nobel Committee generally does not make posthumous nominations.

After finishing her work on DNA, Franklin led pioneering work at Birkbeck on the molecular structures of viruses. Her team member **Aaron Klug (https://en.wikipedia.org/wiki/Aaron_Klug)** continued her research, winning the Nobel Prize in Chemistry in 1982. Read more about **Rosalind Franklin’s legacy at Wikipedia (https://en.wikipedia.org/wiki/Rosalind_Franklin)** .

Image and Content Credit: **https://en.wikipedia.org/wiki/Rosalind_Franklin (https://en.wikipedia.org/wiki/Rosalind_Franklin)** , **https://en.wikipedia.org/wiki/Photo_51 (https://en.wikipedia.org/wiki/Photo_51)**

12.3 | DNA Replication

By the end of this section, you will be able to:

- Explain the process of DNA replication
- Explain the importance of telomerase to DNA replication
- Describe mechanisms of DNA repair

When a cell divides, it is important that each daughter cell receives an identical copy of the DNA. This is accomplished by the process of DNA replication. The replication of DNA occurs during the synthesis phase, or S phase, of the cell cycle, before the cell enters mitosis or meiosis.

The elucidation of the structure of the double helix provided a hint as to how DNA is copied. Recall that adenine nucleotides pair with thymine nucleotides, and cytosine with guanine. This means that the two strands are complementary to each other. For example, a strand of DNA with a nucleotide sequence of AGTCATGA will have a complementary strand with the sequence TCAGTACT (Figure 12.13).

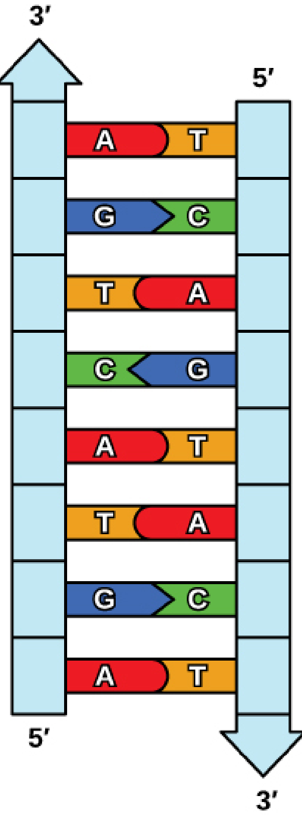


Figure 12.13 The two strands of DNA are complementary, meaning the sequence of bases in one strand can be used to create the correct sequence of bases in the other strand.

Because of the complementarity of the two strands, having one strand means that it is possible to recreate the other strand. This model for replication suggests that the two strands of the double helix separate during replication, and each strand serves as a template from which the new complementary strand is copied (Figure 12.14).

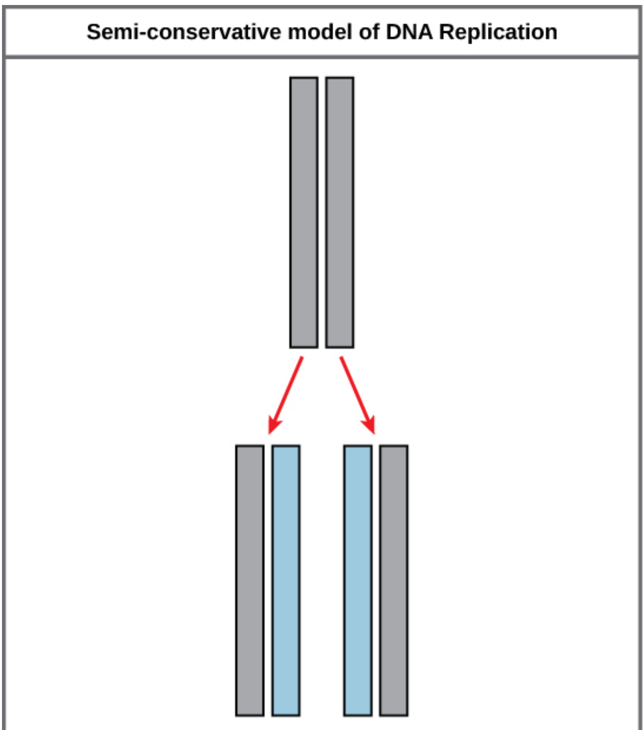


Figure 12.14 The semiconservative model of DNA replication is shown. Gray indicates the original DNA strands, and blue indicates newly synthesized DNA.

During DNA replication, each of the two strands that make up the double helix serves as a template from which new strands are copied. The new strand will be complementary to the parental or "old" strand. Each new double strand consists of one parental strand and one new daughter strand. This is known as **semiconservative replication**. When two DNA copies are formed, they have an identical sequence of nucleotide bases and are divided equally into two daughter cells.

DNA Replication in Eukaryotes

Because eukaryotic genomes are very complex, DNA replication is a very complicated process that involves several enzymes and other proteins. It occurs in three main stages: initiation, elongation, and termination.

Recall that eukaryotic DNA is bound to proteins known as histones to form structures called nucleosomes. During initiation, the DNA is made accessible to the proteins and enzymes involved in the replication process. How does the replication machinery know where on the DNA double helix to begin? It turns out that there are specific nucleotide sequences called origins of replication at which replication begins. Certain proteins bind to the origin of replication while an enzyme called **helicase** unwinds and opens up the DNA helix. As the DNA opens up, Y-shaped structures called **replication forks** are formed (**Figure 12.15**). Two replication forks are formed at the origin of replication, and these get extended in both directions as replication proceeds. There are multiple origins of replication on the eukaryotic chromosome, such that replication can occur simultaneously from several places in the genome.

During elongation, an enzyme called **DNA polymerase** adds DNA nucleotides to the 3' end of the template. Because DNA polymerase can only add new nucleotides at the end of a backbone, a **primer** sequence, which provides this starting point, is added with complementary RNA nucleotides. This primer is removed later, and the nucleotides are replaced with DNA nucleotides. One strand, which is complementary to the parental DNA strand, is synthesized continuously toward the replication fork so the polymerase can add nucleotides in this direction. This continuously synthesized strand is known as the **leading strand**. Because DNA polymerase can only synthesize DNA in a 5' to 3' direction, the other new strand is put together in short pieces called **Okazaki fragments**. The Okazaki fragments each require a primer made of RNA to start the synthesis. The strand with the Okazaki fragments is known as the **lagging strand**. As synthesis proceeds, an enzyme removes the RNA primer, which is then replaced with DNA nucleotides, and the gaps between fragments are sealed by an enzyme called **DNA ligase**.

The process of DNA replication can be summarized as follows:

1. DNA unwinds at the origin of replication.
2. New bases are added to the complementary parental strands. One new strand is made continuously, while the other strand is made in pieces.
3. Primers are removed, new DNA nucleotides are put in place of the primers and the backbone is sealed by DNA ligase.

Visual Connection

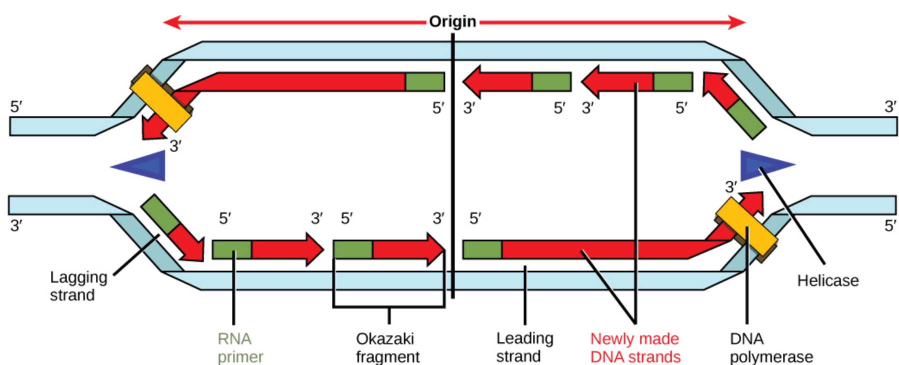


Figure 12.15 A replication fork is formed by the opening of the origin of replication, and helicase separates the DNA strands. An RNA primer is synthesized, and is elongated by the DNA polymerase. On the leading strand, DNA is synthesized continuously, whereas on the lagging strand, DNA is synthesized in short stretches. The DNA fragments are joined by DNA ligase (not shown).

You isolate a cell strain in which the joining together of Okazaki fragments is impaired and suspect that a mutation has occurred in an enzyme found at the replication fork. Which enzyme is most likely to be mutated?

Telomere Replication

Because eukaryotic chromosomes are linear, DNA replication comes to the end of a line in eukaryotic chromosomes. As you have learned, the DNA polymerase enzyme can add nucleotides in only one direction. In the leading strand, synthesis continues until the end of the chromosome is reached; however, on the lagging strand there is no place for a primer to be made for the DNA fragment to be copied at the end of the chromosome. This presents a problem for the cell because the ends remain unpaired, and over time these ends get progressively shorter as cells continue to divide. The ends of the linear chromosomes are known as **telomeres**, which have repetitive sequences that do not code for a particular gene. As a consequence, it is telomeres that are shortened with each round of DNA replication instead of genes. For example, in humans, a six base-pair sequence, TTAGGG, is repeated 100 to 1000 times. The discovery of the enzyme **telomerase** (Figure 12.16) helped in the understanding of how chromosome ends are maintained. The telomerase attaches to the end of the chromosome, and complementary bases to the RNA template are added on the end of the DNA strand. Once the lagging strand template is sufficiently elongated, DNA polymerase can now add nucleotides that are complementary to the ends of the chromosomes. Thus, the ends of the chromosomes are replicated.

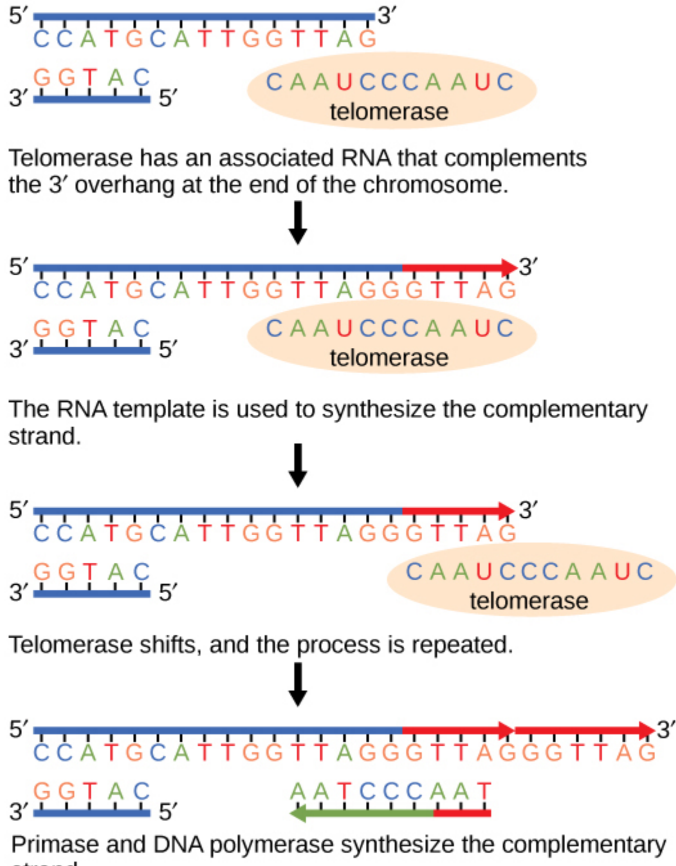


Figure 12.16 The ends of linear chromosomes are maintained by the action of the telomerase enzyme.

Telomerase is typically found to be active in germ cells, adult stem cells, and some cancer cells. For her discovery of telomerase and its action, Elizabeth Blackburn (**Figure 12.17**) received the Nobel Prize for Medicine and Physiology in 2009.

Figure 12.17 Elizabeth Blackburn, 2009 Nobel Laureate, was the scientist who discovered how telomerase works. (credit: U.S. Embassy, Stockholm, Sweden)

Telomerase is not active in adult somatic cells. Adult somatic cells that undergo cell division continue to have their telomeres shortened. This essentially means that telomere shortening is associated with aging. In 2010, scientists found that telomerase can reverse some age-related conditions in mice, and this may have potential in

regenerative medicine.[12] Telomerase-deficient mice were used in these studies; these mice have tissue atrophy, stem-cell depletion, organ system failure, and impaired tissue injury responses. Telomerase reactivation in these mice caused extension of telomeres, reduced DNA damage, reversed neurodegeneration, and improved functioning of the testes, spleen, and intestines. Thus, telomere reactivation may have potential for treating age-related diseases in humans.

DNA Replication in Prokaryotes

Recall that the prokaryotic chromosome is a circular molecule with a less extensive coiling structure than eukaryotic chromosomes. The eukaryotic chromosome is linear and highly coiled around proteins. While there are many similarities in the DNA replication process, these structural differences necessitate some differences in the DNA replication process in these two life forms.

DNA replication has been extremely well-studied in prokaryotes, primarily because of the small size of the genome and large number of variants available. *Escherichia coli* has 4.6 million base pairs in a single circular chromosome, and all of it gets replicated in approximately 42 minutes, starting from a single origin of replication and proceeding around the chromosome in both directions. This means that approximately 1000 nucleotides are added per second. The process is much more rapid than in eukaryotes. **Table 12.1** summarizes the differences between prokaryotic and eukaryotic replications.

Differences between Prokaryotic and Eukaryotic Replications

Property	Prokaryotes	Eukaryotes
Origin of replication	Single	Multiple
Rate of replication	1000 nucleotides/s	50 to 100 nucleotides/s
Chromosome structure	circular	linear
Telomerase	Not present	Present

Table 12.1

Click through a **tutorial (http://openstax.org/l/DNA_replicatio2)** on DNA replication.

DNA Repair

DNA polymerase can make mistakes while adding nucleotides. It edits the DNA by proofreading every newly added base. Incorrect bases are removed and replaced by the correct base, and then polymerization continues (**Figure 12.18a**). Most mistakes are corrected during replication, although when this does not happen, the **mismatch repair** mechanism is employed. Mismatch repair enzymes recognize the wrongly incorporated base and excise it from the DNA, replacing it with the correct base (**Figure 12.18b**). In yet another type of repair, **nucleotide excision repair**, the DNA double strand is unwound and separated, the incorrect bases are removed along with a few bases on the 5' and 3' end, and these are replaced by copying the template with the help of DNA polymerase (**Figure 12.18c**). Nucleotide excision repair is particularly important in correcting thymine dimers, which are primarily caused by ultraviolet light. In a thymine dimer, two thymine nucleotides adjacent to each other on one strand are covalently bonded to each other rather than their complementary bases. If the dimer is not removed and repaired it will lead to a mutation. Individuals with flaws in their nucleotide excision repair genes show extreme sensitivity to sunlight and develop skin cancers early in life.

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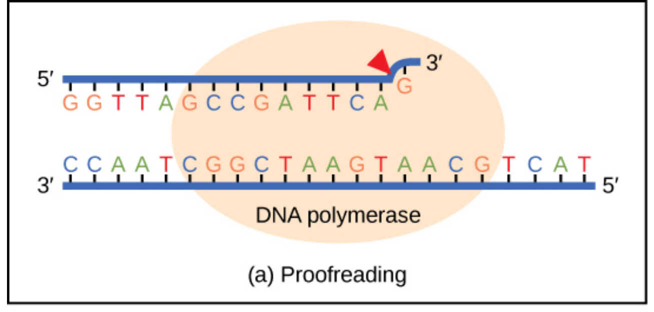
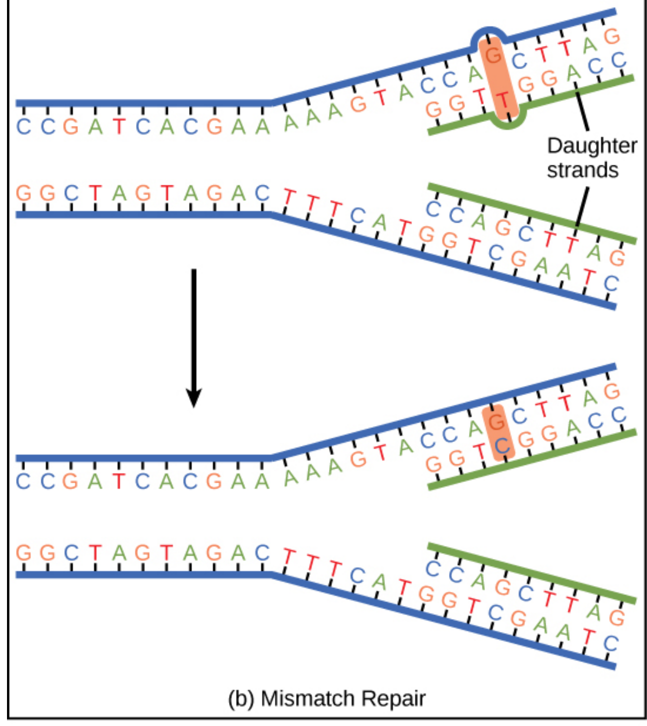
12. Mariella Jaskelioff, et al., "Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice," *Nature*, 469 (2011):102–7.





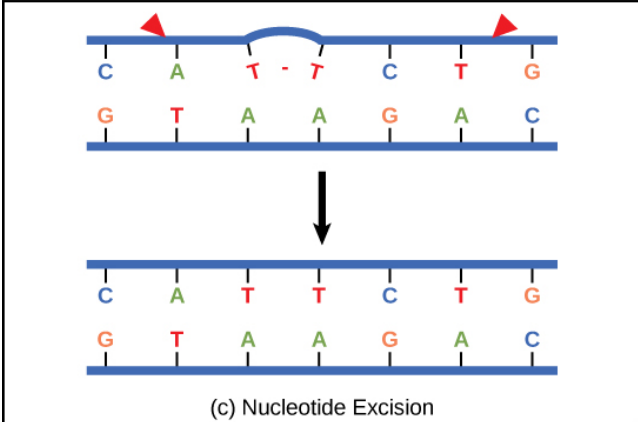


Figure 12.18 Proofreading by DNA polymerase (a) corrects errors during replication. In mismatch repair (b), the incorrectly added base is detected after replication. The mismatch repair proteins detect this base and remove it from the newly synthesized strand by nuclease action. The gap is now filled with the correctly paired base. Nucleotide excision (c) repairs thymine dimers. When exposed to UV, thymines lying adjacent to each other can form thymine dimers. In normal cells, they are excised and replaced.

Most mistakes are corrected; if they are not, they may result in a **mutation**—defined as a permanent change in the DNA sequence. Mutations in repair genes may lead to serious consequences like cancer.

12.4 | Structure and Function of RNA

Learning Objectives

By the end of this section, you will be able to:

- Describe the biochemical structure of ribonucleotides
- Describe the similarities and differences between RNA and DNA
- Describe the functions of the three main types of RNA used in protein synthesis
- Explain how RNA can serve as hereditary information

Structurally speaking, **ribonucleic acid (RNA)**, is quite similar to DNA. However, whereas DNA molecules are typically long and double stranded, RNA molecules are much shorter and are typically single stranded. RNA molecules perform a variety of roles in the cell but are mainly involved in the process of protein synthesis (translation) and its regulation.

RNA Structure

RNA is typically single stranded and is made of **ribonucleotides** that are linked by phosphodiester bonds. A ribonucleotide in the RNA chain contains ribose (the pentose sugar), one of the four nitrogenous bases (A, U, G, and C), and a phosphate group. The subtle structural difference between the sugars gives DNA added stability, making DNA more suitable for storage of genetic information, whereas the relative instability of RNA makes it more suitable for its more short-term functions. The RNA-specific pyrimidine **uracil** forms a complementary base pair with adenine and is used instead of the thymine used in DNA. Even though RNA is single stranded, most types of RNA molecules show extensive intramolecular base pairing between complementary sequences within the RNA strand, creating a predictable three-dimensional structure essential for their function (Figure 12.19 and Figure 12.20).

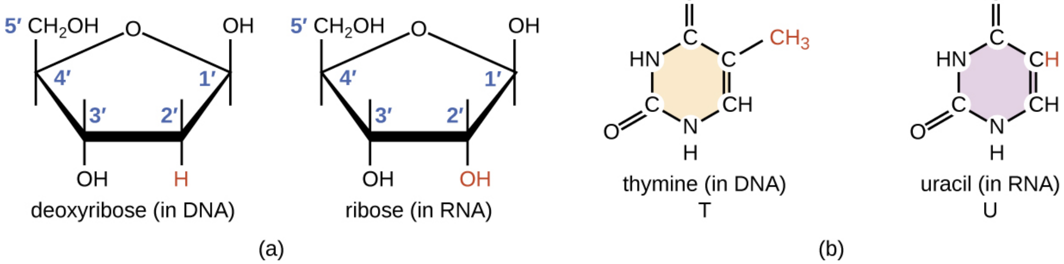


Figure 12.19 (a) Ribonucleotides contain the pentose sugar ribose instead of the deoxyribose found in deoxyribonucleotides. (b) RNA contains the pyrimidine uracil in place of thymine found in DNA.

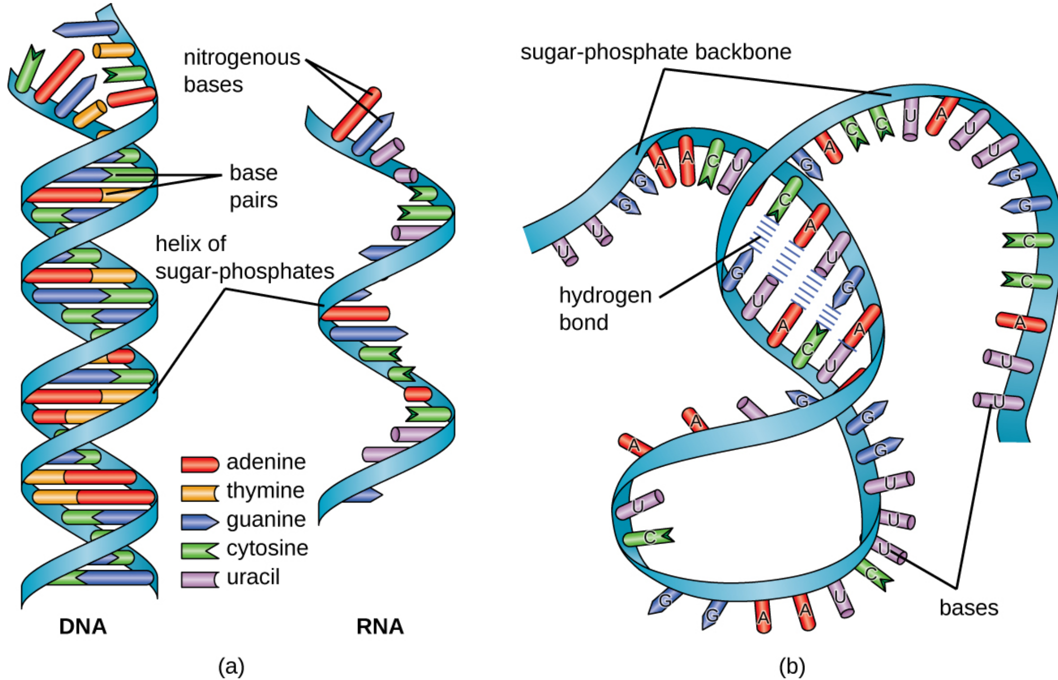


Figure 12.20 (a) DNA is typically double stranded, whereas RNA is typically single stranded. (b) Although it is single stranded, RNA can fold upon itself, with the folds stabilized by short areas of complementary base pairing within the molecule, forming a three-dimensional structure.

- How does the structure of RNA differ from the structure of DNA?

Functions of RNA in Protein Synthesis

Cells access the information stored in DNA by creating RNA to direct the synthesis of proteins through the process of translation. Proteins within a cell have many functions, including building cellular structures and serving as enzyme catalysts for cellular chemical reactions that give cells their specific characteristics. The three main types of RNA directly involved in protein synthesis are **messenger RNA (mRNA)**, **ribosomal RNA (rRNA)**, and **transfer RNA (tRNA)**.

In 1961, French scientists François Jacob and Jacques Monod hypothesized the existence of an intermediary between DNA and its protein products, which they called messenger RNA.[13] Evidence supporting their hypothesis was gathered soon afterwards showing that information from DNA is transmitted to the ribosome for protein synthesis using mRNA. If DNA serves as the complete library of cellular information, mRNA serves as a photocopy of specific information needed at a particular point in time that serves as the instructions to make a protein.

The mRNA carries the message from the DNA, which controls all of the cellular activities in a cell. If a cell requires a certain protein to be synthesized, the gene for this product is "turned on" and the mRNA is synthesized through the process of transcription (see **RNA Transcription (https://legacy.cnx.org/content/m58842/latest/)**). The mRNA then interacts with ribosomes and other cellular machinery (**Figure 12.21**) to direct the synthesis of the protein it encodes during the process of translation (see **Protein Synthesis (https://legacy.cnx.org/content/m58843/latest/)**). mRNA is relatively unstable and short-lived in the cell, especially in prokaryotic cells, ensuring that proteins are only made when needed.

13. A. Rich. "The Era of RNA Awakening: Structural Biology of RNA in the Early Years." *Quarterly Reviews of Biophysics* 42 no. 2 (2009):117–137.

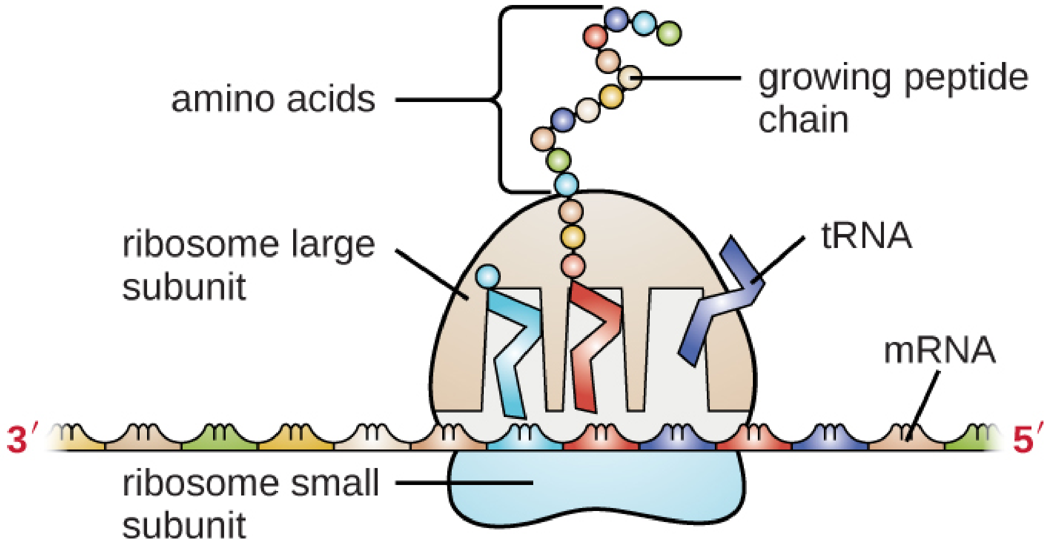


Figure 12.21 A generalized illustration of how mRNA and tRNA are used in protein synthesis within a cell.

rRNA and tRNA are stable types of RNA. In prokaryotes and eukaryotes, tRNA and rRNA are encoded in the DNA, then copied into long RNA molecules that are cut to release smaller fragments containing the individual mature RNA species. In eukaryotes, synthesis, cutting, and assembly of rRNA into ribosomes takes place in the nucleolus region of the nucleus, but these activities occur in the cytoplasm of prokaryotes. Neither of these types of RNA carries instructions to direct the synthesis of a polypeptide, but they play other important roles in protein synthesis.

Ribosomes are composed of rRNA and protein. As its name suggests, rRNA is a major constituent of ribosomes, composing up to about 60% of the ribosome by mass and providing the location where the mRNA binds. The rRNA ensures the proper alignment of the mRNA, tRNA, and the ribosomes; the rRNA of the ribosome also has an enzymatic activity (peptidyl transferase) and catalyzes the formation of the peptide bonds between two aligned amino acids during protein synthesis. Although rRNA had long been thought to serve primarily a structural role, its catalytic role within the ribosome was proven in 2000.[14] Scientists in the laboratories of Thomas Steitz (1940–) and Peter Moore (1939–) at Yale University were able to crystallize the ribosome structure from *Haloarcula marismortui*, a halophilic archaeon isolated from the Dead Sea. Because of the importance of this work, Steitz shared the 2009 Nobel Prize in Chemistry with other scientists who made significant contributions to the understanding of ribosome structure.

Transfer RNA is the third main type of RNA and one of the smallest, usually only 70–90 nucleotides long. It carries the correct amino acid to the site of protein synthesis in the ribosome. It is the base pairing between the tRNA and mRNA that allows for the correct amino acid to be inserted in the polypeptide chain being synthesized (**Figure 12.22**). Any mutations in the tRNA or rRNA can result in global problems for the cell because both are necessary for proper protein synthesis (**Table 12.2**).

14. P. Nissen et al. "The Structural Basis of Ribosome Activity in Peptide Bond Synthesis." *Science* 289 no. 5481 (2000):920–930.

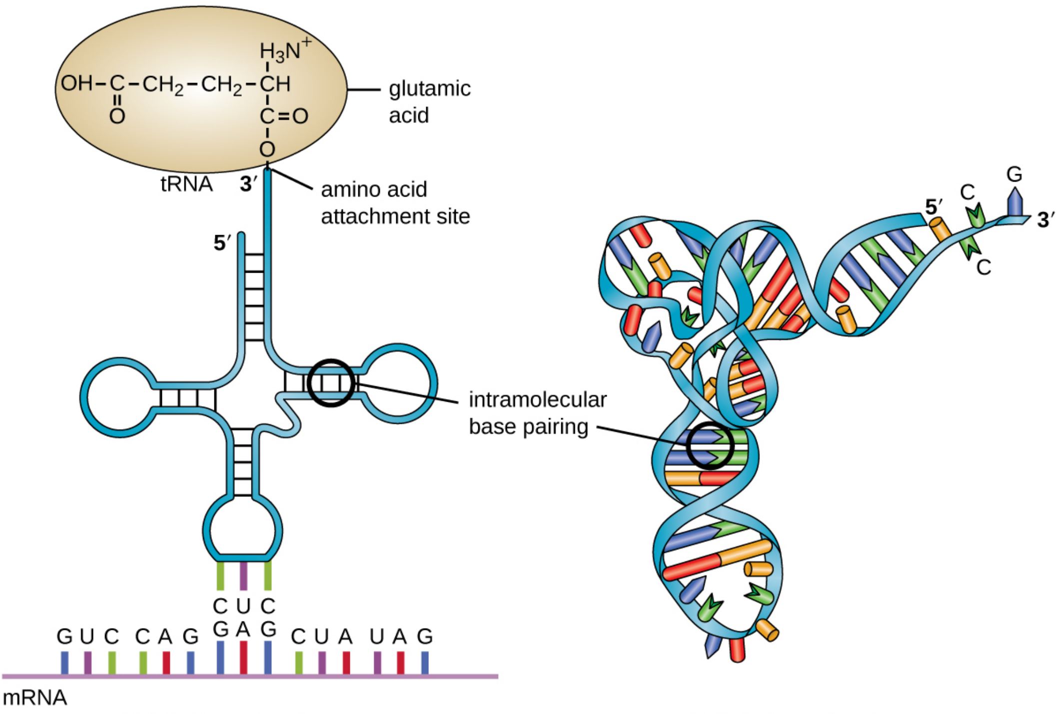


Figure 12.22 A tRNA molecule is a single-stranded molecule that exhibits significant intracellular base pairing, giving it its characteristic three-dimensional shape.

Structure and Function of RNA

	mRNA	rRNA	tRNA
Structure	Short, unstable, single-stranded RNA corresponding to a gene encoded within DNA	Longer, stable RNA molecules composing 60% of ribosome's mass	Short (70-90 nucleotides), stable RNA with extensive intramolecular base pairing; contains an amino acid binding site and an mRNA binding site
Function	Serves as intermediary between DNA and protein; used by ribosome to direct synthesis of protein it encodes	Ensures the proper alignment of mRNA, tRNA, and ribosome during protein synthesis; catalyzes peptide bond formation between amino acids	Carries the correct amino acid to the site of protein synthesis in the ribosome

Table 12.2

- What are the functions of the three major types of RNA molecules involved in protein synthesis?

RNA as Hereditary Information

Although RNA does not serve as the hereditary information in most cells, RNA does hold this function for many viruses that do not contain DNA. Thus, RNA clearly does have the additional capacity to serve as genetic information. Although RNA is typically single stranded within cells, there is significant diversity in viruses. Rhinoviruses, which cause the common cold; influenza viruses; and the Ebola virus are single-stranded RNA viruses. Rotaviruses, which cause severe gastroenteritis in children and other immunocompromised individuals, are examples of double-stranded RNA viruses. Because double-stranded RNA is uncommon in eukaryotic cells, its presence serves as an indicator of viral infection. The implications for a virus having an RNA genome instead

of a DNA genome are discussed in more detail in **Viruses (https://legacy.cnx.org/content/m58808/latest/)** .

Matching

Exercise 12.7

Match the correct molecule with its description:

___tRNA	A. is a major component of ribosome
___rRNA	B. is a copy of the information in a gene
___mRNA	C. carries an amino acid to the ribosome

Solution
C, A, B

True/False

Exercise 12.8

Ribosomes are composed mostly of RNA.

Solution
True

Exercise 12.9

Double-stranded RNA is commonly found inside cells.

Solution
False

Short Answer

Exercise 12.10

What are the differences between DNA nucleotides and RNA nucleotides?

Exercise 12.11

How is the information stored within the base sequence of DNA used to determine a cell's properties?

Exercise 12.12

How do complementary base pairs contribute to intramolecular base pairing within an RNA molecule?

Exercise 12.13

If an antisense RNA has the sequence 5′AUUCGAAUGC3′, what is the sequence of the mRNA to which it will bind? Be sure to label the 5′ and 3′ ends of the molecule you draw.

Exercise 12.14

Why does double-stranded RNA (dsRNA) stimulate RNA interference?

12.5 | Transcription

By the end of this section, you will be able to:

- Explain the central dogma
- Explain the main steps of transcription
- Describe how eukaryotic mRNA is processed

In both prokaryotes and eukaryotes, the second function of DNA (the first was replication) is to provide the information needed to construct the proteins necessary so that the cell can perform all of its functions. To do this, the DNA is “read” or transcribed into an **mRNA** molecule. The mRNA then provides the code to form a protein by a process called translation. Through the processes of transcription and translation, a protein is built with a specific sequence of amino acids that was originally encoded in the DNA. This module discusses the details of transcription.

The Central Dogma: DNA Encodes RNA; RNA Encodes Protein

The flow of genetic information in cells from DNA to mRNA to protein is described by the central dogma (Figure 12.23), which states that genes specify the sequences of mRNAs, which in turn specify the sequences of proteins.

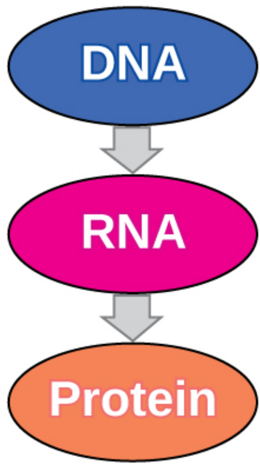


Figure 12.23 The central dogma states that DNA encodes RNA, which in turn encodes protein.

The copying of DNA to mRNA is relatively straightforward, with one nucleotide being added to the mRNA strand for every complementary nucleotide read in the DNA strand. The translation to protein is more complex because groups of three mRNA nucleotides correspond to one amino acid of the protein sequence. However, as we shall see in the next module, the translation to protein is still systematic, such that nucleotides 1 to 3 correspond to amino acid 1, nucleotides 4 to 6 correspond to amino acid 2, and so on.

Transcription: from DNA to mRNA

Both prokaryotes and eukaryotes perform fundamentally the same process of transcription, with the important difference of the membrane-bound nucleus in eukaryotes. With the genes bound in the nucleus, transcription occurs in the nucleus of the cell and the mRNA transcript must be transported to the cytoplasm. The prokaryotes, which include bacteria and archaea, lack membrane-bound nuclei and other organelles, and transcription occurs in the cytoplasm of the cell. In both prokaryotes and eukaryotes, transcription occurs in three main stages: initiation, elongation, and termination.

Initiation

Transcription requires the DNA double helix to partially unwind in the region of mRNA synthesis. The region of unwinding is called a **transcription bubble**. The DNA sequence onto which the proteins and enzymes involved in transcription bind to initiate the process is called a **promoter**. In most cases, promoters exist upstream of the

genes they regulate. The specific sequence of a promoter is very important because it determines whether the corresponding gene is transcribed all of the time, some of the time, or hardly at all (**Figure 12.24**).

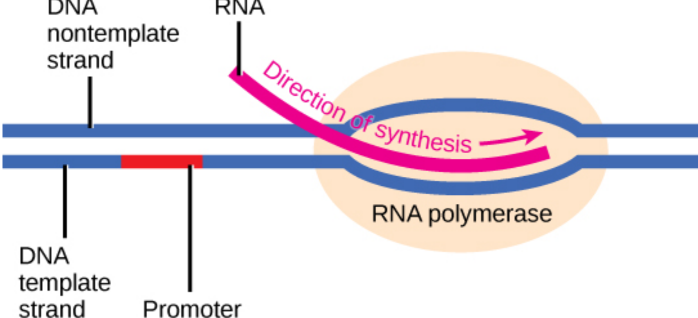


Figure 12.24 The initiation of transcription begins when DNA is unwound, forming a transcription bubble. Enzymes and other proteins involved in transcription bind at the promoter.

Elongation

Transcription always proceeds from one of the two DNA strands, which is called the **template strand**. The mRNA product is complementary to the template strand and is almost identical to the other DNA strand, called the **nontemplate strand**, with the exception that RNA contains a uracil (U) in place of the thymine (T) found in DNA. During elongation, an enzyme called **RNA polymerase** proceeds along the DNA template adding nucleotides by base pairing with the DNA template in a manner similar to DNA replication, with the difference that an RNA strand is being synthesized that does not remain bound to the DNA template. As elongation proceeds, the DNA is continuously unwound ahead of the core enzyme and rewound behind it (**Figure 12.25**).

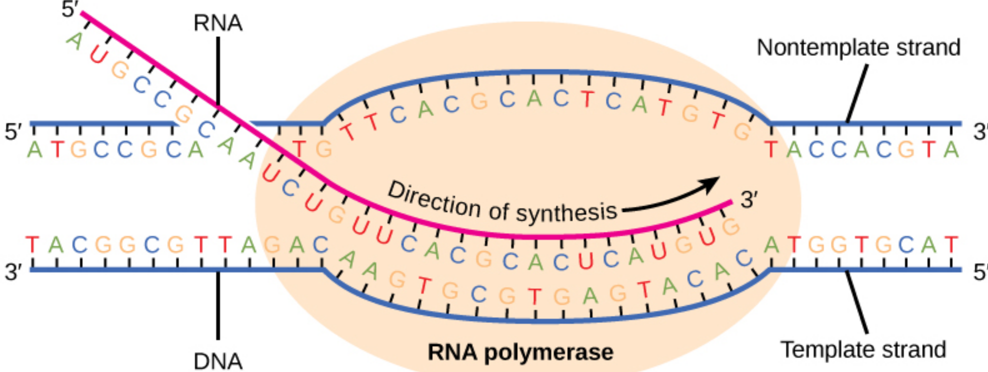


Figure 12.25 During elongation, RNA polymerase tracks along the DNA template, synthesizes mRNA in the 5' to 3' direction, and unwinds then rewinds the DNA as it is read.

Termination

Once a gene is transcribed, the prokaryotic polymerase needs to be instructed to dissociate from the DNA template and liberate the newly made mRNA. Depending on the gene being transcribed, there are two kinds of termination signals, but both involve repeated nucleotide sequences in the DNA template that result in RNA polymerase stalling, leaving the DNA template, and freeing the mRNA transcript.

On termination, the process of transcription is complete. In a prokaryotic cell, by the time termination occurs, the transcript would already have been used to partially synthesize numerous copies of the encoded protein because these processes can occur concurrently using multiple ribosomes (polyribosomes) (**Figure 12.26**). In contrast, the presence of a nucleus in eukaryotic cells precludes simultaneous transcription and translation.

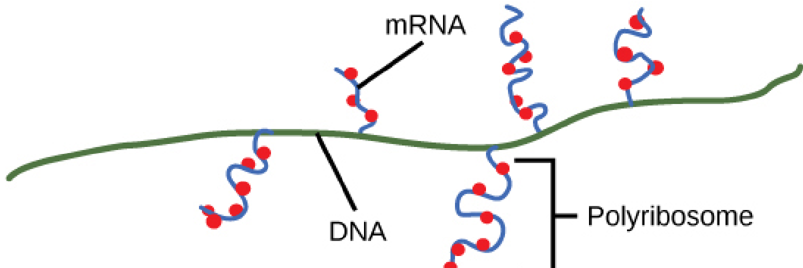


Figure 12.26 Multiple polymerases can transcribe a single bacterial gene while numerous ribosomes concurrently translate the mRNA transcripts into polypeptides. In this way, a specific protein can rapidly reach a high concentration in the bacterial cell.

Eukaryotic RNA Processing

The newly transcribed eukaryotic mRNAs must undergo several processing steps before they can be transferred from the nucleus to the cytoplasm and translated into a protein. The additional steps involved in eukaryotic mRNA maturation create a molecule that is much more stable than a prokaryotic mRNA. For example, eukaryotic mRNAs last for several hours, whereas the typical prokaryotic mRNA lasts no more than five seconds.

The mRNA transcript is first coated in RNA-stabilizing proteins to prevent it from degrading while it is processed and exported out of the nucleus. This occurs while the pre-mRNA still is being synthesized by adding a special nucleotide “cap” to the 5' end of the growing transcript. In addition to preventing degradation, factors involved in protein synthesis recognize the cap to help initiate translation by ribosomes.

Once elongation is complete, an enzyme then adds a string of approximately 200 adenine residues to the 3' end, called the poly-A tail. This modification further protects the pre-mRNA from degradation and signals to cellular factors that the transcript needs to be exported to the cytoplasm.

Eukaryotic genes are composed of protein-coding sequences called **exons** (*ex*-on signifies that they are *ex*pressed) and *int*ervening sequences called **introns** (*int*-ron denotes their *int*ervening role). Introns are removed from the pre-mRNA during processing. Intron sequences in mRNA do not encode functional proteins. It is essential that all of a pre-mRNA’s introns be completely and precisely removed before protein synthesis so that the exons join together to code for the correct amino acids. If the process errs by even a single nucleotide, the sequence of the rejoined exons would be shifted, and the resulting protein would be nonfunctional. The process of removing introns and reconnecting exons is called **splicing** (Figure 12.27). Introns are removed and degraded while the pre-mRNA is still in the nucleus.

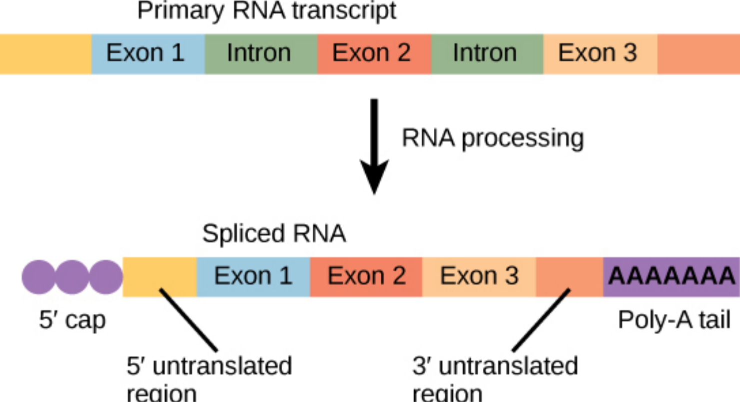


Figure 12.27 Eukaryotic mRNA contains introns that must be spliced out. A 5' cap and 3' tail are also added.

12.6 | Translation

By the end of this section, you will be able to:

- Describe the different steps in protein synthesis
- Discuss the role of ribosomes in protein synthesis
- Describe the genetic code and how the nucleotide sequence determines the amino acid and the protein sequence

The synthesis of proteins is one of a cell's most energy-consuming metabolic processes. In turn, proteins account for more mass than any other component of living organisms (with the exception of water), and proteins perform a wide variety of the functions of a cell. The process of translation, or protein synthesis, involves decoding an mRNA message into a polypeptide product. Amino acids are covalently strung together in lengths ranging from approximately 50 amino acids to more than 1,000.

The Protein Synthesis Machinery

In addition to the mRNA template, many other molecules contribute to the process of translation. The composition of each component may vary across species; for instance, ribosomes may consist of different numbers of ribosomal RNAs (**rRNA**) and polypeptides depending on the organism. However, the general structures and functions of the protein synthesis machinery are comparable from bacteria to human cells. Translation requires the input of an mRNA template, ribosomes, tRNAs, and various enzymatic factors (**Figure 12.28**).

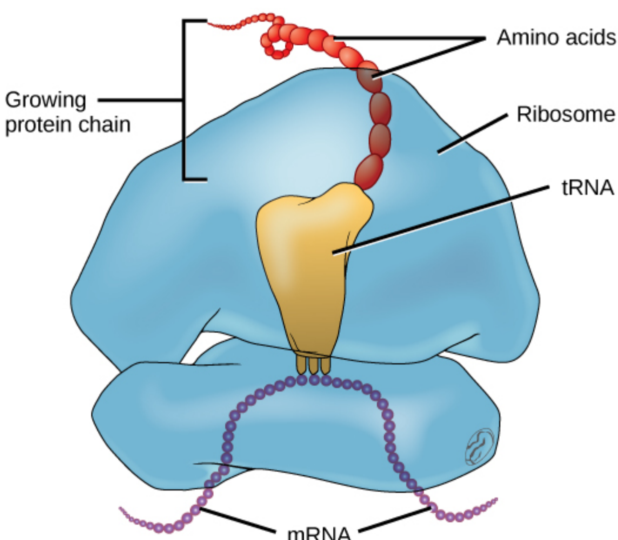


Figure 12.28 The protein synthesis machinery includes the large and small subunits of the ribosome, mRNA, and tRNA. (credit: modification of work by NIGMS, NIH)

In *E. coli*, there are 200,000 ribosomes present in every cell at any given time. A ribosome is a complex macromolecule composed of structural and catalytic rRNAs, and many distinct polypeptides. In eukaryotes, the nucleolus is completely specialized for the synthesis and assembly of rRNAs.

Ribosomes are located in the cytoplasm in prokaryotes and in the cytoplasm and endoplasmic reticulum of eukaryotes. Ribosomes are made up of a large and a small subunit that come together for translation. The small subunit is responsible for binding the mRNA template, whereas the large subunit sequentially binds **tRNAs**, a type of RNA molecule that brings amino acids to the growing chain of the polypeptide. Each mRNA molecule is simultaneously translated by many ribosomes, all synthesizing protein in the same direction.

Depending on the species, 40 to 60 types of tRNA exist in the cytoplasm. Serving as adaptors, specific tRNAs bind to sequences on the mRNA template and add the corresponding amino acid to the polypeptide chain. Therefore, tRNAs are the molecules that actually “translate” the language of RNA into the language of proteins.

For each tRNA to function, it must have its specific amino acid bonded to it. In the process of tRNA “charging,” each tRNA molecule is bonded to its correct amino acid.

The Genetic Code

To summarize what we know to this point, the cellular process of transcription generates messenger RNA (mRNA), a mobile molecular copy of one or more genes with an alphabet of A, C, G, and uracil (U). Translation of the mRNA template converts nucleotide-based genetic information into a protein product. Protein sequences consist of 20 commonly occurring amino acids; therefore, it can be said that the protein alphabet consists of 20 letters. Each amino acid is defined by a three-nucleotide sequence called the triplet **codon**. The relationship between a nucleotide codon and its corresponding amino acid is called the **genetic code**.

Given the different numbers of “letters” in the mRNA and protein “alphabets,” combinations of nucleotides corresponded to single amino acids. Using a three-nucleotide code means that there are a total of 64 (4 × 4 × 4) possible combinations; therefore, a given amino acid is encoded by more than one nucleotide triplet (**Figure 12.29**).

Second letter

First letter	U	C	A	G	Third letter
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

Figure 12.29 This figure shows the genetic code for translating each nucleotide triplet, or codon, in mRNA into an amino acid or a termination signal in a nascent protein. (credit: modification of work by NIH)

Three of the 64 codons terminate protein synthesis and release the polypeptide from the translation machinery. These triplets are called **stop codons**. Another codon, AUG, also has a special function. In addition to specifying the amino acid methionine, it also serves as the **start codon** to initiate translation. The reading frame for translation is set by the AUG start codon near the 5' end of the mRNA. The genetic code is universal. With a few exceptions, virtually all species use the same genetic code for protein synthesis, which is powerful evidence that all life on Earth shares a common origin.

The Mechanism of Protein Synthesis

Just as with mRNA synthesis, protein synthesis can be divided into three phases: initiation, elongation, and termination. The process of translation is similar in prokaryotes and eukaryotes. Here we will explore how translation occurs in *E. coli*, a representative prokaryote, and specify any differences between prokaryotic and eukaryotic translation.

Protein synthesis begins with the formation of an initiation complex. In *E. coli*, this complex involves the small ribosome subunit, the mRNA template, three initiation factors, and a special initiator tRNA. The initiator tRNA interacts with the AUG start codon, and links to a special form of the amino acid methionine that is typically removed from the polypeptide after translation is complete.

In prokaryotes and eukaryotes, the basics of polypeptide elongation are the same, so we will review elongation from the perspective of *E. coli*. The large ribosomal subunit of *E. coli* consists of three compartments: the A site binds incoming charged tRNAs (tRNAs with their attached specific amino acids). The P site binds charged tRNAs

carrying amino acids that have formed bonds with the growing polypeptide chain but have not yet dissociated from their corresponding tRNA. The E site releases dissociated tRNAs so they can be recharged with free amino acids. The ribosome shifts one codon at a time, catalyzing each process that occurs in the three sites. With each step, a charged tRNA enters the complex, the polypeptide becomes one amino acid longer, and an uncharged tRNA departs. The energy for each bond between amino acids is derived from GTP, a molecule similar to ATP (**Figure 12.30**). Amazingly, the *E. coli* translation apparatus takes only 0.05 seconds to add each amino acid, meaning that a 200-amino acid polypeptide could be translated in just 10 seconds.

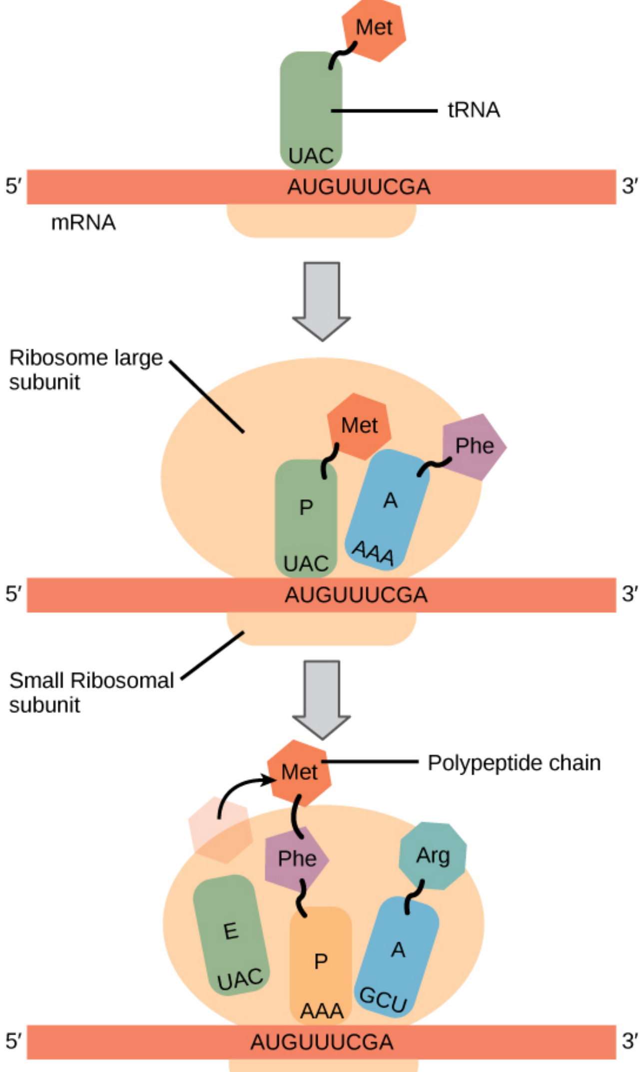


Figure 12.30 Translation begins when a tRNA anticodon recognizes a codon on the mRNA. The large ribosomal subunit joins the small subunit, and a second tRNA is recruited. As the mRNA moves relative to the ribosome, the polypeptide chain is formed. Entry of a release factor into the A site terminates translation and the components dissociate.

Termination of translation occurs when a stop codon (UAA, UAG, or UGA) is encountered. When the ribosome encounters the stop codon, the growing polypeptide is released and the ribosome subunits dissociate and leave the mRNA. After many ribosomes have completed translation, the mRNA is degraded so the nucleotides can be reused in another transcription reaction.

Transcribe a gene and translate it to protein using complementary pairing and the genetic code at **this site (http://openstax.org/l/create_protein2)** .

12.7 | How Genes Are Regulated

By the end of this section, you will be able to:

- Discuss why every cell does not express all of its genes
- Describe how prokaryotic gene expression occurs at the transcriptional level
- Understand that eukaryotic gene expression occurs at the epigenetic, transcriptional, post-transcriptional, translational, and post-translational levels

For a cell to function properly, necessary proteins must be synthesized at the proper time. All organisms and cells control or regulate the transcription and translation of their DNA into protein. The process of turning on a gene to produce RNA and protein is called **gene expression**. Whether in a simple unicellular organism or in a complex multicellular organism, each cell controls when and how its genes are expressed. For this to occur, there must be a mechanism to control when a gene is expressed to make RNA and protein, how much of the protein is made, and when it is time to stop making that protein because it is no longer needed.

Cells in multicellular organisms are specialized; cells in different tissues look very different and perform different functions. For example, a muscle cell is very different from a liver cell, which is very different from a skin cell. These differences are a consequence of the expression of different sets of genes in each of these cells. All cells have certain basic functions they must perform for themselves, such as converting the energy in sugar molecules into energy in ATP. Each cell also has many genes that are not expressed, and expresses many that are not expressed by other cells, such that it can carry out its specialized functions. In addition, cells will turn on or off certain genes at different times in response to changes in the environment or at different times during the development of the organism. Unicellular organisms, both eukaryotic and prokaryotic, also turn on and off genes in response to the demands of their environment so that they can respond to special conditions.

The control of gene expression is extremely complex. Malfunctions in this process are detrimental to the cell and can lead to the development of many diseases, including cancer.

Prokaryotic versus Eukaryotic Gene Expression

To understand how gene expression is regulated, we must first understand how a gene becomes a functional protein in a cell. The process occurs in both prokaryotic and eukaryotic cells, just in slightly different fashions.

Because prokaryotic organisms lack a cell nucleus, the processes of transcription and translation occur almost simultaneously. When the protein is no longer needed, transcription stops. As a result, the primary method to control what type and how much protein is expressed in a prokaryotic cell is through the regulation of DNA transcription into RNA. All the subsequent steps happen automatically. When more protein is required, more transcription occurs. Therefore, in prokaryotic cells, the control of gene expression is almost entirely at the transcriptional level.

The first example of such control was discovered using *E*. *coli* in the 1950s and 1960s by French researchers and is called the *lac* operon. The *lac* operon is a stretch of DNA with three adjacent genes that code for proteins that participate in the absorption and metabolism of lactose, a food source for *E*. *coli*. When lactose is not present in the bacterium’s environment, the *lac* genes are transcribed in small amounts. When lactose is present, the genes are transcribed and the bacterium is able to use the lactose as a food source. The operon also contains a promoter sequence to which the RNA polymerase binds to begin transcription; between the promoter and the three genes is a region called the operator. When there is no lactose present, a protein known as a repressor

binds to the operator and prevents RNA polymerase from binding to the promoter, except in rare cases. Thus very little of the protein products of the three genes is made. When lactose is present, an end product of lactose metabolism binds to the repressor protein and prevents it from binding to the operator. This allows RNA polymerase to bind to the promoter and freely transcribe the three genes, allowing the organism to metabolize the lactose.

Eukaryotic cells, in contrast, have intracellular organelles and are much more complex. Recall that in eukaryotic cells, the DNA is contained inside the cell's nucleus and it is transcribed into mRNA there. The newly synthesized mRNA is then transported out of the nucleus into the cytoplasm, where ribosomes translate the mRNA into protein. The processes of transcription and translation are physically separated by the nuclear membrane; transcription occurs only within the nucleus, and translation only occurs outside the nucleus in the cytoplasm. The regulation of gene expression can occur at all stages of the process (Figure 12.31). Regulation may occur when the DNA is uncoiled and loosened from nucleosomes to bind transcription factors (**epigenetic** level), when the RNA is transcribed (transcriptional level), when RNA is processed and exported to the cytoplasm after it is transcribed (**post-transcriptional** level), when the RNA is translated into protein (translational level), or after the protein has been made (**post-translational** level).

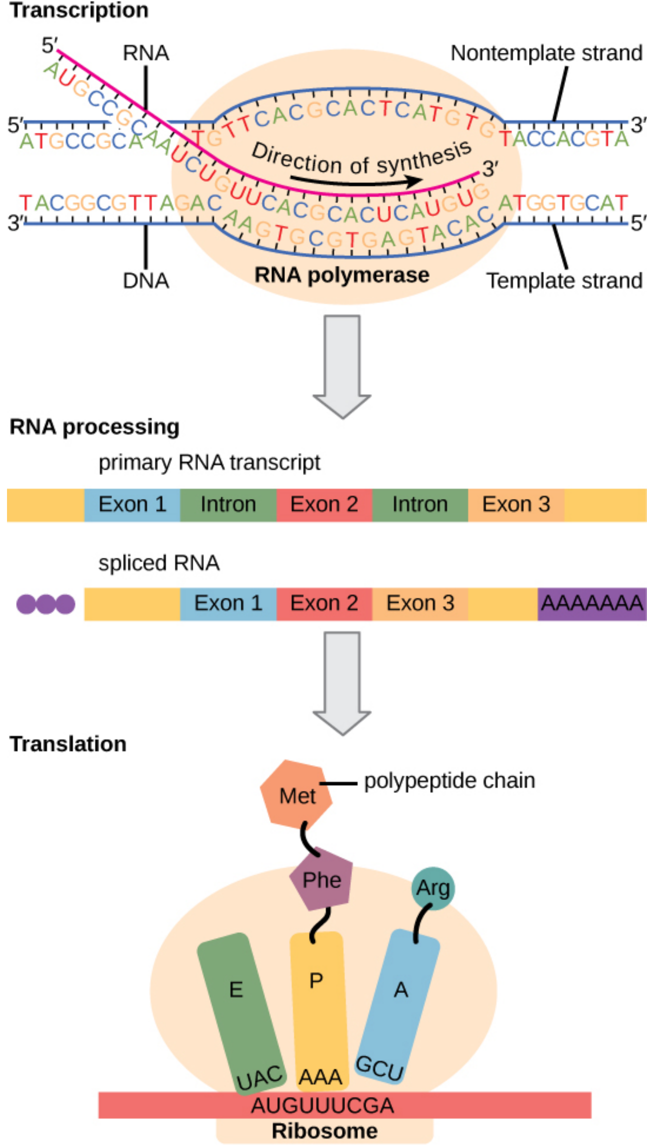


Figure 12.31 Eukaryotic gene expression is regulated during transcription and RNA processing, which take place in the nucleus, as well as during protein translation, which takes place in the cytoplasm. Further regulation may occur through post-translational modifications of proteins.

The differences in the regulation of gene expression between prokaryotes and eukaryotes are summarized in

Table 12.3.

Differences in the Regulation of Gene Expression of Prokaryotic and Eukaryotic Organisms

Prokaryotic organisms	Eukaryotic organisms
Lack nucleus	Contain nucleus
RNA transcription and protein translation occur almost simultaneously	RNA transcription occurs prior to protein translation, and it takes place in the nucleus. RNA translation to protein occurs in the cytoplasm. RNA post-processing includes addition of a 5' cap, poly-A tail, and excision of introns and splicing of exons.
Gene expression is regulated primarily at the transcriptional level	Gene expression is regulated at many levels (epigenetic, transcriptional, post-transcriptional, translational, and post-translational)

Table 12.3

evolution IN ACTION

Alternative RNA Splicing

In the 1970s, genes were first observed that exhibited **alternative RNA splicing**. Alternative RNA splicing is a mechanism that allows different protein products to be produced from one gene when different combinations of introns (and sometimes exons) are removed from the transcript (Figure 12.32). This alternative splicing can be haphazard, but more often it is controlled and acts as a mechanism of gene regulation, with the frequency of different splicing alternatives controlled by the cell as a way to control the production of different protein products in different cells, or at different stages of development. Alternative splicing is now understood to be a common mechanism of gene regulation in eukaryotes; according to one estimate, 70% of genes in humans are expressed as multiple proteins through alternative splicing.

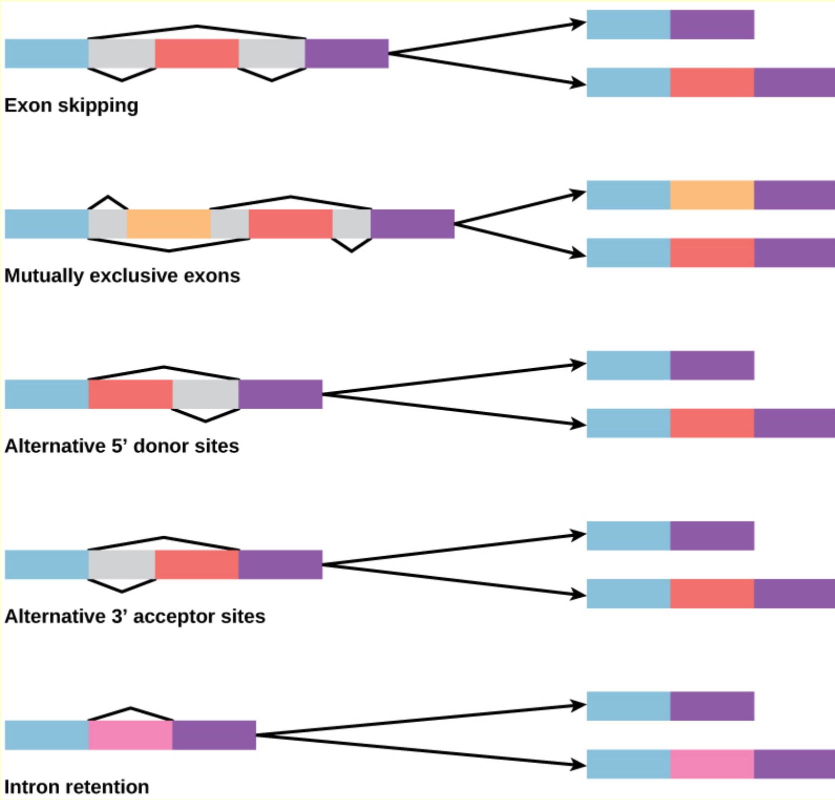


Figure 12.32 There are five basic modes of alternative splicing. Segments of pre-mRNA with exons shown in blue, red, orange, and pink can be spliced to produce a variety of new mature mRNA segments.

How could alternative splicing evolve? Introns have a beginning and ending recognition sequence, and it is easy to imagine the failure of the splicing mechanism to identify the end of an intron and find the end of the next intron, thus removing two introns and the intervening exon. In fact, there are mechanisms in place to prevent such exon skipping, but mutations are likely to lead to their failure. Such “mistakes” would more than likely produce a nonfunctional protein. Indeed, the cause of many genetic diseases is alternative splicing rather than mutations in a sequence. However, alternative splicing would create a protein variant without the loss of the original protein, opening up possibilities for adaptation of the new variant to new functions. Gene duplication has played an important role in the evolution of new functions in a similar way—by providing genes that may evolve without eliminating the original functional protein.

12.8 | Structure and Function of Cellular Genomes

Learning Objectives

By the end of this section, you will be able to:

- Define gene and genotype and differentiate genotype from phenotype
- Describe chromosome structure and packaging
- Compare prokaryotic and eukaryotic chromosomes
- Explain why extrachromosomal DNA is important in a cell

Thus far, we have discussed the structure and function of individual pieces of DNA and RNA. In this section, we will discuss how all of an organism's genetic material—collectively referred to as its **genome**—is organized inside of the cell. Since an organism's genetics to a large extent dictate its characteristics, it should not be surprising that organisms differ in the arrangement of their DNA and RNA.

Genotype versus Phenotype

All cellular activities are encoded within a cell's DNA. The sequence of bases within a DNA molecule represents the genetic information of the cell. Segments of DNA molecules are called **gene**s, and individual genes contain the instructional code necessary for synthesizing various proteins, enzymes, or stable RNA molecules.

The full collection of genes that a cell contains within its genome is called its **genotype**. However, a cell does not express all of its genes simultaneously. Instead, it turns on (expresses) or turns off certain genes when necessary. The set of genes being expressed at any given point in time determines the cell's activities and its observable characteristics, referred to as its **phenotype**. Genes that are always expressed are known as constitutive genes; some constitutive genes are known as housekeeping genes because they are necessary for the basic functions of the cell.

While the genotype of a cell remains constant, the phenotype may change in response to environmental signals (e.g., changes in temperature or nutrient availability) that affect which nonconstitutive genes are expressed. For example, the oral bacterium *Streptococcus mutans* produces a sticky slime layer that allows it to adhere to teeth, forming dental plaque; however, the genes that control the production of the slime layer are only expressed in the presence of sucrose (table sugar). Thus, while the genotype of *S. mutans* is constant, its phenotype changes depending on the presence and absence of sugar in its environment. Temperature can also regulate gene expression. For example, the gram-negative bacterium *Serratia marcescens*, a pathogen frequently associated with hospital-acquired infections, produces a red pigment at 28 °C but not at 37 °C, the normal internal temperature of the human body (**Figure 12.33**).

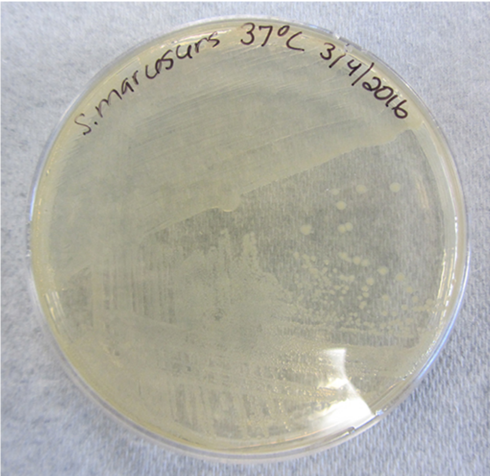


Figure 12.33 Both plates contain strains of *Serratia marcescens* that have the gene for red pigment. However, this gene is expressed at 28 °C (left) but not at 37 °C (right). (credit: modification of work by Ann Auman)

Organization of Genetic Material

The vast majority of an organism's genome is organized into the cell's **chromosomes**, which are discrete DNA structures within cells that control cellular activity. Recall that while eukaryotic chromosomes are housed in the membrane-bound nucleus, most prokaryotes contain a single, circular chromosome that is found in an area of

the cytoplasm called the nucleoid (see **Unique Characteristics of Prokaryotic Cells (https://legacy.cnx.org/content/m58792/latest/)**). A chromosome may contain several thousand genes.

Organization of Eukaryotic Chromosome

Chromosome structure differs somewhat between eukaryotic and prokaryotic cells. Eukaryotic chromosomes are typically linear, and eukaryotic cells contain multiple distinct chromosomes. Many eukaryotic cells contain two copies of each chromosome and, therefore, are **diploid**.

The length of a chromosome greatly exceeds the length of the cell, so a chromosome needs to be packaged into a very small space to fit within the cell. For example, the combined length of all of the 3 billion base pairs[15] of DNA of the human genome would measure approximately 2 meters if completely stretched out, and some eukaryotic genomes are many times larger than the human genome. DNA **supercoiling** refers to the process by which DNA is twisted to fit inside the cell. Supercoiling may result in DNA that is either underwound (less than one turn of the helix per 10 base pairs) or overwound (more than one turn per 10 base pairs) from its normal relaxed state. Proteins known to be involved in supercoiling include **topoisomerases**; these enzymes help maintain the structure of supercoiled chromosomes, preventing overwinding of DNA during certain cellular processes like DNA replication.

During **DNA packaging**, DNA-binding proteins called **histones** perform various levels of DNA wrapping and attachment to scaffolding proteins. The combination of DNA with these attached proteins is referred to as **chromatin**. In eukaryotes, the packaging of DNA by histones may be influenced by environmental factors that affect the presence of methyl groups on certain cytosine nucleotides of DNA. The influence of environmental factors on DNA packaging is called epigenetics. Epigenetics is another mechanism for regulating gene expression without altering the sequence of nucleotides. Epigenetic changes can be maintained through multiple rounds of cell division and, therefore, can be heritable.

View this **animation (https://www.openstax.org/l/22dnapackanim)** from the DNA Learning Center to learn more about on DNA packaging in eukaryotes.

Organization of Prokaryotic Chromosomes

Chromosomes in bacteria and archaea are usually circular, and a prokaryotic cell typically contains only a single chromosome within the nucleoid. Because the chromosome contains only one copy of each gene, prokaryotes are **haploid**. As in eukaryotic cells, DNA supercoiling is necessary for the genome to fit within the prokaryotic cell. The DNA in the bacterial chromosome is arranged in several supercoiled domains. As with eukaryotes, topoisomerases are involved in supercoiling DNA. DNA gyrase is a type of topoisomerase, found in bacteria and some archaea, that helps prevent the overwinding of DNA. (Some antibiotics kill bacteria by targeting DNA gyrase.) In addition, histone-like proteins bind DNA and aid in DNA packaging. Other proteins bind to the origin of replication, the location in the chromosome where DNA replication initiates. Because different regions of DNA are packaged differently, some regions of chromosomal DNA are more accessible to enzymes and thus may be used more readily as templates for gene expression. Interestingly, several bacteria, including *Helicobacter pylori* and *Shigella flexneri*, have been shown to induce epigenetic changes in their hosts upon infection, leading to chromatin remodeling that may cause long-term effects on host immunity.[16]

- What is the difference between a cell's genotype and its phenotype?
- How does DNA fit inside cells?

Noncoding DNA

In addition to genes, a genome also contains many regions of **noncoding DNA** that do not encode proteins or stable RNA products. Noncoding DNA is commonly found in areas prior to the start of coding sequences of genes as well as in intergenic regions (i.e., DNA sequences located between genes) (**Figure 12.34**).

15. National Human Genome Research Institute. "The Human Genome Project Completion: Frequently Asked Questions." https://www.genome.gov/11006943. Accessed June 10, 2016

16. H. Bierne et al. "Epigenetics and Bacterial Infections." *Cold Spring Harbor Perspectives in Medicine* 2 no. 12 (2012):a010272.

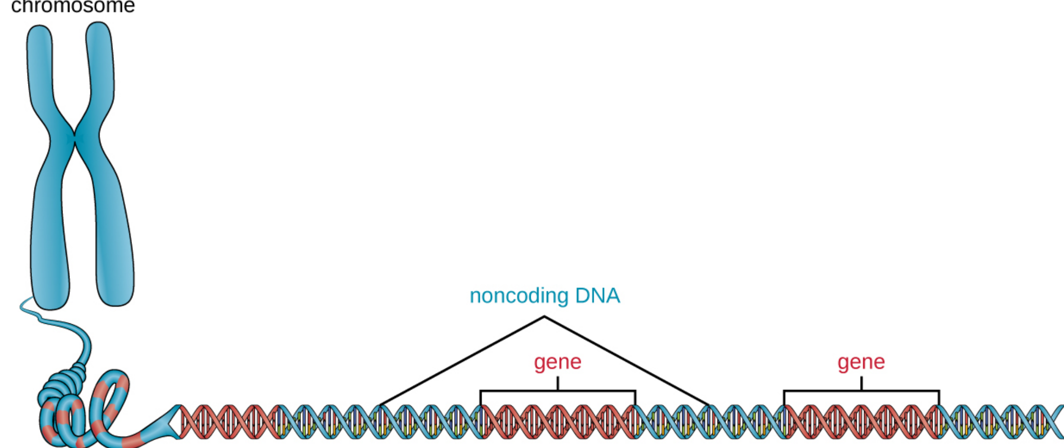


Figure 12.34 Chromosomes typically have a significant amount of noncoding DNA, often found in intergenic regions.

Prokaryotes appear to use their genomes very efficiently, with only an average of 12% of the genome being taken up by noncoding sequences. In contrast, noncoding DNA can represent about 98% of the genome in eukaryotes, as seen in humans, but the percentage of noncoding DNA varies between species.[17] These noncoding DNA regions were once referred to as “junk DNA”; however, this terminology is no longer widely accepted because scientists have since found roles for some of these regions, many of which contribute to the regulation of transcription or translation through the production of small noncoding RNA molecules, DNA packaging, and chromosomal stability. Although scientists may not fully understand the roles of all noncoding regions of DNA, it is generally believed that they do have purposes within the cell.

- What is the role of noncoding DNA?

Extrachromosomal DNA

Although most DNA is contained within a cell’s chromosomes, many cells have additional molecules of DNA outside the chromosomes, called **extrachromosomal DNA**, that are also part of its genome. The genomes of eukaryotic cells would also include the chromosomes from any organelles such as mitochondria and/or chloroplasts that these cells maintain (**Figure 12.35**). The maintenance of circular chromosomes in these organelles is a vestige of their prokaryotic origins and supports the endosymbiotic theory (see **Foundations of Modern Cell Theory (https://legacy.cnx.org/content/m58791/latest/)**). In some cases, genomes of certain DNA viruses can also be maintained independently in host cells during latent viral infection. In these cases, these viruses are another form of extrachromosomal DNA. For example, the human papillomavirus (HPV) may be maintained in infected cells in this way.

17. R.J. Taft et al. “The Relationship between Non-Protein-Coding DNA and Eukaryotic Complexity.” *Bioessays* 29 no. 3 (2007):288–299.

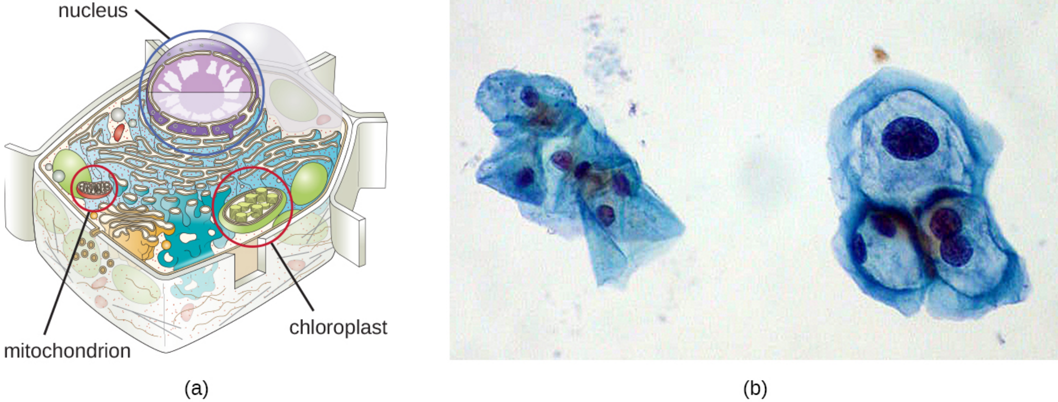


Figure 12.35 The genome of a eukaryotic cell consists of the chromosome housed in the nucleus, and extrachromosomal DNA found in the mitochondria (all cells) and chloroplasts (plants and algae). The cells shown in (b) represent cells obtained from a pap smear. The cells on the left are normal squamous cells whereas the cells on the right are infected with human papillomavirus and show enlarged nuclei with increased staining (hyperchromasia).

Besides chromosomes, some prokaryotes also have smaller loops of DNA called plasmids that may contain one or a few genes not essential for normal growth (**m58792 (https://legacy.cnx.org/content/m58792/latest/#OSC_Microbio_03_03_ProkCell)**). Bacteria can exchange these plasmids with other bacteria in a process known as horizontal gene transfer **(**HGT). The exchange of genetic material on plasmids sometimes provides microbes with new genes beneficial for growth and survival under special conditions. In some cases, genes obtained from plasmids may have clinical implications, encoding virulence factors that give a microbe the ability to cause disease or make a microbe resistant to certain antibiotics. Plasmids are also used heavily in genetic engineering and biotechnology as a way to move genes from one cell to another. The role of plasmids in horizontal gene transfer and biotechnology will be discussed further in **Mechanisms of Microbial Genetics (https://legacy.cnx.org/content/m58839/latest/)** and **Modern Applications of Microbial Genetics**.

- How are plasmids involved in antibiotic resistance?

Lethal Plasmids

Maria, a 20-year-old anthropology student from Texas, recently became ill in the African nation of Botswana, where she was conducting research as part of a study-abroad program. Maria’s research was focused on traditional African methods of tanning hides for the production of leather. Over a period of three weeks, she visited a tannery daily for several hours to observe and participate in the tanning process. One day, after returning from the tannery, Maria developed a fever, chills, and a headache, along with chest pain, muscle aches, nausea, and other flu-like symptoms. Initially, she was not concerned, but when her fever spiked and she began to cough up blood, her African host family became alarmed and rushed her to the hospital, where her condition continued to worsen.

After learning about her recent work at the tannery, the physician suspected that Maria had been exposed to anthrax. He ordered a chest X-ray, a blood sample, and a spinal tap, and immediately started her on a course of intravenous penicillin. Unfortunately, lab tests confirmed the physician’s presumptive diagnosis. Maria’s chest X-ray exhibited pleural effusion, the accumulation of fluid in the space between the pleural membranes, and a Gram stain of her blood revealed the presence of gram-positive, rod-shaped bacteria in short chains, consistent with *Bacillus anthracis*. Blood and bacteria were also shown to be present in her cerebrospinal fluid, indicating that the infection had progressed to meningitis. Despite supportive treatment and aggressive antibiotic therapy, Maria slipped into an unresponsive state and died three days later.

Anthrax is a disease caused by the introduction of endospores from the gram-positive bacterium *B. anthracis* into the body. Once infected, patients typically develop meningitis, often with fatal results. In Maria’s case, she inhaled the endospores while handling the hides of animals that had been infected.

The genome of *B. anthracis* illustrates how small structural differences can lead to major differences in virulence. In 2003, the genomes of *B. anthracis* and *Bacillus cereus*, a similar but less pathogenic bacterium of the same genus, were sequenced and compared.[18] Researchers discovered that the 16S rRNA gene

18. N. Ivanova et al. “Genome Sequence of *Bacillus cereus* and Comparative Analysis with *Bacillus anthracis.” Nature* 423 no. 6935

sequences of these bacteria are more than 99% identical, meaning that they are actually members of the same species despite their traditional classification as separate species. Although their chromosomal sequences also revealed a great deal of similarity, several virulence factors of *B. anthracis* were found to be encoded on two large plasmids not found in *B. cereus*. The plasmid pX01 encodes a three-part toxin that suppresses the host immune system, whereas the plasmid pX02 encodes a capsular polysaccharide that further protects the bacterium from the host immune system (**Figure 12.36**). Since *B. cereus* lacks these plasmids, it does not produce these virulence factors, and although it is still pathogenic, it is typically associated with mild cases of diarrhea from which the body can quickly recover. Unfortunately for Maria, the presence of these toxin-encoding plasmids in *B. anthracis* gives it its lethal virulence.

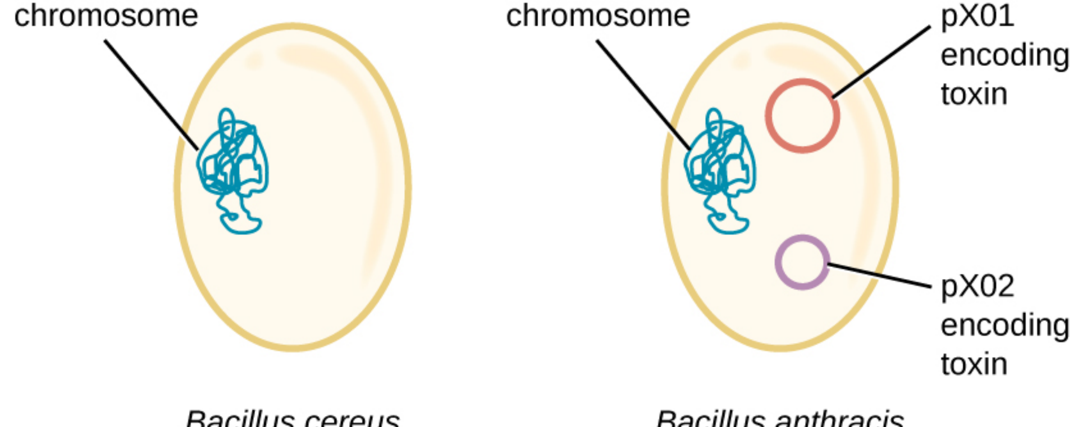


Figure 12.36 Genome sequencing of *Bacillus anthracis* and its close relative *B. cereus* reveals that the pathogenicity of *B. anthracis* is due to the maintenance of two plasmids, pX01 and pX02, which encode virulence factors.

- What do you think would happen to the pathogenicity of *B. anthracis* if it lost one or both of its plasmids?

Resolution

Within 24 hours, the results of the diagnostic test analysis of Alex's stool sample revealed that it was positive for heat-labile enterotoxin (LT), heat-stabile enterotoxin (ST), and colonization factor (CF), confirming the hospital physician's suspicion of ETEC. During a follow-up with Alex's family physician, this physician noted that Alex's symptoms were not resolving quickly and he was experiencing discomfort that was preventing him from returning to classes. The family physician prescribed Alex a course of ciprofloxacin to resolve his symptoms. Fortunately, the ciprofloxacin resolved Alex's symptoms within a few days.

Alex likely got his infection from ingesting contaminated food or water. Emerging industrialized countries like Mexico are still developing sanitation practices that prevent the contamination of water with fecal material. Travelers in such countries should avoid the ingestion of undercooked foods, especially meats, seafood, vegetables, and unpasteurized dairy products. They should also avoid use of water that has not been treated; this includes drinking water, ice cubes, and even water used for brushing teeth. Using bottled water for these purposes is a good alternative. Good hygiene (handwashing) can also aid the prevention of an ETEC infection. Alex had not been careful about his food or water consumption, which led to his illness.

Alex's symptoms were very similar to those of cholera, caused by the gram-negative bacterium *Vibrio cholerae*, which also produces a toxin similar to ST and LT. At some point in the evolutionary history of ETEC, a nonpathogenic strain of *E. coli* similar to those typically found in the gut may have acquired the genes encoding the ST and LT toxins from *V. cholerae*. The fact that the genes encoding those toxins are encoded on extrachromosomal plasmids in ETEC supports the idea that these genes were acquired by *E. coli* and are likely maintained in bacterial populations through horizontal gene transfer.

*Go back to the **previous** Clinical Focus box.*

(2003):87–91.

Viral Genomes

Viral genomes exhibit significant diversity in structure. Some viruses have genomes that consist of DNA as their genetic material. This DNA may be single stranded, as exemplified by human parvoviruses, or double stranded, as seen in the herpesviruses and poxviruses. Additionally, although all cellular life uses DNA as its genetic material, some viral genomes are made of either single-stranded or double-stranded RNA molecules, as we have discussed. Viral genomes are typically smaller than most bacterial genomes, encoding only a few genes, because they rely on their hosts to carry out many of the functions required for their replication. The diversity of viral genome structures and their implications for viral replication life cycles are discussed in more detail in **The Viral Life Cycle (https://legacy.cnx.org/content/m58809/latest/)** .

- Why do viral genomes vary widely among viruses?

Genome Size Matters

There is great variation in size of genomes among different organisms. Most eukaryotes maintain multiple chromosomes; humans, for example have 23 pairs, giving them 46 chromosomes. Despite being large at 3 billion base pairs, the human genome is far from the largest genome. Plants often maintain very large genomes, up to 150 billion base pairs, and commonly are polyploid, having multiple copies of each chromosome.

The size of bacterial genomes also varies considerably, although they tend to be smaller than eukaryotic genomes (**Figure 12.37**). Some bacterial genomes may be as small as only 112,000 base pairs. Often, the size of a bacterium's genome directly relates to how much the bacterium depends on its host for survival. When a bacterium relies on the host cell to carry out certain functions, it loses the genes encoding the abilities to carry out those functions itself. These types of bacterial endosymbionts are reminiscent of the prokaryotic origins of mitochondria and chloroplasts.

From a clinical perspective, obligate and facultative intracellular pathogens also tend to have small genomes (some around 1 million base pairs). Because host cells can supply most of their nutrients, they tend to have a reduced number of genes encoding metabolic functions, making their cultivation in the laboratory difficult if not impossible Due to their small sizes, the genomes of organisms like *Mycoplasma genitalium* (580,000 base pairs), *Chlamydia trachomatis* (1.0 million), *Rickettsia prowazekii* (1.1 million), and *Treponema pallidum* (1.1 million) were some of the earlier bacterial genomes sequenced. Respectively, these pathogens cause urethritis and pelvic inflammation, chlamydia, typhus, and syphilis.

Whereas obligate intracellular pathogens have unusually small genomes, other bacteria with a great variety of metabolic and enzymatic capabilities have unusually large bacterial genomes. *Pseudomonas aeruginosa*, for example, is a bacterium commonly found in the environment and is able to grow on a wide range of substrates. Its genome contains 6.3 million base pairs, giving it a high metabolic ability and the ability to produce virulence factors that cause several types of opportunistic infections.

Interestingly, there has been significant variability in genome size in viruses as well, ranging from 3,500 base pairs to 2.5 million base pairs, significantly exceeding the size of many bacterial genomes. The great variation observed in viral genome sizes further contributes to the great diversity of viral genome characteristics already discussed.

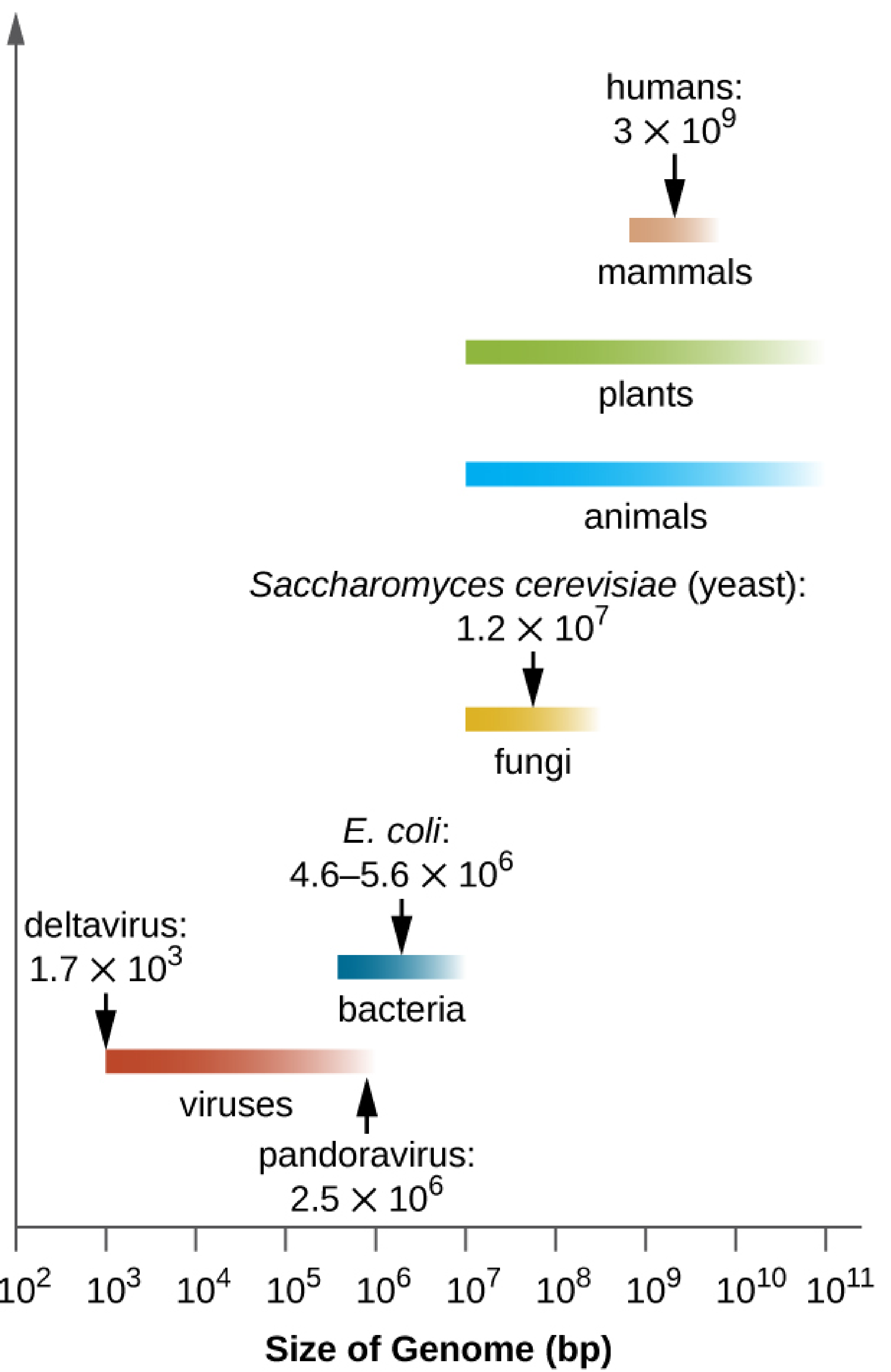


Figure 12.37 There is great variability as well as overlap among the genome sizes of various groups of organisms and viruses.

Visit the **genome database (https://www.openstax.org/l/22NCBIgendata)** of the National Center for Biotechnology Information (NCBI) to see the genomes that have been sequenced and their sizes.

True/False

Exercise 12.15

Within an organism, phenotypes may change while genotypes remain constant.

Solution
True

Exercise 12.16

Noncoding DNA has no biological purpose.

Solution
False

Fill in the Blank

Exercise 12.17

Plasmids are typically transferred among members of a bacterial community by ________ gene transfer.

Solution
horizontal

Short Answer

Exercise 12.18

What are some differences in chromosomal structures between prokaryotes and eukaryotes?

Exercise 12.19

How do prokaryotes and eukaryotes manage to fit their lengthy DNA inside of cells? Why is this necessary?

Exercise 12.20

What are some functions of noncoding DNA?

Exercise 12.21

In the chromatin of eukaryotic cells, which regions of the chromosome would you expect to be more compact: the regions that contain genes being actively copied into RNA or those that contain inactive genes?

12.9 | Mapping Genomes

By the end of this section, you will be able to:

- Define genomics
- Describe genetic and physical maps
- Describe genomic mapping methods

Genomics is the study of entire genomes, including the complete set of genes, their nucleotide sequence and

organization, and their interactions within a species and with other species. **Genome mapping** is the process of finding the locations of genes on each chromosome. The maps created by genome mapping are comparable to the maps that we use to navigate streets. A **genetic map** is an illustration that lists genes and their location on a chromosome. Genetic maps provide the big picture (similar to a map of interstate highways) and use genetic markers (similar to landmarks). A **genetic marker** is a gene or sequence on a chromosome that co-segregates (shows genetic linkage) with a specific trait. Early geneticists called this linkage analysis. Physical maps present the intimate details of smaller regions of the chromosomes (similar to a detailed road map). A **physical map** is a representation of the physical distance, in nucleotides, between genes or genetic markers. Both genetic linkage maps and physical maps are required to build a complete picture of the genome. Having a complete map of the genome makes it easier for researchers to study individual genes. Human genome maps help researchers in their efforts to identify human disease-causing genes related to illnesses like cancer, heart disease, and cystic fibrosis. Genome mapping can be used in a variety of other applications, such as using live microbes to clean up pollutants or even prevent pollution. Research involving plant genome mapping may lead to producing higher crop yields or developing plants that better adapt to climate change.

Genetic Maps

The study of genetic maps begins with **linkage analysis**, a procedure that analyzes the recombination frequency between genes to determine if they are linked or show independent assortment. The term *linkage* was used before the discovery of DNA. Early geneticists relied on the observation of phenotypic changes to understand the genotype of an organism. Shortly after Gregor Mendel (the father of modern genetics) proposed that traits were determined by what are now known as genes, other researchers observed that different traits were often inherited together, and thereby deduced that the genes were physically linked by being located on the same chromosome. The mapping of genes relative to each other based on linkage analysis led to the development of the first genetic maps.

Observations that certain traits were always linked and certain others were not linked came from studying the offspring of crosses between parents with different traits. For example, in experiments performed on the garden pea, it was discovered that the color of the flower and shape of the plant’s pollen were linked traits, and therefore the genes encoding these traits were in close proximity on the same chromosome. The exchange of DNA between homologous pairs of chromosomes is called **genetic recombination**, which occurs by the crossing over of DNA between homologous strands of DNA, such as nonsister chromatids. Linkage analysis involves studying the recombination frequency between any two genes. The greater the distance between two genes, the higher the chance that a recombination event will occur between them, and the higher the recombination frequency between them. Two possibilities for recombination between two nonsister chromatids during meiosis are shown in **Figure 12.38**. If the recombination frequency between two genes is less than 50 percent, they are said to be linked.

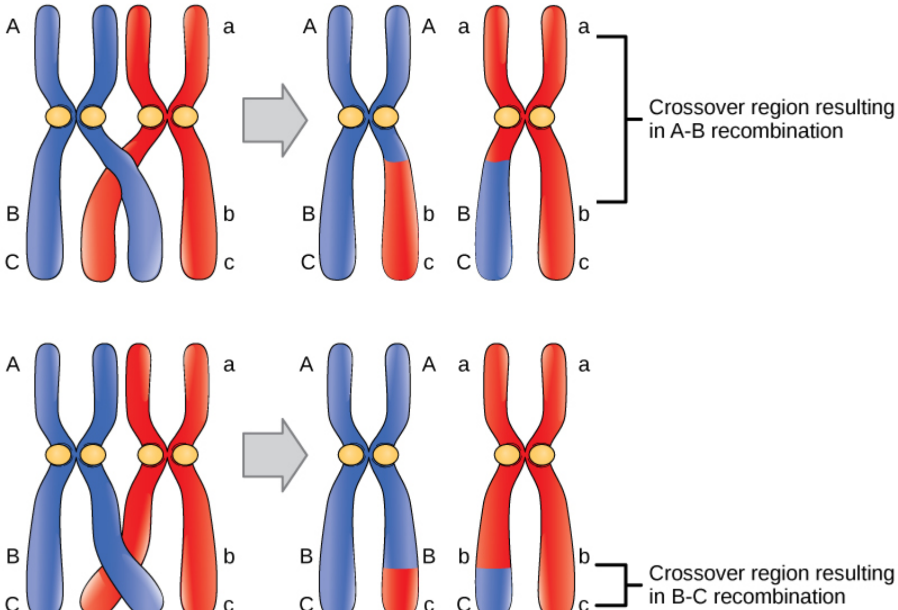


Figure 12.38 Crossover may occur at different locations on the chromosome. Recombination between genes *A* and *B* is more frequent than recombination between genes *B* and *C* because genes *A* and *B* are farther apart; a crossover is therefore more likely to occur between them.

The generation of genetic maps requires markers, just as a road map requires landmarks (such as rivers and mountains). Early genetic maps were based on the use of known genes as markers. More sophisticated markers, including those based on non-coding DNA, are now used to compare the genomes of individuals in a population. Although individuals of a given species are genetically similar, they are not identical; every individual has a unique set of traits. These minor differences in the genome between individuals in a population are useful for the purposes of genetic mapping. In general, a good genetic marker is a region on the chromosome that shows variability or polymorphism (multiple forms) in the population.

Some genetic markers used in generating genetic maps are **restriction fragment length polymorphisms** (RFLP), variable number of tandem repeats (VNTRs), **microsatellite polymorphisms**, and the **single nucleotide polymorphisms** (SNPs). RFLPs (sometimes pronounced "rif-lips") are detected when the DNA of an individual is cut with a restriction endonuclease that recognizes specific sequences in the DNA to generate a series of DNA fragments, which are then analyzed by gel electrophoresis. The DNA of every individual will give rise to a unique pattern of bands when cut with a particular set of restriction endonucleases; this is sometimes referred to as an individual's DNA "fingerprint." Certain regions of the chromosome that are subject to polymorphism will lead to the generation of the unique banding pattern. VNTRs are repeated sets of nucleotides present in the non-coding regions of DNA. Non-coding, or "junk," DNA has no known biological function; however, research shows that much of this DNA is actually transcribed. While its function is uncertain, it is certainly active, and it may be involved in the regulation of coding genes. The number of repeats may vary in individual organisms of a population. Microsatellite polymorphisms are similar to VNTRs, but the repeat unit is very small. SNPs are variations in a single nucleotide.

Because genetic maps rely completely on the natural process of recombination, mapping is affected by natural increases or decreases in the level of recombination in any given area of the genome. Some parts of the genome are recombination hotspots, whereas others do not show a propensity for recombination. For this reason, it is important to look at mapping information developed by multiple methods.

Physical Maps

A physical map provides detail of the actual physical distance between genetic markers, as well as the number of nucleotides. There are three methods used to create a physical map: cytogenetic mapping, radiation hybrid mapping, and sequence mapping. **Cytogenetic mapping** uses information obtained by microscopic analysis of stained sections of the chromosome (**Figure 12.39**). It is possible to determine the approximate distance between genetic markers using cytogenetic mapping, but not the exact distance (number of base pairs). **Radiation hybrid mapping** uses radiation, such as x-rays, to break the DNA into fragments. The amount of radiation can be adjusted to create smaller or larger fragments. This technique overcomes the limitation of

genetic mapping and is not affected by increased or decreased recombination frequency. **Sequence mapping** resulted from DNA sequencing technology that allowed for the creation of detailed physical maps with distances measured in terms of the number of base pairs. The creation of **genomic libraries** and **complementary DNA (cDNA) libraries** (collections of cloned sequences or all DNA from a genome) has sped up the process of physical mapping. A genetic site used to generate a physical map with sequencing technology (a sequence-tagged site, or STS) is a unique sequence in the genome with a known exact chromosomal location. An **expressed sequence tag (EST)** and a single sequence length polymorphism (SSLP) are common STSs. An EST is a short STS that is identified with cDNA libraries, while SSLPs are obtained from known genetic markers and provide a link between genetic maps and physical maps.

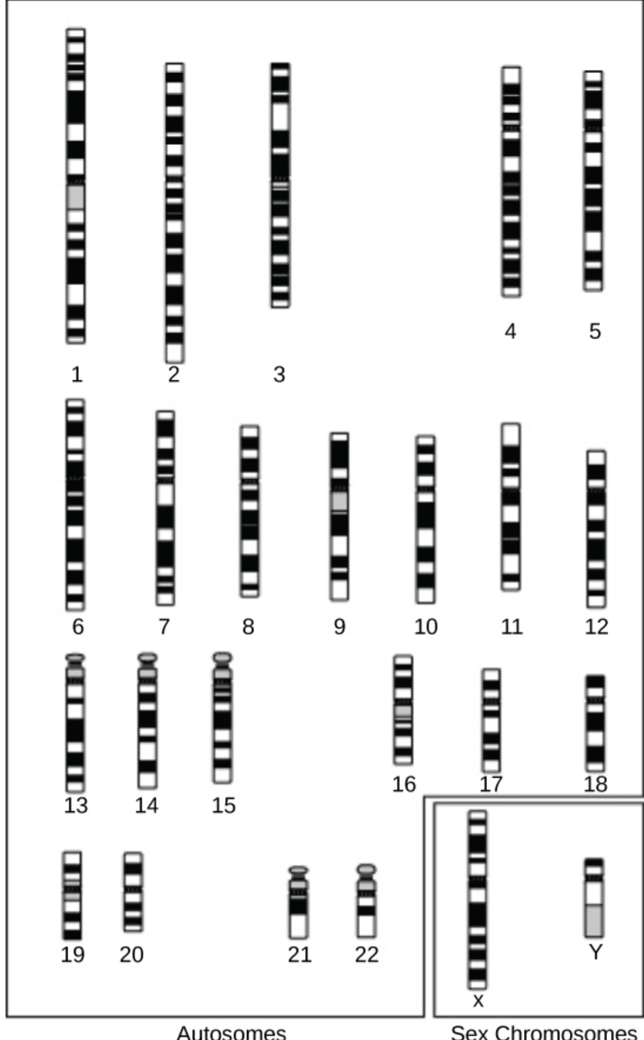


Figure 12.39 A cytogenetic map shows the appearance of a chromosome after it is stained and examined under a microscope. (credit: National Human Genome Research Institute)

Integration of Genetic and Physical Maps

Genetic maps provide the outline and physical maps provide the details. It is easy to understand why both types of genome mapping techniques are important to show the big picture. Information obtained from each technique is used in combination to study the genome. Genomic mapping is being used with different model organisms that are used for research. Genome mapping is still an ongoing process, and as more advanced techniques are developed, more advances are expected. Genome mapping is similar to completing a complicated puzzle using every piece of available data. Mapping information generated in laboratories all over the world is entered into central databases, such as GenBank at the National Center for Biotechnology Information (NCBI). Efforts are being made to make the information more easily accessible to researchers and the general public. Just as we use global positioning systems instead of paper maps to navigate through roadways, NCBI has created a genome viewer tool to simplify the data-mining process.

scientific method CONNECTION

How to Use a Genome Map Viewer

Problem statement: Do the human, macaque, and mouse genomes contain common DNA sequences?

Develop a hypothesis.

To test the hypothesis, click this **link (http://www.ncbi.nlm.nih.gov/mapview/)** .

In Search box on the left panel, type any gene name or phenotypic characteristic, such as iris pigmentation (eye color). Select the species you want to study, and then press Enter. The genome map viewer will indicate which chromosome encodes the gene in your search. Click each hit in the genome viewer for more detailed information. This type of search is the most basic use of the genome viewer; it can also be used to compare sequences between species, as well as many other complicated tasks.

Is the hypothesis correct? Why or why not?

Online Mendelian Inheritance in Man (OMIM) is a searchable online catalog of human genes and genetic disorders. This website shows genome mapping information, and also details the history and research of each trait and disorder. Click this **link (http://openstaxcollege.org/l/OMIM)** to search for traits (such as handedness) and genetic disorders (such as diabetes).

12.10 | Applying Genomics

By the end of this section, you will be able to:

- Explain pharmacogenomics
- Define polygenic

The introduction of DNA sequencing and whole genome sequencing projects, particularly the Human Genome project, has expanded the applicability of DNA sequence information. Genomics is now being used in a wide variety of fields, such as metagenomics, pharmacogenomics, and mitochondrial genomics. The most commonly known application of genomics is to understand and find cures for diseases.

Predicting Disease Risk at the Individual Level

Predicting the risk of disease involves screening currently healthy individuals by genome analysis at the individual level. Intervention with lifestyle changes and drugs can be recommended before disease onset. However, this approach is most applicable when the problem resides within a single gene defect. Such defects only account for approximately 5 percent of diseases in developed countries. Most of the common diseases, such as heart disease, are multi-factored or **polygenic**, which is a phenotypic characteristic that involves two or more genes, and also involve environmental factors such as diet. In April 2010, scientists at Stanford University published the genome analysis of a healthy individual (Stephen Quake, a scientist at Stanford University, who had his genome sequenced); the analysis predicted his propensity to acquire various diseases. A risk assessment was performed to analyze Quake's percentage of risk for 55 different medical conditions. A rare genetic mutation was found, which showed him to be at risk for sudden heart attack. He was also predicted to have a 23 percent risk of developing prostate cancer and a 1.4 percent risk of developing Alzheimer's. The scientists used databases and several publications to analyze the genomic data. Even though

genomic sequencing is becoming more affordable and analytical tools are becoming more reliable, ethical issues surrounding genomic analysis at a population level remain to be addressed.

Art Connection

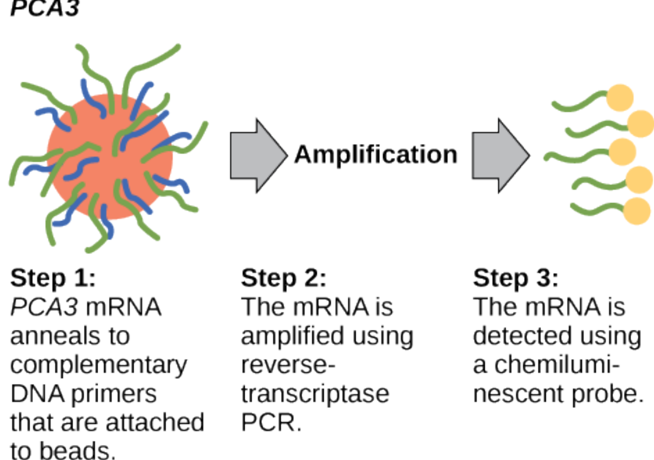


Figure 12.40 *PCA3* is a gene that is expressed in prostate epithelial cells and overexpressed in cancerous cells. A high concentration of *PCA3* in urine is indicative of prostate cancer. The *PCA3* test is considered to be a better indicator of cancer than the more well know PSA test, which measures the level of PSA (prostate-specific antigen) in the blood.

In 2011, the United States Preventative Services Task Force recommended against using the PSA test to screen healthy men for prostate cancer. Their recommendation is based on evidence that screening does not reduce the risk of death from prostate cancer. Prostate cancer often develops very slowly and does not cause problems, while the cancer treatment can have severe side effects. The *PCA3* test is considered to be more accurate, but screening may still result in men who would not have been harmed by the cancer itself suffering side effects from treatment. What do you think? Should all healthy men be screened for prostate cancer using the *PCA3* or PSA test? Should people in general be screened to find out if they have a genetic risk for cancer or other diseases?

Pharmacogenomics and Toxicogenomics

Pharmacogenomics, also called toxicogenomics, involves evaluating the effectiveness and safety of drugs on the basis of information from an individual's genomic sequence. Genomic responses to drugs can be studied using experimental animals (such as laboratory rats or mice) or live cells in the laboratory before embarking on studies with humans. Studying changes in gene expression could provide information about the transcription profile in the presence of the drug, which can be used as an early indicator of the potential for toxic effects. For example, genes involved in cellular growth and controlled cell death, when disturbed, could lead to the growth of cancerous cells. Genome-wide studies can also help to find new genes involved in drug toxicity. Personal genome sequence information can be used to prescribe medications that will be most effective and least toxic on the basis of the individual patient's genotype. The gene signatures may not be completely accurate, but can be tested further before pathologic symptoms arise.

Microbial Genomics: Metagenomics

Traditionally, microbiology has been taught with the view that microorganisms are best studied under **pure culture** conditions, which involves isolating a single type of cell and culturing it in the laboratory. Because microorganisms can go through several generations in a matter of hours, their gene expression profiles adapt to the new laboratory environment very quickly. In addition, the vast majority of bacterial species resist being cultured in isolation. Most microorganisms do not live as isolated entities, but in microbial communities known as biofilms. For all of these reasons, pure culture is not always the best way to study microorganisms. **Metagenomics** is the study of the collective genomes of multiple species that grow and interact in an environmental niche. Metagenomics can be used to identify new species more rapidly and to analyze the effect of pollutants on the environment (**Figure 12.41**).

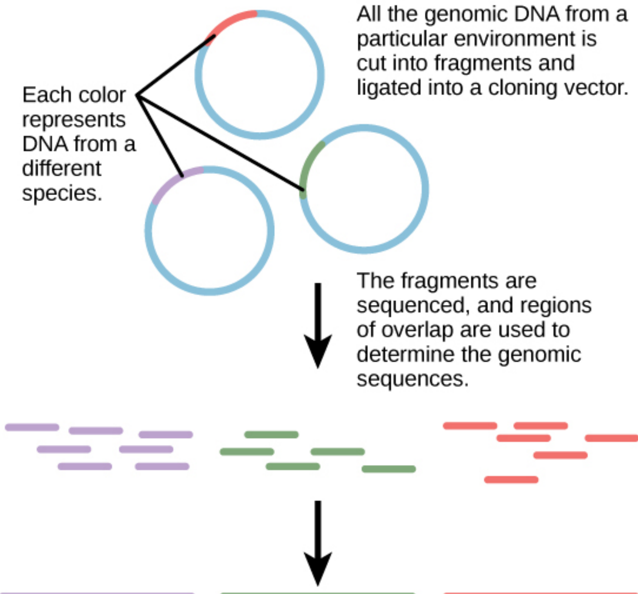


Figure 12.41 Metagenomics involves isolating DNA from multiple species within an environmental niche.

Microbial Genomics: Creation of New Biofuels

Knowledge of the genomics of microorganisms is being used to find better ways to harness biofuels from algae and cyanobacteria. The primary sources of fuel today are coal, oil, wood, and other plant products, such as ethanol. Although plants are renewable resources, there is still a need to find more alternative renewable sources of energy to meet our population's energy demands. The microbial world is one of the largest resources for genes that encode new enzymes and produce new organic compounds, and it remains largely untapped. Microorganisms are used to create products, such as enzymes that are used in research, antibiotics, and other anti-microbial mechanisms. Microbial genomics is helping to develop diagnostic tools, improved vaccines, new disease treatments, and advanced environmental cleanup techniques.

Mitochondrial Genomics

Mitochondria are intracellular organelles that contain their own DNA. Mitochondrial DNA mutates at a rapid rate and is often used to study evolutionary relationships. Another feature that makes studying the mitochondrial genome interesting is that the mitochondrial DNA in most multicellular organisms is passed on from the mother during the process of fertilization. For this reason, mitochondrial genomics is often used to trace genealogy.

Information and clues obtained from DNA samples found at crime scenes have been used as evidence in court cases, and genetic markers have been used in forensic analysis. Genomic analysis has also become useful in this field. In 2001, the first use of genomics in forensics was published. It was a collaborative attempt between academic research institutions and the FBI to solve the mysterious cases of anthrax communicated via the US Postal Service. Using microbial genomics, researchers determined that a specific strain of anthrax was used in all the mailings.

Genomics in Agriculture

Genomics can reduce the trials and failures involved in scientific research to a certain extent, which could improve the quality and quantity of crop yields in agriculture. Linking traits to genes or gene signatures helps to improve crop breeding to generate hybrids with the most desirable qualities. Scientists use genomic data to identify desirable traits, and then transfer those traits to a different organism. Scientists are discovering how genomics can improve the quality and quantity of agricultural production. For example, scientists could use desirable traits to create a useful product or enhance an existing product, such as making a drought-sensitive crop more tolerant of the dry season.

KEY TERMS

alternative RNA splicing a post-transcriptional gene regulation mechanism in eukaryotes in which multiple protein products are produced by a single gene through alternative splicing combinations of the RNA transcript

cDNA library collection of cloned cDNA sequences

codon three consecutive nucleotides in mRNA that specify the addition of a specific amino acid or the release of a polypeptide chain during translation

cytogenetic mapping technique that uses a microscope to create a map from stained chromosomes

DNA ligase the enzyme that catalyzes the joining of DNA fragments together

DNA polymerase an enzyme that synthesizes a new strand of DNA complementary to a template strand

epigenetic describing non-genetic regulatory factors, such as changes in modifications to histone proteins and DNA that control accessibility to genes in chromosomes

exon a sequence present in protein-coding mRNA after completion of pre-mRNA splicing

expressed sequence tag (EST) short STS that is identified with cDNA

gene expression processes that control whether a gene is expressed

genetic code the amino acids that correspond to three-nucleotide codons of mRNA

genetic map outline of genes and their location on a chromosome

genetic marker gene or sequence on a chromosome with a known location that is associated with a specific trait

genetic recombination exchange of DNA between homologous pairs of chromosomes

genome mapping process of finding the location of genes on each chromosome

genomic library collection of cloned DNA which represents all of the sequences and fragments from a genome

genomics study of entire genomes including the complete set of genes, their nucleotide sequence and organization, and their interactions within a species and with other species

helicase an enzyme that helps to open up the DNA helix during DNA replication by breaking the hydrogen bonds

intron non–protein-coding intervening sequences that are spliced from mRNA during processing

lagging strand during replication of the 3' to 5' strand, the strand that is replicated in short fragments and away from the replication fork

leading strand the strand that is synthesized continuously in the 5' to 3' direction that is synthesized in the direction of the replication fork

linkage analysis procedure that analyzes the recombination of genes to determine if they are linked

metagenomics study of the collective genomes of multiple species that grow and interact in an environmental niche

microsatellite polymorphism variation between individuals in the sequence and number of repeats of microsatellite DNA

mismatch repair a form of DNA repair in which non-complementary nucleotides are recognized, excised, and replaced with correct nucleotides

mRNA messenger RNA; a form of RNA that carries the nucleotide sequence code for a protein sequence that is translated into a polypeptide sequence

mutation a permanent variation in the nucleotide sequence of a genome

nontemplate strand the strand of DNA that is not used to transcribe mRNA; this strand is identical to the mRNA except that T nucleotides in the DNA are replaced by U nucleotides in the mRNA

nucleotide excision repair a form of DNA repair in which the DNA molecule is unwound and separated in the region of the nucleotide damage, the damaged nucleotides are removed and replaced with new nucleotides using the complementary strand, and the DNA strand is resealed and allowed to rejoin its complement

Okazaki fragments the DNA fragments that are synthesized in short stretches on the lagging strand

pharmacogenomics study of drug interactions with the genome or proteome; also called toxicogenomics

physical map representation of the physical distance between genes or genetic markers

polygenic phenotypic characteristic caused by two or more genes

post-transcriptional control of gene expression after the RNA molecule has been created but before it is translated into protein

post-translational control of gene expression after a protein has been created

primer a short stretch of RNA nucleotides that is required to initiate replication and allow DNA polymerase to bind and begin replication

promoter a sequence on DNA to which RNA polymerase and associated factors bind and initiate transcription

pure culture growth of a single type of cell in the laboratory

radiation hybrid mapping information obtained by fragmenting the chromosome with x-rays

replication fork the Y-shaped structure formed during the initiation of replication

restriction fragment length polymorphism (RFLP) variation between individuals in the length of DNA fragments generated by restriction endonucleases

RNA polymerase an enzyme that synthesizes an RNA strand from a DNA template strand

rRNA ribosomal RNA; molecules of RNA that combine to form part of the ribosome

semiconservative replication the method used to replicate DNA in which the double-stranded molecule is separated and each strand acts as a template for a new strand to be synthesized, so the resulting DNA molecules are composed of one new strand of nucleotides and one old strand of nucleotides

sequence mapping mapping information obtained after DNA sequencing

single nucleotide polymorphism (SNP) variation between individuals in a single nucleotide

splicing the process of removing introns and reconnecting exons in a pre-mRNA

start codon the AUG (or, rarely GUG) on an mRNA from which translation begins; always specifies methionine

stop codon one of the three mRNA codons that specifies termination of translation

telomerase an enzyme that contains a catalytic part and an inbuilt RNA template; it functions to maintain telomeres at chromosome ends

telomere the DNA at the end of linear chromosomes

template strand the strand of DNA that specifies the complementary mRNA molecule

transcription bubble the region of locally unwound DNA that allows for transcription of mRNA

tRNA transfer RNA; an RNA molecule that contains a specific three-nucleotide anticodon sequence to pair with the mRNA codon and also binds to a specific amino acid

variable number of tandem repeats (VNTRs) variation in the number of tandem repeats between individuals in the population

CHAPTER SUMMARY

12.1 Structure and Function of DNA

- **Nucleic acids** are composed of **nucleotides**, each of which contains a pentose sugar, a phosphate group, and a **nitrogenous base**. **Deoxyribonucleotides** within DNA contain **deoxyribose** as the pentose sugar.
- DNA contains the **pyrimidines cytosine** and **thymine**, and the **purines adenine** and **guanine**.
- **Nucleotides** are linked together by phosphodiester bonds between the 5′ phosphate group of one nucleotide and the 3′ hydroxyl group of another. A **nucleic acid strand** has a free phosphate group at the 5′ end and a free hydroxyl group at the 3′ end.
- Chargaff discovered that the amount of **adenine** is approximately equal to the amount of **thymine** in DNA, and that the amount of the **guanine** is approximately equal to **cytosine**. These relationships were later determined to be due to complementary base pairing.
- Watson and Crick, building on the work of Chargaff, Franklin and Gosling, and Wilkins, proposed the double helix model and base pairing for DNA structure.
- DNA is composed of two complementary strands oriented **antiparallel** to each other with the **phosphodiester backbones** on the exterior of the molecule. The nitrogenous bases of each strand face each other and complementary bases hydrogen bond to each other, stabilizing the double helix.
- Heat or chemicals can break the hydrogen bonds between complementary bases, denaturing DNA. Cooling or removing chemicals can lead to renaturation or reannealing of DNA by allowing hydrogen bonds to reform between complementary bases.
- DNA stores the instructions needed to build and control the cell. This information is transmitted from parent to offspring through **vertical gene transfer**.

12.3 DNA Replication

DNA replicates by a semi-conservative method in which each of the two parental DNA strands act as a template for new DNA to be synthesized. After replication, each DNA has one parental or “old” strand, and one daughter or “new” strand.

Replication in eukaryotes starts at multiple origins of replication, while replication in prokaryotes starts from a single origin of replication. The DNA is opened with enzymes, resulting in the formation of the replication fork. Primase synthesizes an RNA primer to initiate synthesis by DNA polymerase, which can add nucleotides in only one direction. One strand is synthesized continuously in the direction of the replication fork; this is called the leading strand. The other strand is synthesized in a direction away from the replication fork, in short stretches of DNA known as Okazaki fragments. This strand is known as the lagging strand. Once replication is completed, the RNA primers are replaced by DNA nucleotides and the DNA is sealed with DNA ligase.

The ends of eukaryotic chromosomes pose a problem, as polymerase is unable to extend them without a primer. Telomerase, an enzyme with an inbuilt RNA template, extends the ends by copying the RNA template and extending one end of the chromosome. DNA polymerase can then extend the DNA using the primer. In this way, the ends of the chromosomes are protected. Cells have mechanisms for repairing DNA when it becomes damaged or errors are made in replication. These mechanisms include mismatch repair to replace nucleotides that are paired with a non-complementary base and nucleotide excision repair, which removes bases that are damaged such as thymine dimers.

12.4 Structure and Function of RNA

- **Ribonucleic acid (RNA)** is typically single stranded and contains ribose as its pentose sugar and the pyrimidine uracil instead of thymine. An RNA strand can undergo significant intramolecular base pairing to take on a three-dimensional structure.
- There are three main types of RNA, all involved in protein synthesis.
- Messenger RNA (**mRNA**) serves as the intermediary between DNA and the synthesis of protein products during translation.
- Ribosomal RNA (**rRNA**) is a type of stable RNA that is a major constituent of ribosomes. It ensures the proper alignment of the mRNA and the ribosomes during protein synthesis and catalyzes the formation of the peptide bonds between two aligned amino acids during protein synthesis.
- Transfer RNA (**tRNA**) is a small type of stable RNA that carries an amino acid to the corresponding site of protein synthesis in the ribosome. It is the base pairing between the tRNA and mRNA that allows for the correct amino acid to be inserted in the polypeptide chain being synthesized.
- Although RNA is not used for long-term genetic information in cells, many viruses do use RNA as their genetic material.

12.5 Transcription

In prokaryotes, mRNA synthesis is initiated at a promoter sequence on the DNA template. Elongation synthesizes new mRNA. Termination liberates the mRNA and occurs by mechanisms that stall the RNA polymerase and cause it to fall off the DNA template. Newly transcribed eukaryotic mRNAs are modified with a cap and a poly-A tail. These structures protect the mature mRNA from degradation and help export it from the nucleus. Eukaryotic mRNAs also undergo splicing, in which introns are removed and exons are reconnected with single-nucleotide accuracy. Only finished mRNAs are exported from the nucleus to the cytoplasm.

12.6 Translation

The central dogma describes the flow of genetic information in the cell from genes to mRNA to proteins. Genes are used to make mRNA by the process of transcription; mRNA is used to synthesize proteins by the process of translation. The genetic code is the correspondence between the three-nucleotide mRNA codon and an amino acid. The genetic code is “translated” by the tRNA molecules, which associate a specific codon with a specific amino acid. The genetic code is degenerate because 64 triplet codons in mRNA specify only 20 amino acids and three stop codons. This means that more than one codon corresponds to an amino acid. Almost every species on the planet uses the same genetic code.

The players in translation include the mRNA template, ribosomes, tRNAs, and various enzymatic factors. The small ribosomal subunit binds to the mRNA template. Translation begins at the initiating AUG on the mRNA. The formation of bonds occurs between sequential amino acids specified by the mRNA template according to the genetic code. The ribosome accepts charged tRNAs, and as it steps along the mRNA, it catalyzes bonding between the new amino acid and the end of the growing polypeptide. The entire mRNA is translated in three-nucleotide “steps” of the ribosome. When a stop codon is encountered, a release factor binds and dissociates the components and frees the new protein.

12.7 How Genes Are Regulated

While all somatic cells within an organism contain the same DNA, not all cells within that organism express the same proteins. Prokaryotic organisms express the entire DNA they encode in every cell, but not necessarily all at the same time. Proteins are expressed only when they are needed. Eukaryotic organisms express a subset of the DNA that is encoded in any given cell. In each cell type, the type and amount of protein is regulated by controlling gene expression. To express a protein, the DNA is first transcribed into RNA, which is then translated into proteins. In prokaryotic cells, these processes occur almost simultaneously. In eukaryotic cells, transcription occurs in the nucleus and is separate from the translation that occurs in the cytoplasm. Gene expression in prokaryotes is regulated only at the transcriptional level, whereas in eukaryotic cells, gene expression is regulated at the epigenetic, transcriptional, post-transcriptional, translational, and post-translational levels.

12.8 Structure and Function of Cellular Genomes

- The entire genetic content of a cell is its **genome**.
- **Genes** code for proteins, or stable RNA molecules, each of which carries out a specific function in the cell.
- Although the **genotype** that a cell possesses remains constant, expression of genes is dependent on environmental conditions.
- A **phenotype** is the observable characteristics of a cell (or organism) at a given point in time and results from the complement of genes currently being used.
- The majority of genetic material is organized into **chromosomes** that contain the DNA that controls cellular activities.
- Prokaryotes are typically haploid, usually having a single circular chromosome found in the nucleoid. Eukaryotes are diploid; DNA is organized into multiple linear chromosomes found in the nucleus.
- Supercoiling and DNA packaging using DNA binding proteins allows lengthy molecules to fit inside a cell. Eukaryotes and archaea use histone proteins, and bacteria use different proteins with similar function.
- Prokaryotic and eukaryotic genomes both contain **noncoding DNA**, the function of which is not well understood. Some noncoding DNA appears to participate in the formation of small noncoding RNA molecules that influence gene expression; some appears to play a role in maintaining chromosomal structure and in DNA packaging.
- **Extrachromosomal DNA** in eukaryotes includes the chromosomes found within organelles of prokaryotic origin (mitochondria and chloroplasts) that evolved by endosymbiosis. Some viruses may also maintain themselves extrachromosomally.
- Extrachromosomal DNA in prokaryotes is commonly maintained as **plasmids** that encode a few nonessential genes that may be helpful under specific conditions. Plasmids can be spread through a bacterial community by horizontal gene transfer.
- Viral genomes show extensive variation and may be composed of either RNA or DNA, and may be either double or single stranded.

12.9 Mapping Genomes

Genome mapping is similar to solving a big, complicated puzzle with pieces of information coming from laboratories all over the world. Genetic maps provide an outline for the location of genes within a genome, and they estimate the distance between genes and genetic markers on the basis of recombination frequencies during meiosis. Physical maps provide detailed information about the physical distance between the genes. The most detailed information is available through sequence mapping. Information from all mapping and sequencing sources is combined to study an entire genome.

12.10 Applying Genomics

Imagination is the only barrier to the applicability of genomics. Genomics is being applied to most fields of biology; it is being used for personalized medicine, prediction of disease risks at an individual level, the study of drug interactions before the conduct of clinical trials, and the study of microorganisms in the environment as opposed to the laboratory. It is also being applied to developments such as the generation of new biofuels, genealogical assessment using mitochondria, advances in forensic science, and improvements in agriculture.

VISUAL CONNECTION QUESTIONS

12.3 DNA Replication

22. Figure 12.15 You isolate a cell strain in which the joining together of Okazaki fragments is impaired and suspect that a mutation has occurred in an enzyme found at the replication fork. Which enzyme is most likely to be mutated?

12.10 Applying Genomics

23. Figure 12.40 In 2011, the United States Preventative Services Task Force recommended

against using the PSA test to screen healthy men for prostate cancer. Their recommendation is based on evidence that screening does not reduce the risk of death from prostate cancer. Prostate cancer often develops very slowly and does not cause problems, while the cancer treatment can have severe side effects. The *PCA3* test is considered to be more accurate, but screening may still result in men who would not have been harmed by the cancer itself suffering side effects from treatment. What do you think? Should all healthy men be screened for prostate cancer using the *PCA3* or PSA test? Should people in general be screened to find out if they have a genetic risk for cancer or other diseases?

REVIEW QUESTIONS

24. Which of the following is not found within DNA?

A. thymine
B. phosphodiester bonds
C. complementary base pairing
D. amino acids

25. If 30% of the bases within a DNA molecule are adenine, what is the percentage of thymine?

A. 20%
B. 25%
C. 30%
D. 35%

26. Which of the following statements about base pairing in DNA is incorrect?

A. Purines always base pairs with pyrimidines.
B. Adenine binds to guanine.
C. Base pairs are stabilized by hydrogen bonds.
D. Base pairing occurs at the interior of the double helix.

27. If a DNA strand contains the sequence 5′-ATTCCGGATCGA-3′, which of the following is the sequence of the complementary strand of DNA?

A. 5′-TAAGGCCTAGCT-3′
B. 5′-ATTCCGGATCGA-3′
C. 3′-TAACCGGTACGT-5′
D. 5′-TCGATCCGGAAT-3′

28. During denaturation of DNA, which of the following happens?

A. Hydrogen bonds between complementary bases break.
B. Phosphodiester bonds break within the sugar-phosphate backbone.
C. Hydrogen bonds within the sugar-phosphate backbone break.
D. Phosphodiester bonds between complementary bases break.

29. DNA replicates by which of the following models?

a. conservative
b. semiconservative
c. dispersive
d. none of the above

30. The initial mechanism for repairing nucleotide errors in DNA is ________.

a. mismatch repair
b. DNA polymerase proofreading
c. nucleotide excision repair
d. thymine dimers

31. Which of the following types of RNA codes for a protein?

a. dsRNA
b. mRNA
c. rRNA
d. tRNA

32. A nucleic acid is purified from a mixture. The molecules are relatively small, contain uracil, and most are covalently bound to an amino acid. Which of the following was purified?

A. DNA
B. mRNA
C. rRNA
D. tRNA

33. Which of the following types of RNA is known for its catalytic abilities?

A. dsRNA
B. mRNA
C. rRNA
D. tRNA

34. Ribosomes are composed of rRNA and what other component?

A. protein
B. carbohydrates
C. DNA
D. mRNA

35. Which of the following may use RNA as its genome?

A. a bacterium
B. an archaeon
C. a virus
D. a eukaryote

36. A promoter is ________.

a. a specific sequence of DNA nucleotides
b. a specific sequence of RNA nucleotides
c. a protein that binds to DNA
d. an enzyme that synthesizes RNA

37. Portions of eukaryotic mRNA sequence that are removed during RNA processing are ________.

a. exons
b. caps
c. poly-A tails
d. introns

38. The RNA components of ribosomes are synthesized in the ________.
a. cytoplasm
b. nucleus
c. nucleolus
d. endoplasmic reticulum

39. How long would the peptide be that is translated from this MRNA sequence: 5'-AUGGGCUACCGA-3'?

a. 0
b. 2
c. 3
d. 4

40. Control of gene expression in eukaryotic cells occurs at which level(s)?
a. only the transcriptional level
b. epigenetic and transcriptional levels
c. epigenetic, transcriptional, and translational levels
d. epigenetic, transcriptional, post-transcriptional, translational, and post-translational levels

41. Post-translational control refers to:
a. regulation of gene expression after transcription
b. regulation of gene expression after translation
c. control of epigenetic activation
d. period between transcription and translation

42. Which of the following correctly describes the structure of the typical eukaryotic genome?
A. diploid
B. linear
C. singular
D. double stranded

43. Which of the following is typically found as part of the prokaryotic genome?
A. chloroplast DNA
B. linear chromosomes
C. plasmids
D. mitochondrial DNA

44. *Serratia marcescens* cells produce a red pigment at room temperature. The red color of the colonies is an example of which of the following?
A. genotype
B. phenotype
C. change in DNA base composition
D. adaptation to the environment

45. Which of the following genes would not likely be encoded on a plasmid?
A. genes encoding toxins that damage host tissue
B. genes encoding antibacterial resistance
C. gene encoding enzymes for glycolysis
D. genes encoding enzymes for the degradation of an unusual substrate

46. Histones are DNA binding proteins that are important for DNA packaging in which of the following?
A. double-stranded and single-stranded DNA viruses
B. archaea and bacteria
C. bacteria and eukaryotes
D. eukaryotes and archaea

47. ESTs are ________.
a. generated after a cDNA library is made
b. unique sequences in the genome
c. useful for mapping using sequence information
d. all of the above

48. Linkage analysis ________.
a. is used to create a physical map
b. is based on the natural recombination process
c. requires radiation hybrid mapping
d. involves breaking and re-joining of DNA artificially

49. Genetic recombination occurs by which process?

a. independent assortment
b. crossing over
c. chromosome segregation
d. sister chromatids

50. Individual genetic maps in a given species are:

a. genetically similar
b. genetically identical
c. genetically dissimilar
d. not useful in species analysis

51. Information obtained by microscopic analysis of stained chromosomes is used in:
a. radiation hybrid mapping
b. sequence mapping
c. RFLP mapping
d. cytogenetic mapping

52. Genomics can be used in agriculture to:
a. generate new hybrid strains
b. improve disease resistance
c. improve yield
d. all of the above

53. Genomics can be used on a personal level to:

a. decrease transplant rejection
b. Predict genetic diseases that a person may have inherited
c. Determine the risks of genetic diseases for an individual's children
d. All the above

CRITICAL THINKING QUESTIONS

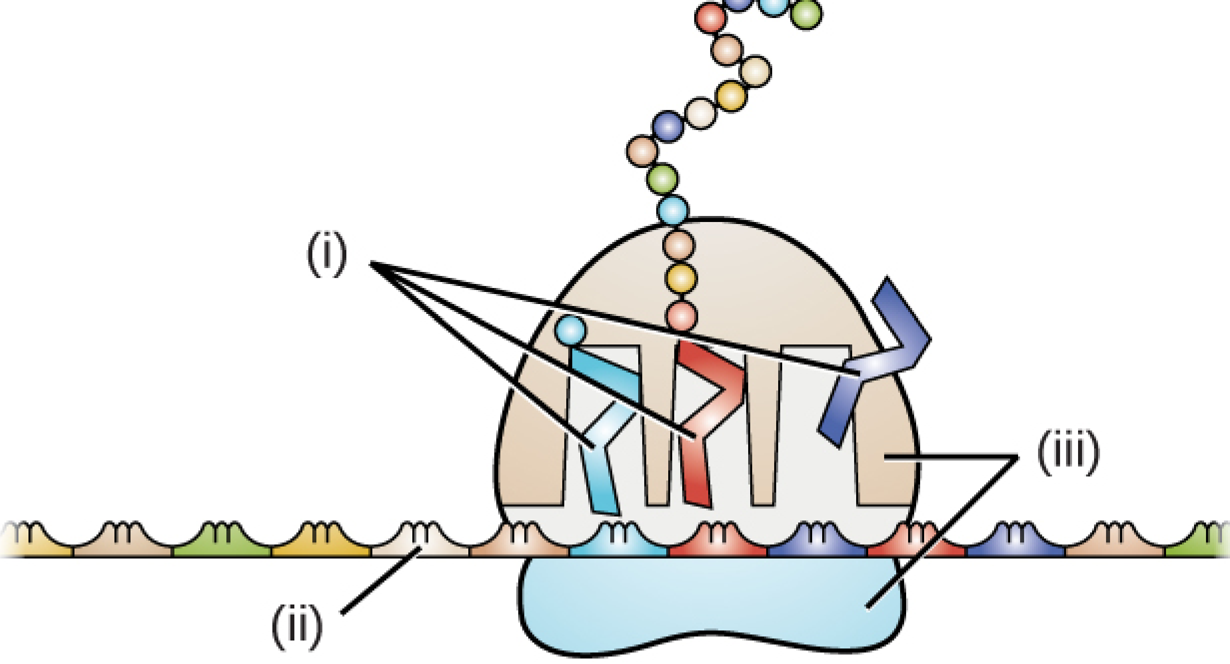
54. A certain DNA sample is found to have a makeup consisting of 22% thymine. Use Chargaff's rules to fill in the percentages for the other three nitrogenous bases.

adenine	guanine	thymine	cytosine
___%	___%	22%	___%

55. In considering the structure of the DNA double helix, how would you expect the structure to differ if there was base pairing between two purines? Between two pyrimidines?

56. How do the linear chromosomes in eukaryotes ensure that its ends are replicated completely?

57. Identify the location of mRNA, rRNA, and tRNA in the figure.



58. Why does it make sense that tRNA and rRNA molecules are more stable than mRNA molecules?

59. Transcribe and translate the following DNA sequence (nontemplate strand): 5'-ATGGCCGGTTATTAAGCA-3'

60. Describe how controlling gene expression will alter the overall protein levels in the cell.

61. A new type of bacteriophage has been isolated and you are in charge of characterizing its genome. The base composition of the bacteriophage is A (15%), C (20%), T (35%), and G (30%). What can you conclude about the genome of the virus?

62. Why is so much effort being poured into genome mapping applications?

63. How could a genetic map of the human genome help find a cure for cancer?

64. Explain why metagenomics is probably the most revolutionary application of genomics.

65. How can genomics be used to predict disease risk and treatment options?

13 | PROTEIN STRUCTURE AND FUNCTION

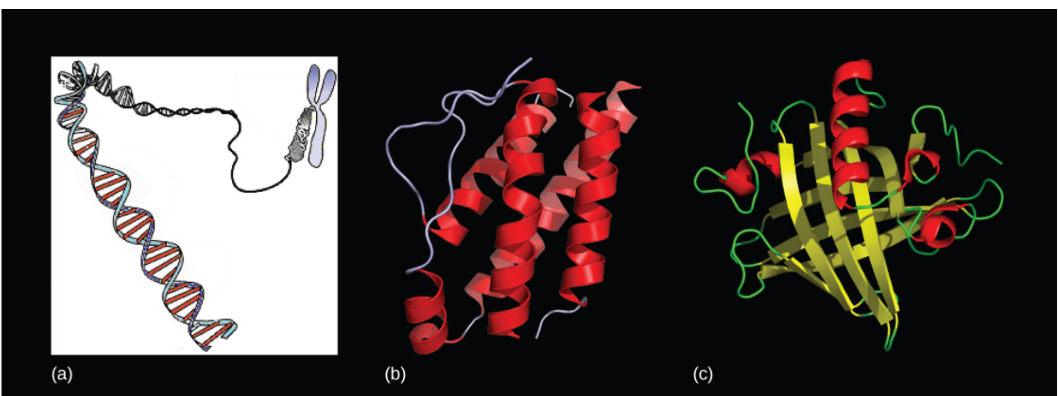


Figure 13.1 Genes, which are carried on (a) chromosomes, are linearly organized instructions for making the RNA and protein molecules that are necessary for all of the processes of life. The (b) interleukin-2 protein and (c) alpha-2u-globulin protein are just two examples of the array of different molecular structures that are encoded by genes. (credit "chromosome: National Human Genome Research Institute; credit "interleukin-2": Ramin Herati/Created from PDB 1M47 and rendered with Pymol; credit "alpha-2u-globulin": Darren Logan/rendered with AISMIG)

Chapter Outline

13.1: Proteins

13.2: Enzymes

13.3: Applying Proteomics

Introduction

Since the rediscovery of Mendel's work in 1900, the definition of the gene has progressed from an abstract unit of heredity to a tangible molecular entity capable of replication, expression, and mutation (Figure 13.1). Genes are composed of DNA and are linearly arranged on chromosomes. Genes specify the sequences of amino acids, which are the building blocks of proteins. In turn, proteins are responsible for orchestrating nearly every function of the cell. Both genes and the proteins they encode are absolutely essential to life as we know it.

13.1 | Proteins

By the end of this section, you will be able to:

- Describe the functions proteins perform in the cell and in tissues
- Discuss the relationship between amino acids and proteins
- Explain the four levels of protein organization
- Describe the ways in which protein shape and function are linked

Proteins are one of the most abundant organic molecules in living systems and have the most diverse range of functions of all macromolecules. Proteins may be structural, regulatory, contractile, or protective; they may serve in transport, storage, or membranes; or they may be toxins or enzymes. Each cell in a living system may contain thousands of proteins, each with a unique function. Their structures, like their functions, vary greatly. They are

all, however, polymers of amino acids, arranged in a linear sequence.

Types and Functions of Proteins

Enzymes, which are produced by living cells, are catalysts in biochemical reactions (like digestion) and are usually complex or conjugated proteins. Each enzyme is specific for the substrate (a reactant that binds to an enzyme) it acts on. The enzyme may help in breakdown, rearrangement, or synthesis reactions. Enzymes that break down their substrates are called catabolic enzymes, enzymes that build more complex molecules from their substrates are called anabolic enzymes, and enzymes that affect the rate of reaction are called catalytic enzymes. It should be noted that all enzymes increase the rate of reaction and, therefore, are considered to be organic catalysts. An example of an enzyme is salivary amylase, which hydrolyzes its substrate amylose, a component of starch.

Hormones are chemical-signaling molecules, usually small proteins or steroids, secreted by endocrine cells that act to control or regulate specific physiological processes, including growth, development, metabolism, and reproduction. For example, insulin is a protein hormone that helps to regulate the blood glucose level. The primary types and functions of proteins are listed in Table 13.1.

Protein Types and Functions

Type	Examples	Functions
Digestive Enzymes	Amylase, lipase, pepsin, trypsin	Help in digestion of food by catabolizing nutrients into monomeric units
Transport	Hemoglobin, albumin	Carry substances in the blood or lymph throughout the body
Structural	Actin, tubulin, keratin	Construct different structures, like the cytoskeleton
Hormones	Insulin, thyroxine	Coordinate the activity of different body systems
Defense	Immunoglobulins	Protect the body from foreign pathogens
Contractile	Actin, myosin	Effect muscle contraction
Storage	Legume storage proteins, egg white (albumin)	Provide nourishment in early development of the embryo and the seedling

Table 13.1

Proteins have different shapes and molecular weights; some proteins are globular in shape whereas others are fibrous in nature. For example, hemoglobin is a globular protein, but collagen, found in our skin, is a fibrous protein. Protein shape is critical to its function, and this shape is maintained by many different types of chemical bonds. Changes in temperature, pH, and exposure to chemicals may lead to permanent changes in the shape of the protein, leading to loss of function, known as **denaturation**. All proteins are made up of different arrangements of the same 20 types of amino acids.

Amino Acids

Amino acids are the monomers that make up proteins. Each amino acid has the same fundamental structure, which consists of a central carbon atom, also known as the alpha (*α*) carbon, bonded to an amino group (NH_2), a carboxyl group (COOH), and to a hydrogen atom. Every amino acid also has another atom or group of atoms bonded to the central atom known as the R group (Figure 13.2).

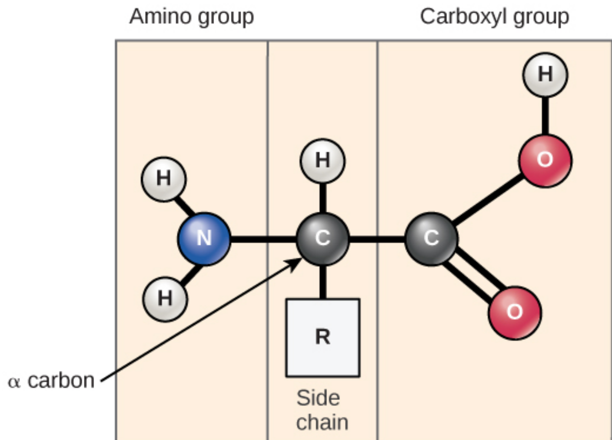


Figure 13.2 Amino acids have a central asymmetric carbon to which an amino group, a carboxyl group, a hydrogen atom, and a side chain (R group) are attached.

The name "amino acid" is derived from the fact that they contain both amino group and carboxyl-acid-group in their basic structure. As mentioned, there are 20 amino acids present in proteins. Nine of these are considered essential amino acids in humans because the human body cannot produce them and they are obtained from the diet. For each amino acid, the R group (or side chain) is different (**Figure 13.3**).

Art Connection

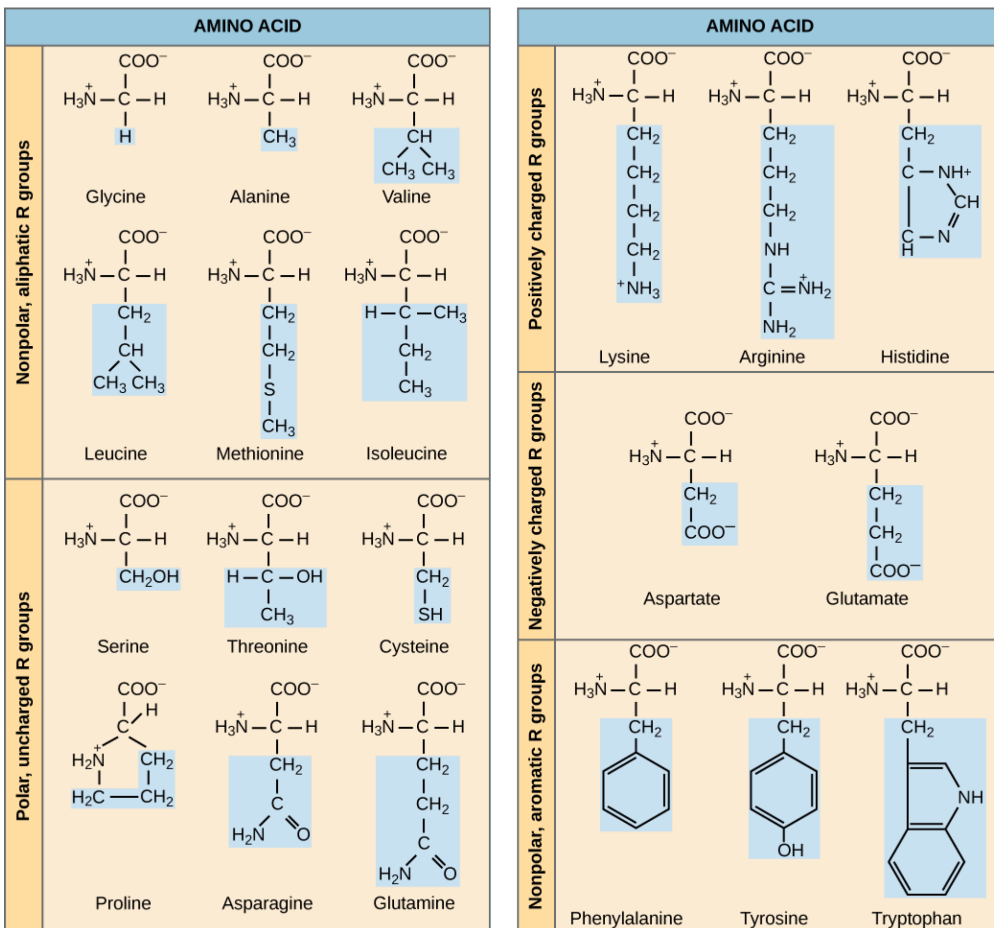


Figure 13.3 There are 20 common amino acids commonly found in proteins, each with a different R group (variant group) that determines its chemical nature.

Which categories of amino acid would you expect to find on the surface of a soluble protein, and which would you expect to find in the interior? What distribution of amino acids would you expect to find in a protein embedded in a lipid bilayer?

The chemical nature of the side chain determines the nature of the amino acid (that is, whether it is acidic, basic, polar, or nonpolar). For example, the amino acid glycine has a hydrogen atom as the R group. Amino acids such as valine, methionine, and alanine are nonpolar or hydrophobic in nature, while amino acids such as serine, threonine, and cysteine are polar and have hydrophilic side chains. The side chains of lysine and arginine are positively charged, and therefore these amino acids are also known as basic amino acids. Proline has an R group that is linked to the amino group, forming a ring-like structure. Proline is an exception to the standard structure of an animo acid since its amino group is not separate from the side chain (**Figure 13.3**).

Amino acids are represented by a single upper case letter or a three-letter abbreviation. For example, valine is known by the letter V or the three-letter symbol val. Just as some fatty acids are essential to a diet, some amino acids are necessary as well. They are known as essential amino acids, and in humans they include isoleucine, leucine, and cysteine. Essential amino acids refer to those necessary for construction of proteins in the body, although not produced by the body; which amino acids are essential varies from organism to organism.

The sequence and the number of amino acids ultimately determine the protein's shape, size, and function. Each amino acid is attached to another amino acid by a covalent bond, known as a **peptide bond**, which is formed by a dehydration reaction. The carboxyl group of one amino acid and the amino group of the incoming amino acid combine, releasing a molecule of water. The resulting bond is the peptide bond (**Figure 13.4**).

Figure 13.4 Peptide bond formation is a dehydration synthesis reaction. The carboxyl group of one amino acid is linked to the amino group of the incoming amino acid. In the process, a molecule of water is released.

The products formed by such linkages are called peptides. As more amino acids join to this growing chain, the resulting chain is known as a polypeptide. Each polypeptide has a free amino group at one end. This end is called the N terminal, or the amino terminal, and the other end has a free carboxyl group, also known as the C or carboxyl terminal. While the terms polypeptide and protein are sometimes used interchangeably, a polypeptide is technically a polymer of amino acids, whereas the term protein is used for a polypeptide or polypeptides that have combined together, often have bound non-peptide prosthetic groups, have a distinct shape, and have a unique function. After protein synthesis (translation), most proteins are modified. These are known as post-translational modifications. They may undergo cleavage, phosphorylation, or may require the addition of other chemical groups. Only after these modifications is the protein completely functional.

Click through the steps of protein synthesis in this **interactive tutorial (http://openstaxcollege.org/l/protein_synth)** .

The Evolutionary Significance of Cytochrome c

Cytochrome c is an important component of the electron transport chain, a part of cellular respiration, and it is normally found in the cellular organelle, the mitochondrion. This protein has a heme prosthetic group, and the central ion of the heme gets alternately reduced and oxidized during electron transfer. Because this essential protein's role in producing cellular energy is crucial, it has changed very little over millions of years. Protein sequencing has shown that there is a considerable amount of cytochrome c amino acid sequence homology among different species; in other words, evolutionary kinship can be assessed by measuring the similarities or differences among various species' DNA or protein sequences.

Scientists have determined that human cytochrome c contains 104 amino acids. For each cytochrome c molecule from different organisms that has been sequenced to date, 37 of these amino acids appear in the same position in all samples of cytochrome c. This indicates that there may have been a common ancestor. On comparing the human and chimpanzee protein sequences, no sequence difference was found. When human and rhesus monkey sequences were compared, the single difference found was in one amino acid. In another comparison, human to yeast sequencing shows a difference in the 44th position.

Protein Structure

As discussed earlier, the shape of a protein is critical to its function. For example, an enzyme can bind to a specific substrate at a site known as the active site. If this active site is altered because of local changes or changes in overall protein structure, the enzyme may be unable to bind to the substrate. To understand how the protein gets its final shape or conformation, we need to understand the four levels of protein structure: primary, secondary, tertiary, and quaternary.

Primary Structure

The unique sequence of amino acids in a polypeptide chain is its **primary structure**. For example, the pancreatic hormone insulin has two polypeptide chains, A and B, and they are linked together by disulfide bonds. The N terminal amino acid of the A chain is glycine, whereas the C terminal amino acid is asparagine (**Figure 13.5**). The sequences of amino acids in the A and B chains are unique to insulin.

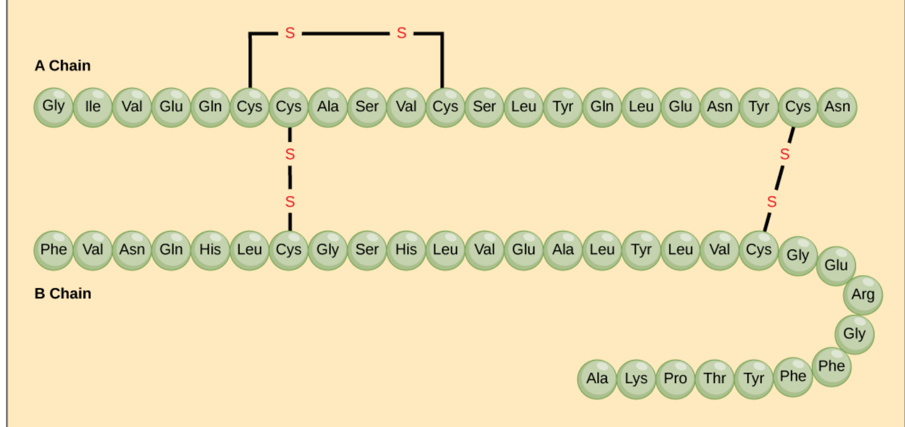


Figure 13.5 Bovine serum insulin is a protein hormone made of two peptide chains, A (21 amino acids long) and B (30 amino acids long). In each chain, primary structure is indicated by three-letter abbreviations that represent the names of the amino acids in the order they are present. The amino acid cysteine (cys) has a sulfhydryl (SH) group as a side chain. Two sulfhydryl groups can react in the presence of oxygen to form a disulfide (S-S) bond. Two disulfide bonds connect the A and B chains together, and a third helps the A chain fold into the correct shape. Note that all disulfide bonds are the same length, but are drawn different sizes for clarity.

The unique sequence for every protein is ultimately determined by the gene encoding the protein. A change in nucleotide sequence of the gene's coding region may lead to a different amino acid being added to the growing polypeptide chain, causing a change in protein structure and function. In sickle cell anemia, the hemoglobin *β* chain (a small portion of which is shown in **Figure 13.6**) has a single amino acid substitution, causing a change in protein structure and function. Specifically, the amino acid glutamic acid is substituted by valine in the *β* chain. What is most remarkable to consider is that a hemoglobin molecule is made up of two alpha chains and two beta chains that each consist of about 150 amino acids. The molecule, therefore, has about 600 amino acids. The structural difference between a normal hemoglobin molecule and a sickle cell molecule—which dramatically decreases life expectancy—is a single amino acid of the 600. What is even more remarkable is that those 600 amino acids are encoded by three nucleotides each, and the mutation is caused by a single base change (point mutation), 1 in 1800 bases.

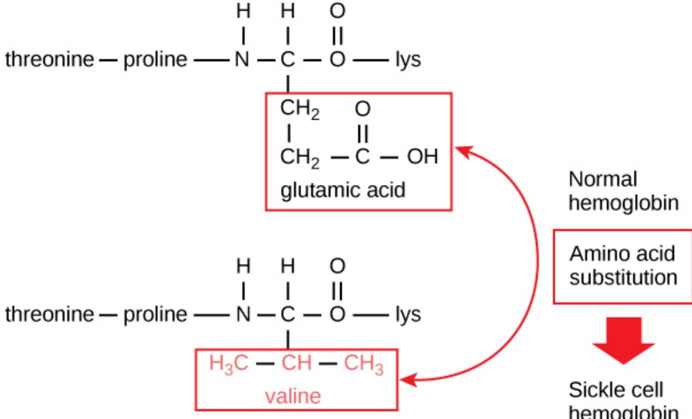


Figure 13.6 The beta chain of hemoglobin is 147 residues in length, yet a single amino acid substitution leads to sickle cell anemia. In normal hemoglobin, the amino acid at position seven is glutamate. In sickle cell hemoglobin, this glutamate is replaced by a valine.

Because of this change of one amino acid in the chain, hemoglobin molecules form long fibers that distort the biconcave, or disc-shaped, red blood cells and assume a crescent or "sickle" shape, which clogs arteries (**Figure 13.7**). This can lead to myriad serious health problems such as breathlessness, dizziness, headaches, and abdominal pain for those affected by this disease.

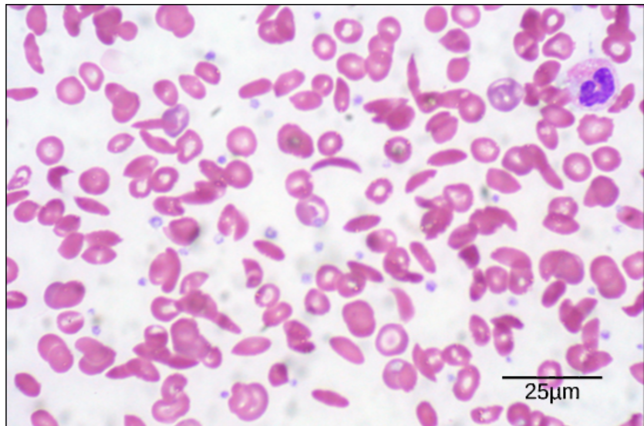


Figure 13.7 In this blood smear, visualized at 535x magnification using bright field microscopy, sickle cells are crescent shaped, while normal cells are disc-shaped. (credit: modification of work by Ed Uthman; scale-bar data from Matt Russell)

Secondary Structure

The local folding of the polypeptide in some regions gives rise to the **secondary structure** of the protein. The most common are the ***α*-helix** and ***β*-pleated sheet** structures (**Figure 13.8**). Both structures are the *α*-helix structure—the helix held in shape by hydrogen bonds. The hydrogen bonds form between the oxygen atom in the carbonyl group in one amino acid and another amino acid that is four amino acids farther along the chain.

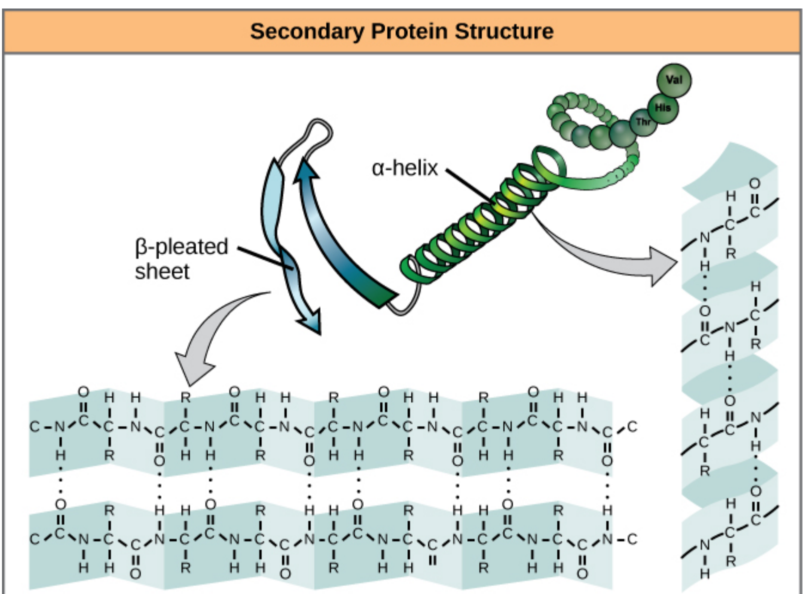


Figure 13.8 The *α*-helix and *β*-pleated sheet are secondary structures of proteins that form because of hydrogen bonding between carbonyl and amino groups in the peptide backbone. Certain amino acids have a propensity to form an *α*-helix, while others have a propensity to form a *β*-pleated sheet.

Every helical turn in an alpha helix has 3.6 amino acid residues. The R groups (the variant groups) of the polypeptide protrude out from the *α*-helix chain. In the *β*-pleated sheet, the “pleats” are formed by hydrogen bonding between atoms on the backbone of the polypeptide chain. The R groups are attached to the carbons and extend above and below the folds of the pleat. The pleated segments align parallel or antiparallel to each other, and hydrogen bonds form between the partially positive nitrogen atom in the amino group and the partially negative oxygen atom in the carbonyl group of the peptide backbone. The *α*-helix and *β*-pleated sheet structures are found in most globular and fibrous proteins and they play an important structural role.

Tertiary Structure

The unique three-dimensional structure of a polypeptide is its **tertiary structure** (**Figure 13.9**). This structure is in part due to chemical interactions at work on the polypeptide chain. Primarily, the interactions among R groups

creates the complex three-dimensional tertiary structure of a protein. The nature of the R groups found in the amino acids involved can counteract the formation of the hydrogen bonds described for standard secondary structures. For example, R groups with like charges are repelled by each other and those with unlike charges are attracted to each other (ionic bonds). When protein folding takes place, the hydrophobic R groups of nonpolar amino acids lay in the interior of the protein, whereas the hydrophilic R groups lay on the outside. The former types of interactions are also known as hydrophobic interactions. Interaction between cysteine side chains forms disulfide linkages in the presence of oxygen, the only covalent bond forming during protein folding.

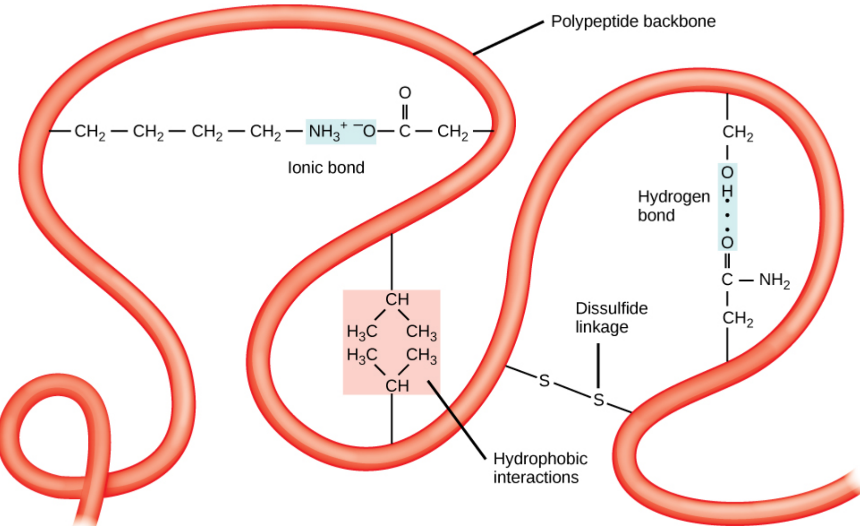


Figure 13.9 The tertiary structure of proteins is determined by a variety of chemical interactions. These include hydrophobic interactions, ionic bonding, hydrogen bonding and disulfide linkages.

All of these interactions, weak and strong, determine the final three-dimensional shape of the protein. When a protein loses its three-dimensional shape, it may no longer be functional.

Quaternary Structure

In nature, some proteins are formed from several polypeptides, also known as subunits, and the interaction of these subunits forms the **quaternary structure**. Weak interactions between the subunits help to stabilize the overall structure. For example, insulin (a globular protein) has a combination of hydrogen bonds and disulfide bonds that cause it to be mostly clumped into a ball shape. Insulin starts out as a single polypeptide and loses some internal sequences in the presence of post-translational modification after the formation of the disulfide linkages that hold the remaining chains together. Silk (a fibrous protein), however, has a β-pleated sheet structure that is the result of hydrogen bonding between different chains.

The four levels of protein structure (primary, secondary, tertiary, and quaternary) are illustrated in **Figure 13.10**.

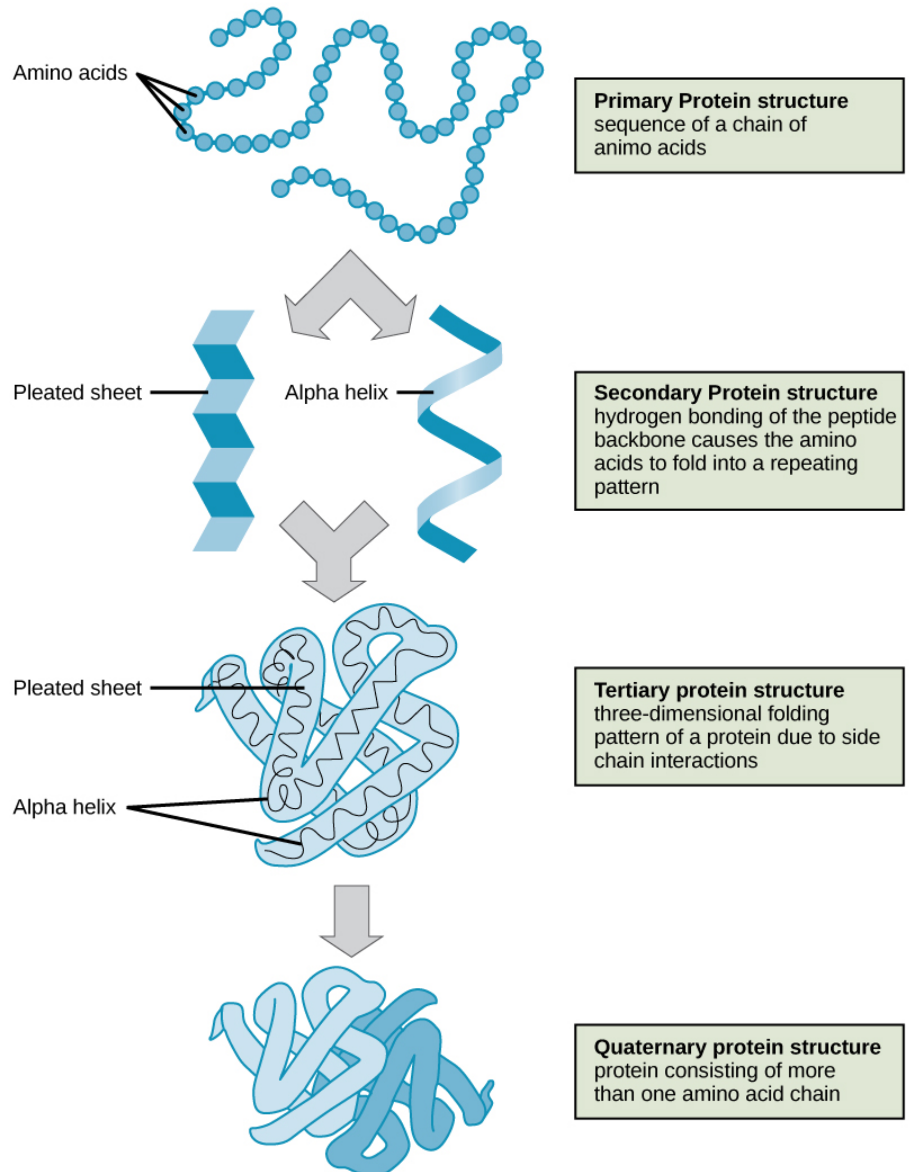


Figure 13.10 The four levels of protein structure can be observed in these illustrations. (credit: modification of work by National Human Genome Research Institute)

Denaturation and Protein Folding

Each protein has its own unique sequence and shape that are held together by chemical interactions. If the protein is subject to changes in temperature, pH, or exposure to chemicals, the protein structure may change, losing its shape without losing its primary sequence in what is known as denaturation. Denaturation is often reversible because the primary structure of the polypeptide is conserved in the process if the denaturing agent is removed, allowing the protein to resume its function. Sometimes denaturation is irreversible, leading to loss of function. One example of irreversible protein denaturation is when an egg is fried. The albumin protein in the liquid egg white is denatured when placed in a hot pan. Not all proteins are denatured at high temperatures; for instance, bacteria that survive in hot springs have proteins that function at temperatures close to boiling. The stomach is also very acidic, has a low pH, and denatures proteins as part of the digestion process; however, the digestive enzymes of the stomach retain their activity under these conditions.

Protein folding is critical to its function. It was originally thought that the proteins themselves were responsible for the folding process. Only recently was it found that often they receive assistance in the folding process from protein helpers known as **chaperones** (or chaperonins) that associate with the target protein during the folding process. They act by preventing aggregation of polypeptides that make up the complete protein structure, and

they disassociate from the protein once t[illegible]ded.

For an additional perspective on proteins, view **this animation (http://openstaxcollege.org/l/proteins)** called "Biomolecules: The Proteins."

13.2 | Enzymes

By the end of this section, you will be able to do the following:

- Describe the role of enzymes in metabolic pathways
- Explain how enzymes function as molecular catalysts
- Discuss enzyme regulation by various factors

A substance that helps a chemical reaction to occur is a catalyst, and the special molecules that catalyze biochemical reactions are enzymes. Almost all enzymes are proteins, comprised of amino acid chains, and they perform the critical task of lowering the activation energies of chemical reactions inside the cell. Enzymes do this by binding to the reactant molecules, and holding them in such a way as to make the chemical bond-breaking and bond-forming processes take place more readily. It is important to remember that enzymes do not change the reaction's ΔG. In other words, they do not change whether a reaction is exergonic (spontaneous) or endergonic. This is because they do not change the reactants' or products' free energy. They only reduce the activation energy required to reach the transition state (**Figure 13.11**).

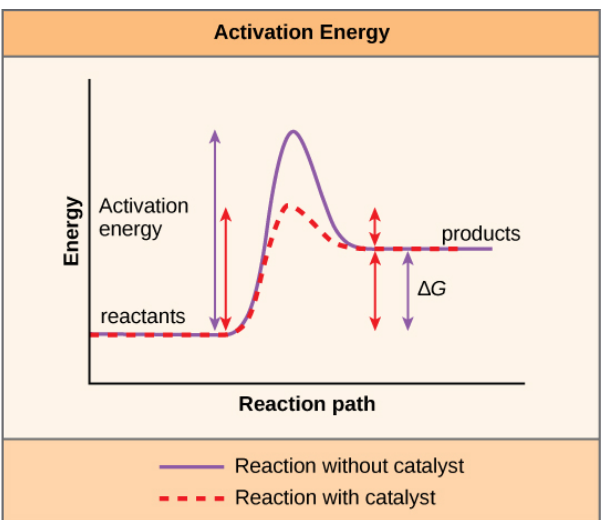


Figure 13.11 Enzymes lower the reaction's activation energy but do not change the reaction's free energy.

Enzyme Active Site and Substrate Specificity

The chemical reactants to which an enzyme binds are the enzyme's **substrates**. There may be one or more substrates, depending on the particular chemical reaction. In some reactions, a single-reactant substrate breaks down into multiple products. In others, two substrates may come together to create one larger molecule. Two reactants might also enter a reaction, both become modified, and leave the reaction as two products. The location within the enzyme where the substrate binds is the enzyme's **active site**. This is where the "action" happens. Since enzymes are proteins, there is a unique combination of amino acid residues (also side chains,

or R groups) within the active site. Different properties characterize each residue. These can be large or small, weakly acidic or basic, hydrophilic or hydrophobic, positively or negatively charged, or neutral. The unique combination of amino acid residues, their positions, sequences, structures, and properties, creates a very specific chemical environment within the active site. This specific environment is suited to bind, albeit briefly, to a specific chemical substrate (or substrates). Due to this jigsaw puzzle-like match between an enzyme and its substrates (which adapts to find the best fit between the transition state and the active site), enzymes are known for their specificity. The “best fit” results from the shape and the amino acid functional group’s attraction to the substrate. There is a specifically matched enzyme for each substrate and, thus, for each chemical reaction; however, there is flexibility as well.

The fact that active sites are so perfectly suited to provide specific environmental conditions also means that they are subject to local environmental influences. It is true that increasing the environmental temperature generally increases reaction rates, enzyme-catalyzed or otherwise. However, increasing or decreasing the temperature outside of an optimal range can affect chemical bonds within the active site in such a way that they are less well suited to bind substrates. High temperatures will eventually cause enzymes, like other biological molecules, to **denature**, a process that changes the substance's natural properties. Likewise, the local environment's pH can also affect enzyme function. Active site amino acid residues have their own acidic or basic properties that are optimal for catalysis. These residues are sensitive to changes in pH that can impair the way substrate molecules bind. Enzymes are suited to function best within a certain pH range, and, as with temperature, extreme environmental pH values (acidic or basic) can cause enzymes to denature.

Induced Fit and Enzyme Function

For many years, scientists thought that enzyme-substrate binding took place in a simple “lock-and-key” fashion. This model asserted that the enzyme and substrate fit together perfectly in one instantaneous step. However, current research supports a more refined view scientists call **induced fit** (**Figure 13.12**). This model expands upon the lock-and-key model by describing a more dynamic interaction between enzyme and substrate. As the enzyme and substrate come together, their interaction causes a mild shift in the enzyme’s structure that confirms an ideal binding arrangement between the enzyme and the substrate's transition state. This ideal binding maximizes the enzyme’s ability to catalyze its reaction.

View an induced fit animation at **this website (http://openstax.org/l/hexokinase)** .

When an enzyme binds its substrate, it forms an enzyme-substrate complex. This complex lowers the reaction's activation energy and promotes its rapid progression in one of many ways. On a basic level, enzymes promote chemical reactions that involve more than one substrate by bringing the substrates together in an optimal orientation. The appropriate region (atoms and bonds) of one molecule is juxtaposed to the other molecule's appropriate region with which it must react. Another way in which enzymes promote substrate reaction is by creating an optimal environment within the active site for the reaction to occur. Certain chemical reactions might proceed best in a slightly acidic or non-polar environment. The chemical properties that emerge from the particular arrangement of amino acid residues within an active site create the perfect environment for an enzyme’s specific substrates to react.

You have learned that the activation energy required for many reactions includes the energy involved in manipulating or slightly contorting chemical bonds so that they can easily break and allow others to reform. Enzymatic action can aid this process. The enzyme-substrate complex can lower the activation energy by contorting substrate molecules in such a way as to facilitate bond-breaking, helping to reach the transition state. Finally, enzymes can also lower activation energies by taking part in the chemical reaction itself. The amino acid residues can provide certain ions or chemical groups that actually form covalent bonds with substrate molecules as a necessary step of the reaction process. In these cases, it is important to remember that the enzyme will always return to its original state at the reaction's completion. One of enzymes' hallmark properties is that they remain ultimately unchanged by the reactions they catalyze. After an enzyme catalyzes a reaction, it releases its product(s).

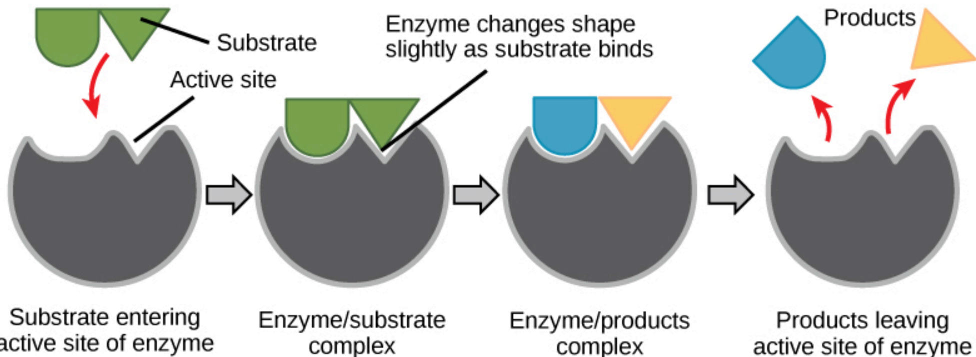


Figure 13.12 According to the induced-fit model, both enzyme and substrate undergo dynamic conformational changes upon binding. The enzyme contorts the substrate into its transition state, thereby increasing the reaction's rate.

Metabolism Control Through Enzyme Regulation

It would seem ideal to have a scenario in which all the encoded enzymes in an organism's genome existed in abundant supply and functioned optimally under all cellular conditions, in all cells, at all times. In reality, this is far from the case. A variety of mechanisms ensure that this does not happen. Cellular needs and conditions vary from cell to cell, and change within individual cells over time. The required enzymes and energetic demands of stomach cells are different from those of fat storage cells, skin cells, blood cells, and nerve cells. Furthermore, a digestive cell works much harder to process and break down nutrients during the time that closely follows a meal compared with many hours after a meal. As these cellular demands and conditions vary, so do the amounts and functionality of different enzymes.

Since the rates of biochemical reactions are controlled by activation energy, and enzymes lower and determine activation energies for chemical reactions, the relative amounts and functioning of the variety of enzymes within a cell ultimately determine which reactions will proceed and at which rates. This determination is tightly controlled. In certain cellular environments, environmental factors like pH and temperature partly control enzyme activity. There are other mechanisms through which cells control enzyme activity and determine the rates at which various biochemical reactions will occur.

Molecular Regulation of Enzymes

Enzymes can be regulated in ways that either promote or reduce their activity. There are many different kinds of molecules that inhibit or promote enzyme function, and various mechanisms exist for doing so. For example, in some cases of enzyme inhibition, an inhibitor molecule is similar enough to a substrate that it can bind to the active site and simply block the substrate from binding. When this happens, the enzyme is inhibited through **competitive inhibition**, because an inhibitor molecule competes with the substrate for active site binding (**Figure 13.13**). On the other hand, in **noncompetitive inhibition**, an inhibitor molecule binds to the enzyme in a location other than the active site, called an allosteric site, but still manages to prevent substrate binding to the active site. Some inhibitor molecules bind to enzymes in a location where their binding induces a conformational change that reduces the enzyme activity as it no longer effectively catalyzes the conversion of the substrate to product.

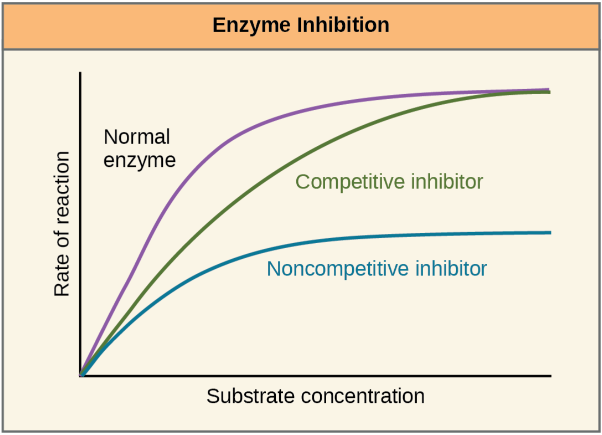


Figure 13.13 Competitive and noncompetitive inhibition affect the reaction's rate differently. Competitive inhibitors affect the initial rate but do not affect the maximal rate; whereas, noncompetitive inhibitors affect the maximal rate.

Some inhibitor molecules bind to enzymes in a location where their binding induces a conformational change that reduces the enzyme's affinity for its substrate. This type of inhibition is an **allosteric inhibition** (Figure 13.14). More than one polypeptide comprise most allosterically regulated enzymes, meaning that they have more than one protein subunit. When an allosteric inhibitor binds to an enzyme, all active sites on the protein subunits change slightly such that they bind their substrates with less efficiency. There are allosteric activators as well as inhibitors. Allosteric activators bind to locations on an enzyme away from the active site, inducing a conformational change that increases the affinity of the enzyme's active site(s) for its substrate(s).

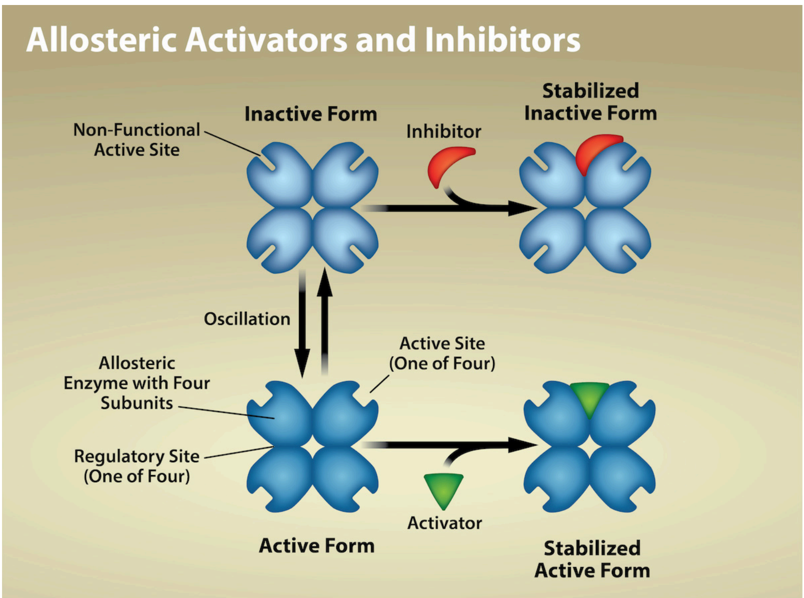


Figure 13.14 Allosteric inhibitors modify the enzyme's active site so that substrate binding is reduced or prevented. In contrast, allosteric activators modify the enzyme's active site so that the affinity for the substrate increases. Credit: Rao, A., Hawkins, A., Fletcher, S. and Tag, A. Department of Biology, Texas A&M University.

everyday CONNECTION

Figure 13.15 Have you ever wondered how pharmaceutical drugs are developed? (credit: Deborah Austin)

Drug Discovery by Looking for Inhibitors of Key Enzymes in Specific Pathways

Enzymes are key components of metabolic pathways. Understanding how enzymes work and how they can be regulated is a key principle behind developing many pharmaceutical drugs (Figure 13.15) on the market today. Biologists working in this field collaborate with other scientists, usually chemists, to design drugs.

Consider statins for example—which is a class of drugs that reduces cholesterol levels. These compounds are essentially inhibitors of the enzyme HMG-CoA reductase. HMG-CoA reductase is the enzyme that synthesizes cholesterol from lipids in the body. By inhibiting this enzyme, the drug reduces cholesterol levels synthesized in the body. Similarly, acetaminophen, popularly marketed under the brand name Tylenol, is an inhibitor of the enzyme cyclooxygenase. While it is effective in providing relief from fever and inflammation (pain), scientists still do not completely understand its mechanism of action.

How are drugs developed? One of the first challenges in drug development is identifying the specific molecule that the drug is intended to target. In the case of statins, HMG-CoA reductase is the drug target. Researchers identify targets through painstaking research in the laboratory. Identifying the target alone is not sufficient. Scientists also need to know how the target acts inside the cell and which reactions go awry in the case of disease. Once researchers identify the target and the pathway, then the actual drug design process begins. During this stage, chemists and biologists work together to design and synthesize molecules that can either block or activate a particular reaction. However, this is only the beginning: both if and when a drug prototype is successful in performing its function, then it must undergo many tests from *in vitro* experiments to clinical trials before it can obtain FDA approval to be on the market.

Many enzymes don't work optimally, or even at all, unless bound to other specific non-protein helper molecules, either temporarily through ionic or hydrogen bonds or permanently through stronger covalent bonds. Two types of helper molecules are **cofactors** and **coenzymes**. Binding to these molecules promotes optimal conformation and function for their respective enzymes. Cofactors are inorganic ions such as iron (Fe++) and magnesium (Mg++). One example of an enzyme that requires a metal ion as a cofactor is the enzyme that builds DNA molecules, DNA polymerase, which requires a bound zinc ion (Zn++) to function. Coenzymes are organic helper molecules, with a basic atomic structure comprised of carbon and hydrogen, which are required for enzyme action. The most common sources of coenzymes are dietary vitamins (Figure 13.16). Some vitamins are precursors to coenzymes and others act directly as coenzymes. Vitamin C is a coenzyme for multiple enzymes that take part in building the important connective tissue component, collagen. An important step in breaking down glucose to yield energy is catalysis by a multi-enzyme complex scientists call pyruvate dehydrogenase. Pyruvate dehydrogenase is a complex of several enzymes that actually requires one cofactor (a magnesium ion) and five different organic coenzymes to catalyze its specific chemical reaction. Therefore, enzyme function is, in part, regulated by an abundance of various cofactors and coenzymes, which the diets of most organisms supply.

Dietary Vitamins	
Vitamin A (retinol)	Folic acid (folate)
Vitamin B_1 (thiamin)	Vitamin C (ascorbic acid)
Vitamin B_2 (riboflavin)	Vitamin D_2 (calciferol)
Vitamin B_6 (pyridoxine)	Vitamin E (α-tocopherol)

Figure 13.16 Vitamins are important coenzymes or precursors of coenzymes, and are required for enzymes to function properly. Multivitamin capsules usually contain mixtures of all the vitamins at different percentages.

Enzyme Compartmentalization

In eukaryotic cells, molecules such as enzymes are usually compartmentalized into different organelles. This allows for yet another level of regulation of enzyme activity. Enzymes required only for certain cellular processes are sometimes housed separately along with their substrates, allowing for more efficient chemical reactions. Examples of this sort of enzyme regulation based on location and proximity include the enzymes involved in the latter stages of cellular respiration, which take place exclusively in the mitochondria, and the enzymes involved in digesting cellular debris and foreign materials, located within lysosomes.

Feedback Inhibition in Metabolic Pathways

Molecules can regulate enzyme function in many ways. However, a major question remains: What are these molecules and from where do they come? Some are cofactors and coenzymes, ions, and organic molecules, as you have learned. What other molecules in the cell provide enzymatic regulation, such as allosteric modulation, and competitive and noncompetitive inhibition? The answer is that a wide variety of molecules can perform these roles. Some include pharmaceutical and non-pharmaceutical drugs, toxins, and poisons from the environment. Perhaps the most relevant sources of enzyme regulatory molecules, with respect to cellular metabolism, are cellular metabolic reaction products themselves. In a most efficient and elegant way, cells have evolved to use their own reactions' products for feedback inhibition of enzyme activity. **Feedback inhibition** involves using a reaction product to regulate its own further production (Figure 13.17). The cell responds to the abundance of specific products by slowing down production during anabolic or catabolic reactions. Such reaction products may inhibit the enzymes that catalyzed their production through the mechanisms that we described above.

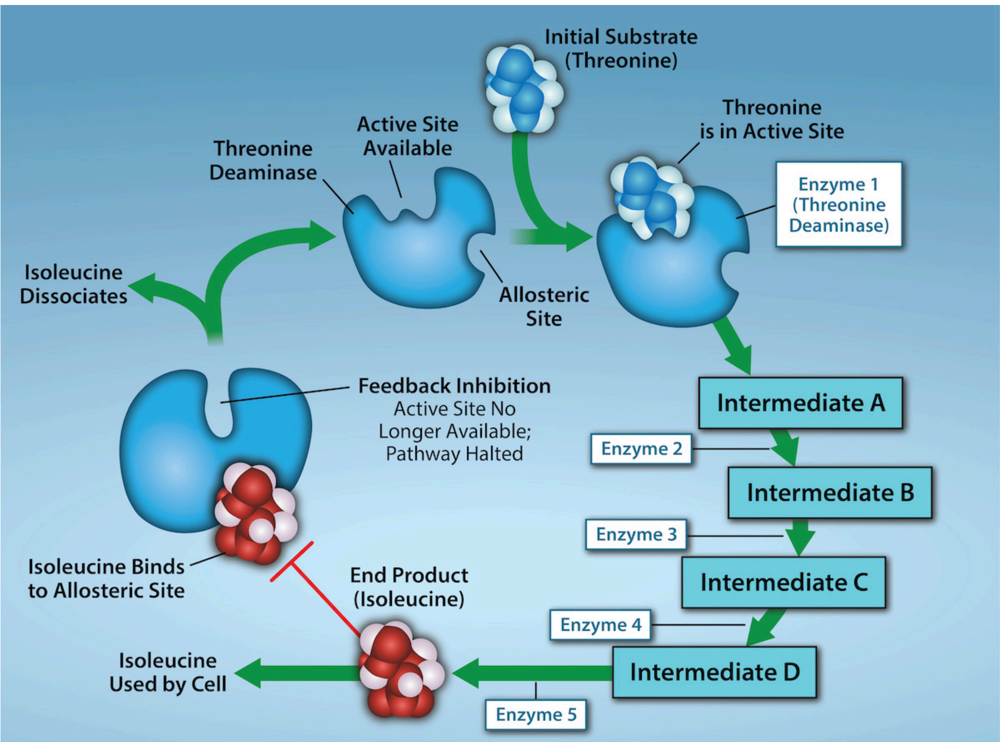


Figure 13.17 Metabolic pathways are a series of reactions that multiple enzymes catalyze. Feedback inhibition, where the pathway's end product inhibits an upstream step, is an important regulatory mechanism in cells. Metabolic pathways are a series of reactions catalyzed by multiple enzymes (Intermediates A – D, Enzymes 1 – 5). Feedback inhibition occurs when the pathway's end product (here isoleucine) inhibits an upstream enzyme (indicated by red bar). In this example, isoleucine will bind to Threonine Deaminase (at the allosteric site) and prevent threonine from binding to this enzyme's active site, effectively blocking this metabolic pathway. When isoleucine levels decrease, Threonine will then bind to Threonine Deaminase's active site and the metabolic pathway will resume. This is an important regulatory mechanism in cells to inhibit the overproduction of a product. Credit: Rao, A., Ryan, K., Tag, A., Hawkins, A. and Fletcher S. Department of Biology, Texas A&M University.

Producing both amino acids and nucleotides is controlled through feedback inhibition. Additionally, ATP is an allosteric regulator of some of the enzymes involved in sugar's catabolic breakdown, the process that produces ATP. In this way, when ATP is abundant, the cell can prevent its further production. Remember that ATP is an unstable molecule that can spontaneously dissociate into ADP and inorganic phosphate. If too much ATP were present in a cell, much of it would go to waste. Alternatively, ADP serves as a positive allosteric regulator (an allosteric activator) for some of the same enzymes that ATP inhibits. Thus, when relative ADP levels are high compared to ATP, the cell is triggered to produce more ATP through sugar catabolism.

13.3 | Applying Proteomics

By the end of this section, you will be able to do the following:

- Explain systems biology
- Describe a proteome
- Define protein signature

Proteins are the final products of genes, which help perform the function that the gene encodes. Amino acids comprise proteins and play important roles in the cell. All enzymes (except ribozymes) are proteins that act as catalysts to affect the rate of reactions. Proteins are also regulatory molecules, and some are hormones. Transport proteins, such as hemoglobin, help transport oxygen to various organs. Antibodies that defend against foreign particles are also proteins. In the diseased state, protein function can be impaired because of changes at the genetic level or because of direct impact on a specific protein.

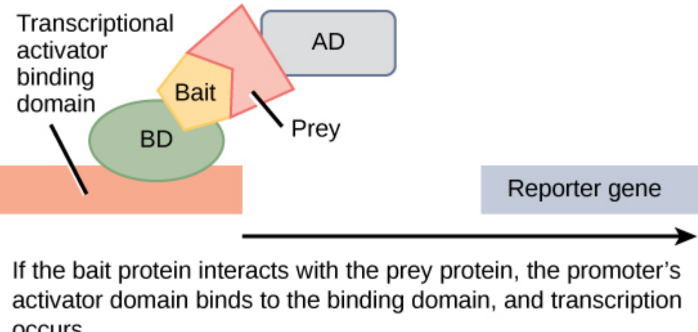
A **proteome** is the entire set of proteins that a cell type produces. We can study proteoms using the knowledge of genomes because genes code for mRNAs, and the mRNAs encode proteins. Although mRNA analysis is a step in the right direction, not all mRNAs are translated into proteins. **Proteomics** is the study of proteomes' function. Proteomics complements genomics and is useful when scientists want to test their hypotheses that they based on genes. Even though all multicellular organisms' cells have the same set of genes, the set of proteins produced in different tissues is different and dependent on gene expression. Thus, the genome is constant, but the proteome varies and is dynamic within an organism. In addition, RNAs can be alternately spliced (cut and pasted to create novel combinations and novel proteins) and many proteins modify themselves after translation by processes such as proteolytic cleavage, phosphorylation, glycosylation, and ubiquitination. There are also protein-protein interactions, which complicate studying proteomes. Although the genome provides a blueprint, the final architecture depends on several factors that can change the progression of events that generate the proteome.

Metabolomics is related to genomics and proteomics. **Metabolomics** involves studying small molecule metabolites in an organism. The **metabolome** is the complete set of metabolites that are related to an organism's genetic makeup. Metabolomics offers an opportunity to compare genetic makeup and physical characteristics, as well as genetic makeup and environmental factors. The goal of metabolome research is to identify, quantify, and catalogue all the metabolites in living organisms' tissues and fluids.

Basic Techniques in Protein Analysis

The ultimate goal of proteomics is to identify or compare the proteins expressed from a given genome under specific conditions, study the interactions between the proteins, and use the information to predict cell behavior or develop drug targets. Just as scientists analyze the genome using the basic DNA sequencing technique, proteomics requires techniques for protein analysis. The basic technique for protein analysis, analogous to DNA sequencing, is mass spectrometry. Mass spectrometry identifies and determines a molecule's characteristics. Advances in spectrometry have allowed researchers to analyze very small protein samples. X-ray crystallography, for example, enables scientists to determine a protein crystal's three-dimensional structure at atomic resolution. Another protein imaging technique, nuclear magnetic resonance (NMR), uses atoms' magnetic properties to determine the protein's three-dimensional structure in aqueous solution. Scientists have also used protein microarrays to study protein interactions. Large-scale adaptations of the basic two-hybrid screen (**Figure 13.18**) have provided the basis for protein microarrays. Scientists use computer software to analyze the vast amount of data for proteomic analysis.

Genomic- and proteomic-scale analyses are part of **systems biology**, which is the study of whole biological systems (genomes and proteomes) based on interactions within the system. The European Bioinformatics Institute and the Human Proteome Organization (HUPO) are developing and establishing effective tools to sort through the enormous pile of systems biology data. Because proteins are the direct products of genes and reflect activity at the genomic level, it is natural to use proteomes to compare the protein profiles of different cells to identify proteins and genes involved in disease processes. Most pharmaceutical drug trials target proteins. Researchers use information that they obtain from proteomics to identify novel drugs and to understand their mechanisms of action.



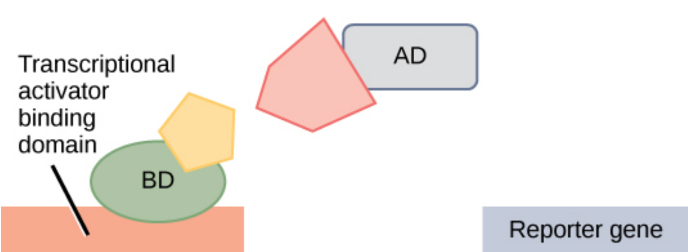


Figure 13.18 Scientists use two-hybrid screening to determine whether two proteins interact. In this method, a transcription factor splits into a DNA-binding domain (BD) and an activator domain (AD). The binding domain is able to bind the promoter in the activator domain's absence, but it does not turn on transcription. The bait protein attaches to the BD, and the prey protein attaches to the AD. Transcription occurs only if the prey "catches" the bait.

Scientists are challenged when implementing proteomic analysis because it is difficult to detect small protein quantities. Although mass spectrometry is good for detecting small protein amounts, variations in protein expression in diseased states can be difficult to discern. Proteins are naturally unstable molecules, which makes proteomic analysis much more difficult than genomic analysis.

Cancer Proteomics

Researchers are studying patients' genomes and proteomes to understand the genetic basis of diseases. The most prominent disease researchers are studying with proteomic approaches is cancer. These approaches improve screening and early cancer detection. Researchers are able to identify proteins whose expression indicates the disease process. An individual protein is a **biomarker**; whereas, a set of proteins with altered expression levels is a **protein signature**. For a biomarker or protein signature to be useful as a candidate for early cancer screening and detection, they must secrete in body fluids, such as sweat, blood, or urine, such that health professionals can perform large-scale screenings in a noninvasive fashion. The current problem with using biomarkers for early cancer detection is the high rate of false-negative results. A **false negative** is an incorrect test result that should have been positive. In other words, many cancer cases go undetected, which makes biomarkers unreliable. Some examples of protein biomarkers in cancer detection are CA-125 for ovarian cancer and PSA for prostate cancer. Protein signatures may be more reliable than biomarkers to detect cancer cells. Researchers are also using proteomics to develop individualized treatment plans, which involves predicting whether or not an individual will respond to specific drugs and the side effects that the individual may experience. Researchers also use proteomics to predict the possibility of disease recurrence.

The National Cancer Institute has developed programs to improve cancer detection and treatment. The Clinical Proteomic Technologies for Cancer and the Early Detection Research Network are efforts to identify protein signatures specific to different cancer types. The Biomedical Proteomics Program identifies protein signatures and designs effective therapies for cancer patients.

KEY TERMS

active site enzyme's specific region to which the substrate binds

allosteric inhibition inhibition by a binding event at a site different from the active site, which induces a conformational change and reduces the enzyme's affinity for its substrate

alpha-helix structure (α-helix) type of secondary structure of proteins formed by folding of the polypeptide into a helix shape with hydrogen bonds stabilizing the structure

amino acid monomer of a protein; has a central carbon or alpha carbon to which an amino group, a carboxyl group, a hydrogen, and an R group or side chain is attached; the R group is different for all 20 amino acids

beta-pleated sheet (β-pleated) secondary structure found in proteins in which “pleats” are formed by hydrogen bonding between atoms on the backbone of the polypeptide chain

biomarker individual protein that is uniquely produced in a diseased state

chaperone (also, chaperonin) protein that helps nascent protein in the folding process

coenzyme small organic molecule, such as a vitamin or its derivative, which is required to enhance an enzyme's activity

cofactor inorganic ion, such as iron and magnesium ions, required for optimal enzyme activity regulation

competitive inhibition type of inhibition in which the inhibitor competes with the substrate molecule by binding to the enzyme's active site

denaturation loss of shape in a protein as a result of changes in temperature, pH, or exposure to chemicals

denature process that changes a substance's natural properties

enzyme catalyst in a biochemical reaction that is usually a complex or conjugated protein

false negative incorrect test result that should have been positive

feedback inhibition a product's effect of a reaction sequence to decrease its further production by inhibiting the first enzyme's activity in the pathway that produces it

hormone chemical signaling molecule, usually protein or steroid, secreted by endocrine cells that act to control or regulate specific physiological processes

induced fit dynamic fit between the enzyme and its substrate, in which both components modify their structures to allow for ideal binding

metabolome complete set of metabolites which are related to an organism's genetic makeup

metabolomics study of small molecule metabolites in an organism

peptide bond bond formed between two amino acids by a dehydration reaction

polypeptide long chain of amino acids linked by peptide bonds

primary structure linear sequence of amino acids in a protein

protein biological macromolecule composed of one or more chains of amino acids

protein signature set of uniquely expressed proteins in the diseased state

proteome entire set of proteins that cell type produces

proteomics study of proteomes' function

quaternary structure association of discrete polypeptide subunits in a protein

secondary structure regular structure formed by proteins by intramolecular hydrogen bonding between the oxygen atom of one amino acid residue and the hydrogen attached to the nitrogen atom of another amino acid residue

substrate molecule on which the enzyme acts

systems biology study of whole biological systems (genomes and proteomes) based on interactions within the system

tertiary structure three-dimensional conformation of a protein, including interactions between secondary structural elements; formed from interactions between amino acid side chains

CHAPTER SUMMARY

13.1 Proteins

Proteins are a class of macromolecules that perform a diverse range of functions for the cell. They help in metabolism by providing structural support and by acting as enzymes, carriers, or hormones. The building blocks of proteins (monomers) are amino acids. Each amino acid has a central carbon that is linked to an amino group, a carboxyl group, a hydrogen atom, and an R group or side chain. There are 20 commonly occurring amino acids, each of which differs in the R group. Each amino acid is linked to its neighbors by a peptide bond. A long chain of amino acids is known as a polypeptide.

Proteins are organized at four levels: primary, secondary, tertiary, and (optional) quaternary. The primary structure is the unique sequence of amino acids. The local folding of the polypeptide to form structures such as the *α* helix and *β*-pleated sheet constitutes the secondary structure. The overall three-dimensional structure is the tertiary structure. When two or more polypeptides combine to form the complete protein structure, the configuration is known as the quaternary structure of a protein. Protein shape and function are intricately linked; any change in shape caused by changes in temperature or pH may lead to protein denaturation and a loss in function.

13.2 Enzymes

Enzymes are chemical catalysts that accelerate chemical reactions at physiological temperatures by lowering their activation energy. Enzymes are usually proteins consisting of one or more polypeptide chains. Enzymes have an active site that provides a unique chemical environment, comprised of certain amino acid R groups (residues). This unique environment is perfectly suited to convert particular chemical reactants for that enzyme, scientists call substrates, into unstable intermediates that they call transition states. Enzymes and substrates bind with an induced fit, which means that enzymes undergo slight conformational adjustments upon substrate contact, leading to full, optimal binding. Enzymes bind to substrates and catalyze reactions in four different ways: bringing substrates together in an optimal orientation, compromising the bond structures of substrates so that bonds can break down more easily, providing optimal environmental conditions for a reaction to occur, or participating directly in their chemical reaction by forming transient covalent bonds with the substrates.

Enzyme action must be regulated so that in a given cell at a given time, the desired reactions catalyze and the undesired reactions are not. Enzymes are regulated by cellular conditions, such as temperature and pH. They are also regulated through their location within a cell, sometimes compartmentalized so that they can only catalyze reactions under certain circumstances. Enzyme inhibition and activation via other molecules are other important ways that enzymes are regulated. Inhibitors can act competitively, noncompetitively, or allosterically. Noncompetitive inhibitors are usually allosteric. Activators can also enhance enzyme function allosterically. The most common method by which cells regulate the enzymes in metabolic pathways is through feedback inhibition. During feedback inhibition, metabolic pathway products serve as inhibitors (usually allosteric) of one or more of the enzymes (usually the first committed enzyme of the pathway) involved in the pathway that produces them.

13.3 Applying Proteomics

Proteomics is the study of the entire set of proteins expressed by a given type of cell under certain environmental conditions. In a multicellular organism, different cell types will have different proteomes, and

these will vary with environmental changes. Unlike a genome, a proteome is dynamic and in constant flux, which makes it both more complicated and more useful than the knowledge of genomes alone.

Proteomics approaches rely on protein analysis. Researchers are constantly upgrading these techniques. Researchers have used proteomics to study different cancer types. Medical professionals are using different biomarkers and protein signatures to analyze each cancer type. The future goal is to have a personalized treatment plan for each individual.

13.1 Proteins

1. Figure 13.3 Which categories of amino acid would you expect to find on the surface of a soluble protein, and which would you expect to find in the interior? What distribution of amino acids would you expect to find in a protein embedded in a lipid bilayer?

REVIEW QUESTIONS

2. The monomers that make up proteins are called ________.

a. nucleotides
b. disaccharides
c. amino acids
d. chaperones

3. The α helix and the β-pleated sheet are part of which protein structure?

a. primary
b. secondary
c. tertiary
d. quaternary

4. Which of the following is not true about enzymes:

a. They increase ΔG of reactions.
b. They are usually made of amino acids.
c. They lower the activation energy of chemical reactions.
d. Each one is specific to the particular substrate(s) to which it binds.

5. An allosteric inhibitor does which of the following?

a. Binds to an enzyme away from the active site and changes the conformation of the active site, increasing its affinity for substrate binding.
b. Binds to the active site and blocks it from binding substrate.
c. Binds to an enzyme away from the active site and changes the conformation of the active site, decreasing its affinity for the substrate.
d. Binds directly to the active site and mimics the substrate.

6. Which of the following analogies best describes the induced-fit model of enzyme-substrate binding?

a. a hug between two people
b. a key fitting into a lock
c. a square peg fitting through the square hole and a round peg fitting through the round hole of a children's toy
d. the fitting together of two jigsaw puzzle pieces

7. What is a biomarker?

a. the color coding of different genes
b. a protein that is uniquely produced in a diseased state
c. a molecule in the genome or proteome
d. a marker that is genetically inherited

8. A protein signature is:

a. the path followed by a protein after it is synthesized in the nucleus
b. the path followed by a protein in the cytoplasm
c. a protein expressed on the cell surface
d. a unique set of proteins present in a diseased state

CRITICAL THINKING QUESTIONS

9. Explain what happens if even one amino acid is substituted for another in a polypeptide chain. Provide a specific example.

10. Describe the differences in the four protein structures.

11. With regard to enzymes, why are vitamins necessary for good health? Give examples.

12. Explain in your own words how enzyme feedback

inhibition benefits a cell.

13. How has proteomics been used in cancer detection and treatment?

14. What is personalized medicine?

14 | LABORATORY TECHNIQUES: NUCLEIC ACIDS AND PROTEINS

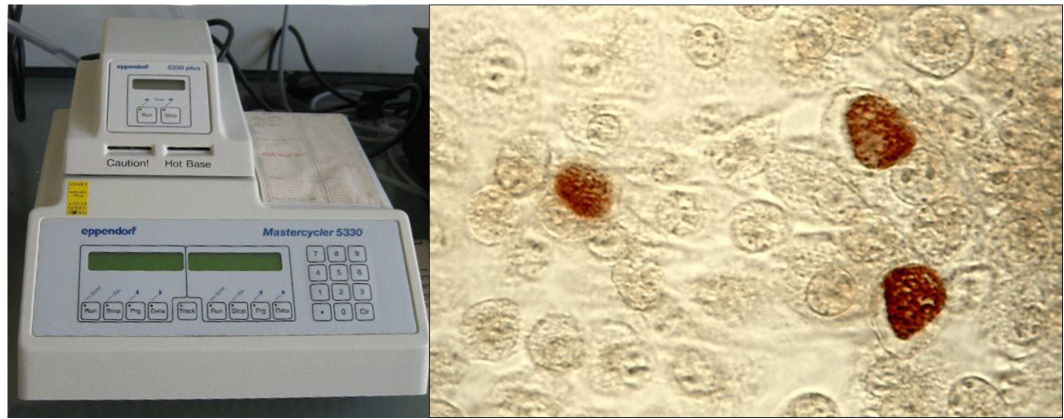


Figure 14.1 A thermal cycler (left) is used during a polymerase chain reaction (PCR). PCR amplifies the number of copies of DNA and can assist in diagnosis of infections caused by microbes that are difficult to culture, such as *Chlamydia trachomatis* (right). *C. trachomatis* causes chlamydia, the most common sexually transmitted disease in the United States, and trachoma, the world's leading cause of preventable blindness. (credit right: modification of work by Centers for Disease Control and Prevention)

Chapter Outline

14.1: Tools of Genetic Engineering
14.2: Pharmaceutical Applications of Genetic Engineering
14.3: Cellular Disruption Techniques
14.4: Column Chromatography
14.5: Advanced Chromatography Applications
14.6: Visualizing and Characterizing DNA, RNA, and Protein
14.7: Genomics and Proteomics
14.8: Gene Therapy

Introduction

Watson and Crick's identification of the structure of DNA in 1953 was the seminal event in the field of genetic engineering. Since the 1970s, there has been a veritable explosion in scientists' ability to manipulate DNA in ways that have revolutionized the fields of biology, medicine, diagnostics, forensics, and industrial manufacturing. Many of the molecular tools discovered in recent decades have been produced using prokaryotic microbes. In this chapter, we will explore some of those tools, especially as they relate to applications in medicine and health care.

As an example, the thermal cycler in **Figure 14.1** is used to perform a diagnostic technique called the polymerase chain reaction (PCR), which relies on DNA polymerase enzymes from thermophilic bacteria. Other molecular tools, such as restriction enzymes and plasmids obtained from microorganisms, allow scientists to insert genes from humans or other organisms into microorganisms. The microorganisms are then grown on an

industrial scale to synthesize products such as insulin, vaccines, and biodegradable polymers. These are just a few of the numerous applications of microbial genetics that we will explore in this chapter.

14.1 | Tools of Genetic Engineering

Learning Objectives

By the end of this section, you will be able to:

- Identify tools of molecular genetics that are derived from microorganisms
- Describe the methods used to create recombinant DNA molecules
- Describe methods used to introduce DNA into prokaryotic cells
- List the types of genomic libraries and describe their uses
- Describe the methods used to introduce DNA into eukaryotic cells

Part 1

Kayla, a 24-year-old electrical engineer and running enthusiast, just moved from Arizona to New Hampshire to take a new job. On her weekends off, she loves to explore her new surroundings, going for long runs in the pine forests. In July she spent a week hiking through the mountains. In early August, Kayla developed a low fever, headache, and mild muscle aches, and she felt a bit fatigued. Not thinking much of it, she took some ibuprofen to combat her symptoms and vowed to get more rest.

- What types of medical conditions might be responsible for Kayla's symptoms?

*Jump to the **next** Clinical Focus box.*

The science of using living systems to benefit humankind is called **biotechnology**. Technically speaking, the domestication of plants and animals through farming and breeding practices is a type of biotechnology. However, in a contemporary sense, we associate biotechnology with the direct alteration of an organism's genetics to achieve desirable traits through the process of **genetic engineering**. Genetic engineering involves the use of **recombinant DNA technology**, the process by which a DNA sequence is manipulated *in vitro,* thus creating **recombinant DNA molecules** that have new combinations of genetic material. The recombinant DNA is then introduced into a host organism. If the DNA that is introduced comes from a different species, the host organism is now considered to be **transgenic**.

One example of a transgenic microorganism is the bacterial strain that produces human insulin (**Figure 14.2**). The insulin gene from humans was inserted into a plasmid. This recombinant DNA plasmid was then inserted into bacteria. As a result, these transgenic microbes are able to produce and secrete human insulin. Many prokaryotes are able to acquire foreign DNA and incorporate functional genes into their own genome through "mating" with other cells (conjugation), viral infection (transduction), and taking up DNA from the environment (transformation). Recall that these mechanisms are examples of horizontal gene transfer—the transfer of genetic material between cells of the same generation.

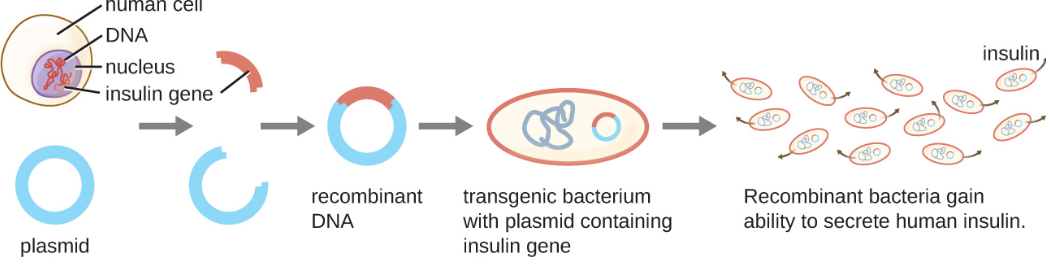


Figure 14.2 Recombinant DNA technology is the artificial recombination of DNA from two organisms. In this example, the human insulin gene is inserted into a bacterial plasmid. This recombinant plasmid can then be used to transform bacteria, which gain the ability to produce the insulin protein.

Molecular Cloning

Herbert Boyer and Stanley Cohen first demonstrated the complete **molecular cloning** process in 1973 when they successfully cloned genes from the African clawed frog (*Xenopus laevis*) into a bacterial plasmid that was then introduced into the bacterial host *Escherichia coli*. Molecular cloning is a set of methods used to construct recombinant DNA and incorporate it into a host organism; it makes use of a number of molecular tools that are derived from microorganisms.

Restriction Enzymes and Ligases

In recombinant DNA technology, DNA molecules are manipulated using naturally occurring enzymes derived mainly from bacteria and viruses. The creation of recombinant DNA molecules is possible due to the use of naturally occurring **restriction endonucleases** (**restriction enzymes**), bacterial enzymes produced as a protection mechanism to cut and destroy foreign cytoplasmic DNA that is most commonly a result of bacteriophage infection. Stewart Linn and Werner Arber discovered restriction enzymes in their 1960s studies of how *E. coli* limits bacteriophage replication on infection. Today, we use restriction enzymes extensively for cutting DNA fragments that can then be spliced into another DNA molecule to form recombinant molecules. Each restriction enzyme cuts DNA at a characteristic **recognition site**, a specific, usually palindromic, DNA sequence typically between four to six base pairs in length. A palindrome is a sequence of letters that reads the same forward as backward. (The word “level” is an example of a palindrome.) Palindromic DNA sequences contain the same base sequences in the 5′ to 3′ direction on one strand as in the 5′ to 3′ direction on the complementary strand. A restriction enzyme recognizes the DNA palindrome and cuts each backbone at identical positions in the palindrome. Some restriction enzymes cut to produce molecules that have complementary overhangs (**sticky ends**) while others cut without generating such overhangs, instead producing **blunt ends** (**Figure 14.3**).

Molecules with complementary sticky ends can easily **anneal**, or form hydrogen bonds between complementary bases, at their sticky ends. The annealing step allows **hybridization** of the single-stranded overhangs. Hybridization refers to the joining together of two complementary single strands of DNA. Blunt ends can also attach together, but less efficiently than sticky ends due to the lack of complementary overhangs facilitating the process. In either case, **ligation** by DNA ligase can then rejoin the two sugar-phosphate backbones of the DNA through covalent bonding, making the molecule a continuous double strand. In 1972, Paul Berg, a Stanford biochemist, was the first to produce a recombinant DNA molecule using this technique, combining the SV40 monkey virus with *E. coli* bacteriophage lambda to create a hybrid.

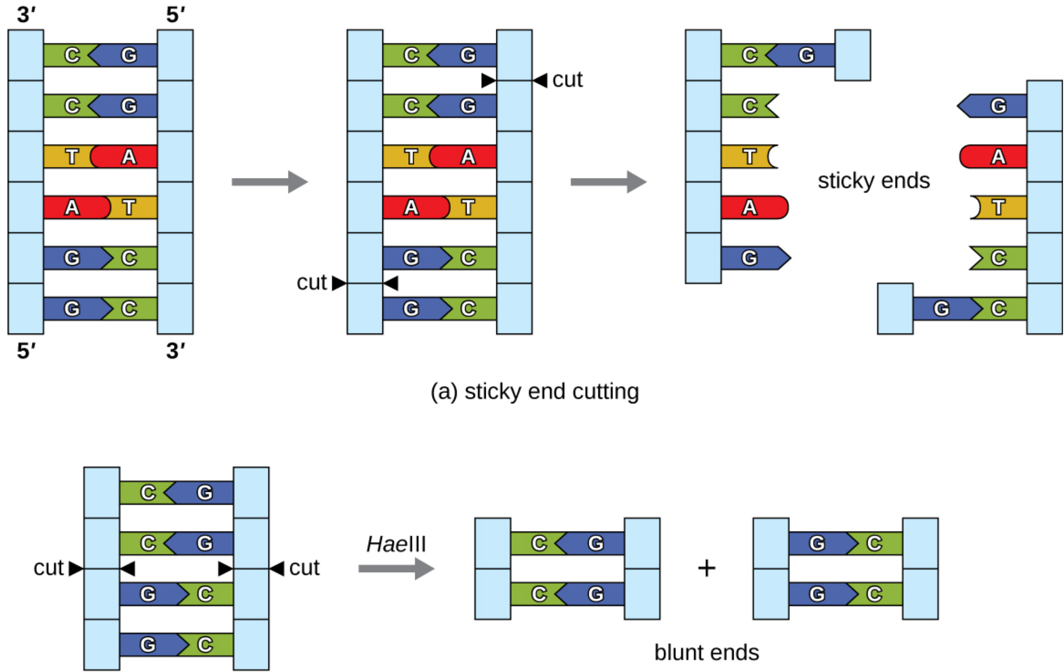


Figure 14.3 (a) In this six-nucleotide restriction enzyme site, recognized by the enzyme *Bam*HI, notice that the sequence reads the same in the 5′ to 3′ direction on both strands. This is known as a palindrome. The cutting of the DNA by the restriction enzyme at the sites (indicated by the black arrows) produces DNA fragments with sticky ends. Another piece of DNA cut with the same restriction enzyme could attach to one of these sticky ends, forming a recombinant DNA molecule. (b) This four-nucleotide recognition site also exhibits a palindromic sequence. The cutting of the DNA by the restriction enzyme *Hae*III at the indicated sites produces DNA fragments with blunt ends. Any other piece of blunt DNA could attach to one of the blunt ends produced, forming a recombinant DNA molecule.

Plasmids

After restriction digestion, genes of interest are commonly inserted into plasmids, small pieces of typically circular, double-stranded DNA that replicate independently of the bacterial chromosome (see **Unique Characteristics of Prokaryotic Cells (https://legacy.cnx.org/content/m58792/latest/)**). In recombinant DNA technology, plasmids are often used as **vectors**, DNA molecules that carry DNA fragments from one organism to another. Plasmids used as vectors can be genetically engineered by researchers and scientific supply companies to have specialized properties, as illustrated by the commonly used plasmid vector pUC19 (**Figure 14.4**). Some plasmid vectors contain genes that confer antibiotic resistance; these resistance genes allow researchers to easily find plasmid-containing colonies by plating them on media containing the corresponding antibiotic. The antibiotic kills all host cells that do not harbor the desired plasmid vector, but those that contain the vector are able to survive and grow.

Plasmid vectors used for cloning typically have a **polylinker site**, or **multiple cloning site (MCS)**. A polylinker site is a short sequence containing multiple unique restriction enzyme recognition sites that are used for inserting DNA into the plasmid after restriction digestion of both the DNA and the plasmid. Having these multiple restriction enzyme recognition sites within the polylinker site makes the plasmid vector versatile, so it can be used for many different cloning experiments involving different restriction enzymes.

This polylinker site is often found within a **reporter gene**, another gene sequence artificially engineered into the plasmid that encodes a protein that allows for visualization of DNA insertion. The reporter gene allows a researcher to distinguish host cells that contain recombinant plasmids with cloned DNA fragments from host cells that only contain the non-recombinant plasmid vector. The most common reporter gene used in plasmid vectors is the bacterial *lacZ* gene encoding beta-galactosidase, an enzyme that naturally degrades lactose but can also degrade a colorless synthetic analog X-gal, thereby producing blue colonies on X-gal–containing media. The *lacZ* reporter gene is disabled when the recombinant DNA is spliced into the plasmid. Because the LacZ protein is not produced when the gene is disabled, X-gal is not degraded and white colonies are produced, which can then be isolated. This **blue-white screening** method is described later and shown in **Figure 14.5**. In addition to these features, some plasmids come pre-digested and with an enzyme linked to the linearized plasmid to aid in ligation after the insertion of foreign DNA fragments.

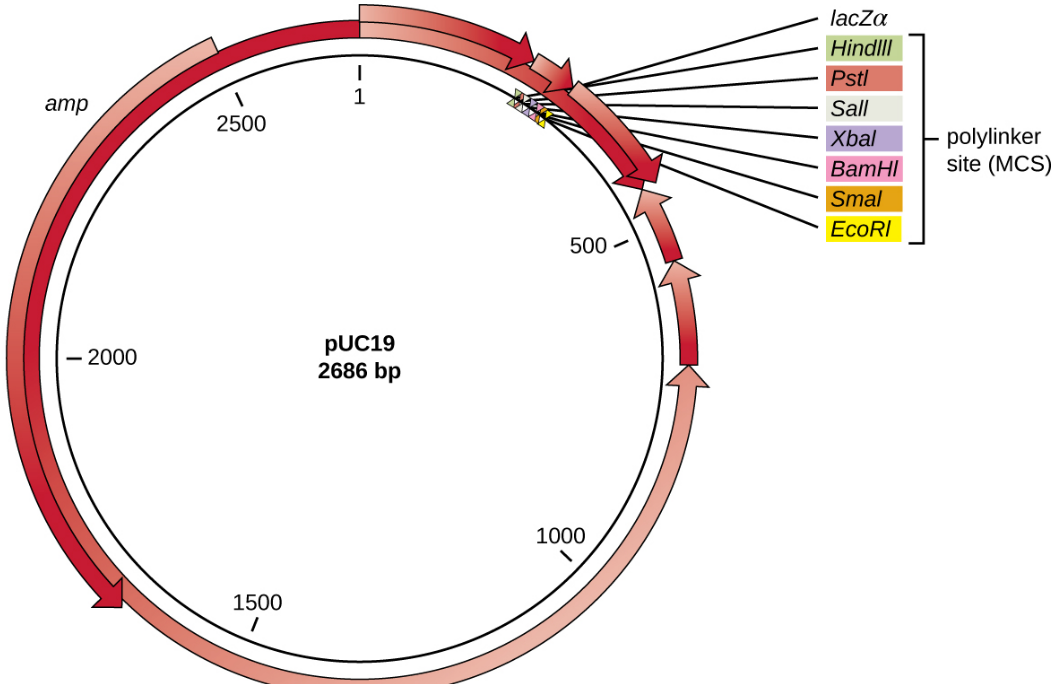


Figure 14.4 The artificially constructed plasmid vector pUC19 is commonly used for cloning foreign DNA. Arrows indicate the directions in which the genes are transcribed. Note the polylinker site, containing multiple unique restriction enzyme recognition sites, found within the *lacZ* reporter gene. Also note the ampicillin (*amp*) resistance gene encoded on the plasmid.

Molecular Cloning using Transformation

The most commonly used mechanism for introducing engineered plasmids into a bacterial cell is transformation, a process in which bacteria take up free DNA from their surroundings. In nature, free DNA typically comes from other lysed bacterial cells; in the laboratory, free DNA in the form of recombinant plasmids is introduced to the cell's surroundings.

Some bacteria, such as *Bacillus* spp., are naturally competent, meaning they are able to take up foreign DNA. However, not all bacteria are naturally competent. In most cases, bacteria must be made artificially competent in the laboratory by increasing the permeability of the cell membrane. This can be achieved through chemical treatments that neutralize charges on the cell membrane or by exposing the bacteria to an electric field that creates microscopic pores in the cell membrane. These methods yield chemically competent or electrocompetent bacteria, respectively.

Following the transformation protocol, bacterial cells are plated onto an antibiotic-containing medium to inhibit the growth of the many host cells that were not transformed by the plasmid conferring antibiotic resistance. A technique called **blue-white screening** is then used for *lacZ*-encoding plasmid vectors such as pUC19. Blue colonies have a functional beta-galactosidase enzyme because the *lacZ* gene is uninterrupted, with no foreign DNA inserted into the polylinker site. These colonies typically result from the digested, linearized plasmid religating to itself. However, white colonies lack a functional beta-galactosidase enzyme, indicating the insertion of foreign DNA within the polylinker site of the plasmid vector, thus disrupting the *lacZ* gene. Thus, white colonies resulting from this blue-white screening contain plasmids with an insert and can be further screened to characterize the foreign DNA. To be sure the correct DNA was incorporated into the plasmid, the DNA insert can then be sequenced.

View an **animation of molecular cloning (https://openstax.org/l/22moleclonani)** from the DNA Learning Center.

- In blue-white screening, what does a blue colony mean and why is it blue?

Molecular Cloning Using Conjugation or Transduction

The bacterial process of conjugation (see **How Asexual Prokaryotes Achieve Genetic Diversity (https://legacy.cnx.org/content/m58845/latest/)**) can also be manipulated for molecular cloning. F plasmids, or fertility plasmids, are transferred between bacterial cells through the process of conjugation. Recombinant DNA can be transferred by conjugation when bacterial cells containing a recombinant F plasmid are mixed with compatible bacterial cells lacking the plasmid. F plasmids encode a surface structure called an F pilus that facilitates contact between a cell containing an F plasmid and one without an F plasmid. On contact, a cytoplasmic bridge forms between the two cells and the F-plasmid-containing cell replicates its plasmid, transferring a copy of the recombinant F plasmid to the recipient cell. Once it has received the recombinant F plasmid, the recipient cell can produce its own F pilus and facilitate transfer of the recombinant F plasmid to an additional cell. The use of conjugation to transfer recombinant F plasmids to recipient cells is another effective way to introduce recombinant DNA molecules into host cells.

Alternatively, bacteriophages can be used to introduce recombinant DNA into host bacterial cells through a manipulation of the transduction process (see **How Asexual Prokaryotes Achieve Genetic Diversity (https://legacy.cnx.org/content/m58845/latest/)**). In the laboratory, DNA fragments of interest can be engineered into **phagemids**, which are plasmids that have phage sequences that allow them to be packaged into bacteriophages. Bacterial cells can then be infected with these bacteriophages so that the recombinant phagemids can be introduced into the bacterial cells. Depending on the type of phage, the recombinant DNA may be integrated into the host bacterial genome (lysogeny), or it may exist as a plasmid in the host's cytoplasm.

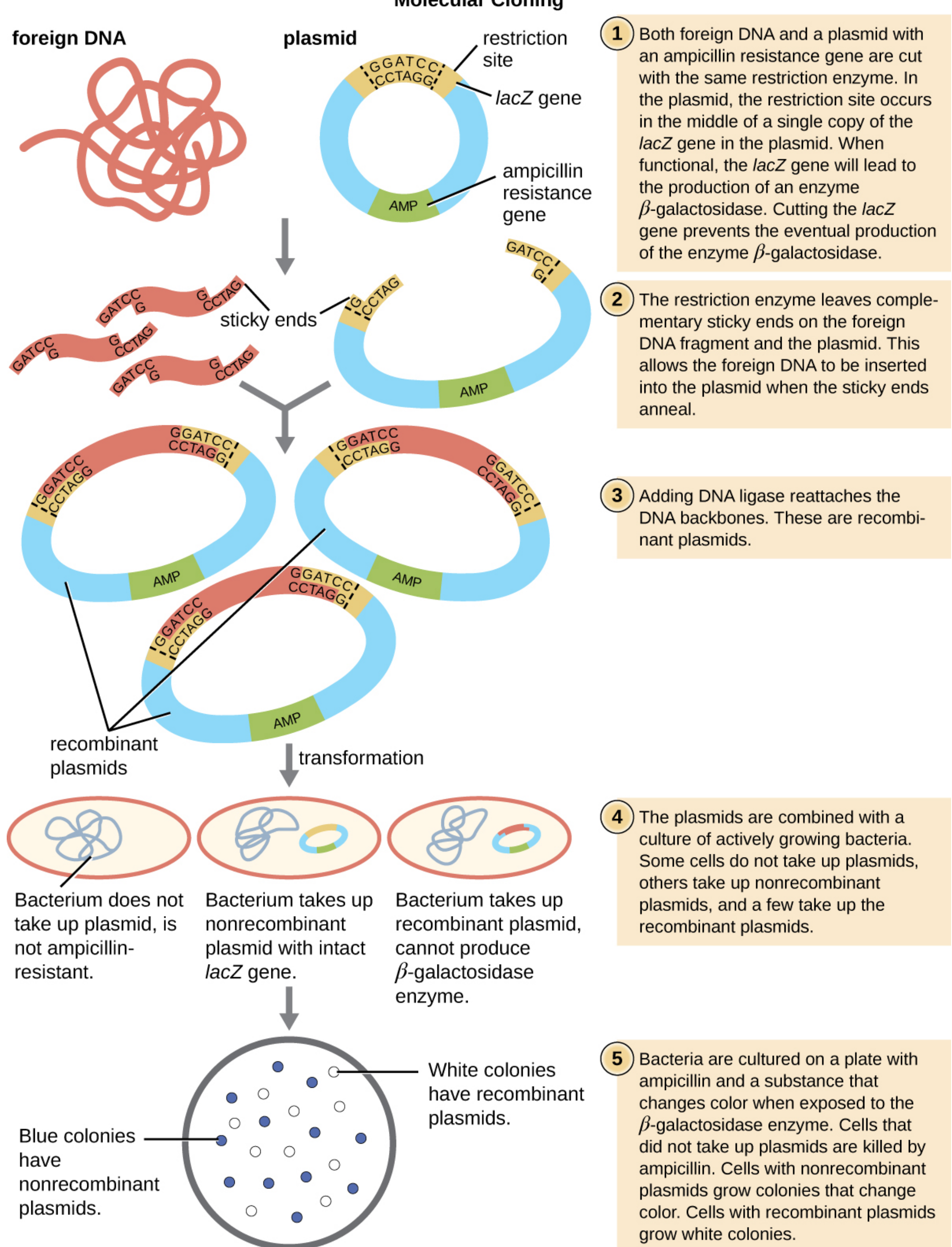


Figure 14.5 The steps involved in molecular cloning using bacterial transformation are outlined in this graphic flowchart.

- What is the original function of a restriction enzyme?
- What two processes are exploited to get recombinant DNA into a bacterial host cell?
- Distinguish the uses of an antibiotic resistance gene and a reporter gene in a plasmid vector.

Creating a Genomic Library

Molecular cloning may also be used to generate a **genomic library**. The library is a complete (or nearly complete) copy of an organism's genome contained as recombinant DNA plasmids engineered into unique

clones of bacteria. Having such a library allows a researcher to create large quantities of each fragment by growing the bacterial host for that fragment. These fragments can be used to determine the sequence of the DNA and the function of any genes present.

One method for generating a genomic library is to ligate individual restriction enzyme-digested genomic fragments into plasmid vectors cut with the same restriction enzyme (Figure 14.6). After transformation into a bacterial host, each transformed bacterial cell takes up a single recombinant plasmid and grows into a colony of cells. All of the cells in this colony are identical **clones** and carry the same recombinant plasmid. The resulting library is a collection of colonies, each of which contains a fragment of the original organism's genome, that are each separate and distinct and can each be used for further study. This makes it possible for researchers to screen these different clones to discover the one containing a gene of interest from the original organism's genome.

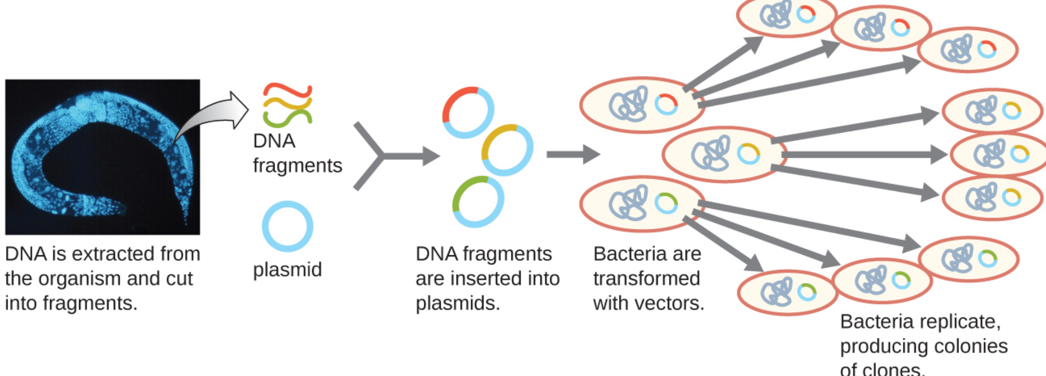


Figure 14.6 The generation of a genomic library facilitates the discovery of the genomic DNA fragment that contains a gene of interest. (credit "micrograph": modification of work by National Institutes of Health)

To construct a genomic library using larger fragments of genomic DNA, an *E. coli* bacteriophage, such as lambda, can be used as a host (Figure 14.7). Genomic DNA can be sheared or enzymatically digested and ligated into a pre-digested bacteriophage lambda DNA vector. Then, these recombinant phage DNA molecules can be packaged into phage particles and used to infect *E. coli* host cells on a plate. During infection within each cell, each recombinant phage will make many copies of itself and lyse the *E. coli* lawn, forming a plaque. Thus, each plaque from a phage library represents a unique recombinant phage containing a distinct genomic DNA fragment. Plaques can then be screened further to look for genes of interest. One advantage to producing a library using phages instead of plasmids is that a phage particle holds a much larger insert of foreign DNA compared with a plasmid vector, thus requiring a much smaller number of cultures to fully represent the entire genome of the original organism.

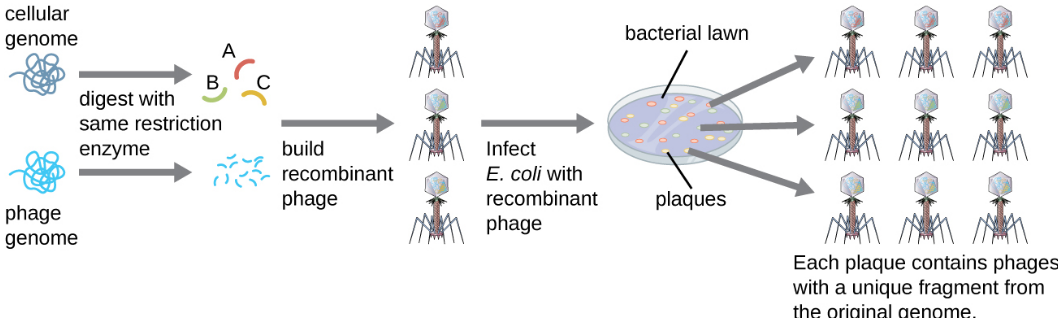


Figure 14.7 Recombinant phage DNA molecules are made by ligating digested phage particles with fragmented genomic DNA molecules. These recombinant phage DNA molecules are packaged into phage particles and allowed to infect a bacterial lawn. Each plaque represents a unique recombinant DNA molecule that can be further screened for genes of interest.

To focus on the expressed genes in an organism or even a tissue, researchers construct libraries using the organism's messenger RNA (mRNA) rather than its genomic DNA. Whereas all cells in a single organism will have the same genomic DNA, different tissues express different genes, producing different complements of mRNA. For example, all human cells' genomic DNA contains the gene for insulin, but only cells in the pancreas express mRNA directing the production of insulin. Because mRNA cannot be cloned directly, in the laboratory

mRNA must be used as a template by the retroviral enzyme reverse transcriptase to make **complementary DNA (cDNA)**. A cell's full complement of mRNA can be reverse-transcribed into cDNA molecules, which can be used as a template for DNA polymerase to make double-stranded DNA copies; these fragments can subsequently be ligated into either plasmid vectors or bacteriophage to produce a cDNA library. The benefit of a cDNA library is that it contains DNA from only the expressed genes in the cell. This means that the introns, control sequences such as promoters, and DNA not destined to be translated into proteins are not represented in the library. The focus on translated sequences means that the library cannot be used to study the sequence and structure of the genome in its entirety. The construction of a cDNA genomic library is shown in **Figure 14.8**.

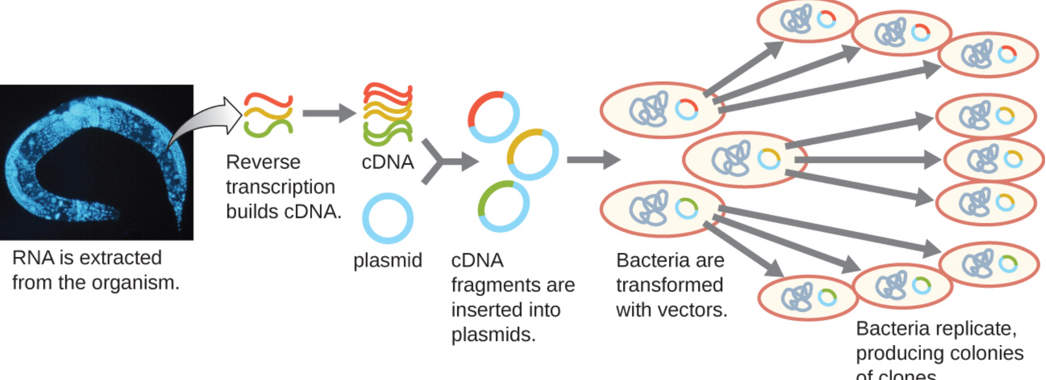


Figure 14.8 Complementary DNA (cDNA) is made from mRNA by the retroviral enzyme reverse transcriptase, converted into double-stranded copies, and inserted into either plasmid vectors or bacteriophage, producing a cDNA library. (credit "micrograph": modification of work by National Institutes of Health)

- What are the hosts for the genomic libraries described?
- What is cDNA?

Introducing Recombinant Molecules into Eukaryotic Hosts

The use of bacterial hosts for genetic engineering laid the foundation for recombinant DNA technology; however, researchers have also had great interest in genetically engineering eukaryotic cells, particularly those of plants and animals. The introduction of recombinant DNA molecules into eukaryotic hosts is called **transfection**. Genetically engineered plants, called transgenic plants, are of significant interest for agricultural and pharmaceutical purposes. The first transgenic plant sold commercially was the Flavr Savr delayed-ripening tomato, which came to market in 1994. Genetically engineered livestock have also been successfully produced, resulting, for example, in pigs with increased nutritional value[1] and goats that secrete pharmaceutical products in their milk.[2]

Electroporation

Compared to bacterial cells, eukaryotic cells tend to be less amenable as hosts for recombinant DNA molecules. Because eukaryotes are typically neither competent to take up foreign DNA nor able to maintain plasmids, transfection of eukaryotic hosts is far more challenging and requires more intrusive techniques for success. One method used for transfecting cells in cell culture is called **electroporation**. A brief electric pulse induces the formation of transient pores in the phospholipid bilayers of cells through which the gene can be introduced. At the same time, the electric pulse generates a short-lived positive charge on one side of the cell's interior and a negative charge on the opposite side; the charge difference draws negatively charged DNA molecules into the cell (**Figure 14.9**).

1. Liangxue Lai, Jing X. Kang, Rongfeng Li, Jingdong Wang, William T. Witt, Hwan Yul Yong, Yanhong Hao et al. "Generation of Cloned Transgenic Pigs Rich in Omega-3 Fatty Acids." *Nature Biotechnology 24* no. 4 (2006): 435–436.
2. Raylene Ramos Moura, Luciana Magalhães Melo, and Vicente José de Figueirêdo Freitas. "Production of Recombinant Proteins in Milk of Transgenic and Non-Transgenic Goats." *Brazilian Archives of Biology and Technology 54* no. 5 (2011): 927–938.

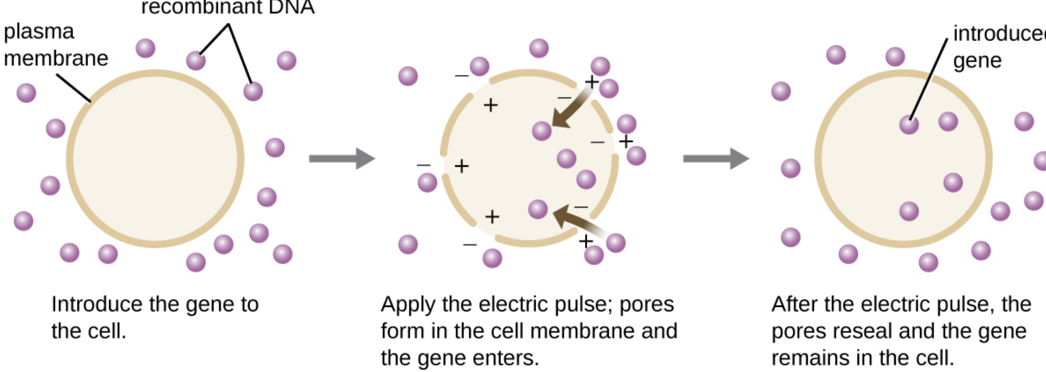


Figure 14.9 Electroporation is one laboratory technique used to introduce DNA into eukaryotic cells.

Microinjection

An alternative method of transfection is called **microinjection**. Because eukaryotic cells are typically larger than those of prokaryotes, DNA fragments can sometimes be directly injected into the cytoplasm using a glass micropipette, as shown in **Figure 14.10**.

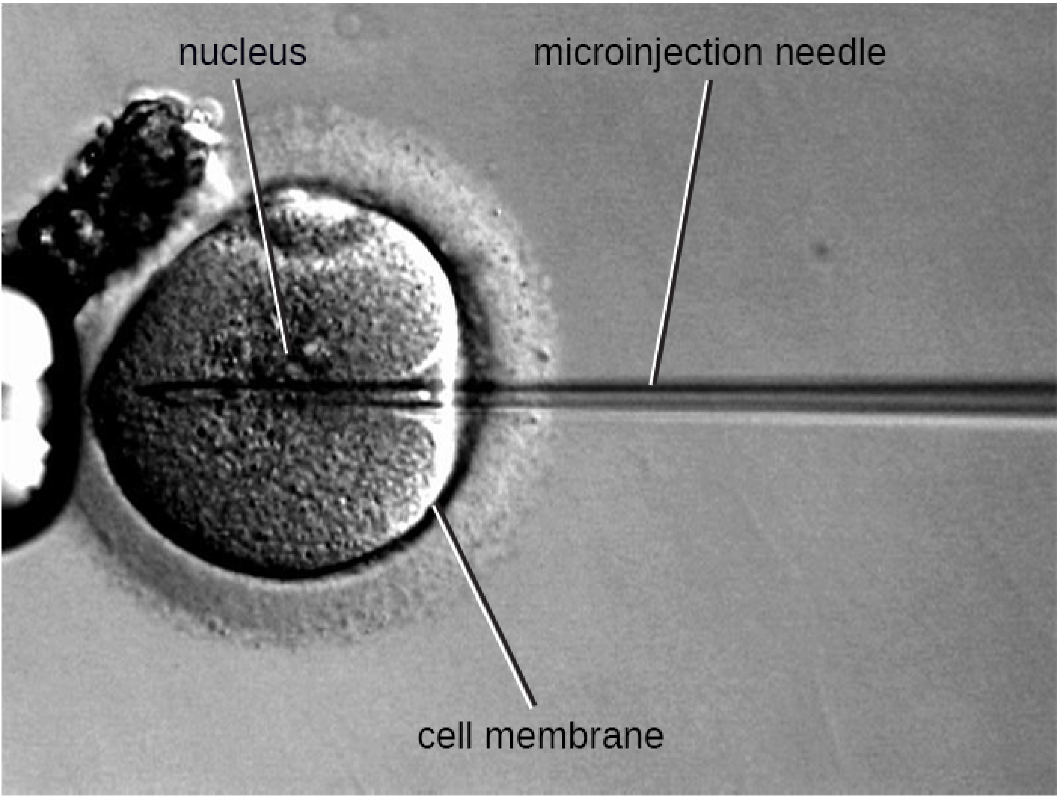


Figure 14.10 Microinjection is another technique for introducing DNA into eukaryotic cells. A microinjection needle containing recombinant DNA is able to penetrate both the cell membrane and nuclear envelope.

Gene Guns

Transfecting plant cells can be even more difficult than animal cells because of their thick cell walls. One approach involves treating plant cells with enzymes to remove their cell walls, producing protoplasts. Then, a **gene gun** is used to shoot gold or tungsten particles coated with recombinant DNA molecules into the plant protoplasts at high speeds. Recipient protoplast cells can then recover and be used to generate new transgenic plants (**Figure 14.11**).

Figure 14.11 Heavy-metal particles coated with recombinant DNA are shot into plant protoplasts using a gene gun. The resulting transformed cells are allowed to recover and can be used to generate recombinant plants. (a) A schematic of a gene gun. (b) A photograph of a gene gun. (credit a, b: modification of work by JA O'Brien, SC Lummis)

Shuttle Vectors

Another method of transfecting plants involves **shuttle vectors**, plasmids that can move between bacterial and eukaryotic cells. The **tumor-inducing (T_i) plasmids** originating from the bacterium *Agrobacterium tumefaciens* are commonly used as shuttle vectors for incorporating genes into plants (Figure 14.12). In nature, the T_i plasmids of *A. tumefaciens* cause plants to develop tumors when they are transferred from bacterial cells to plant cells. Researchers have been able to manipulate these naturally occurring plasmids to remove their tumor-causing genes and insert desirable DNA fragments. The resulting recombinant T_i plasmids can be transferred into the plant genome through the natural transfer of T_i plasmids from the bacterium to the plant host. Once inside the plant host cell, the gene of interest recombines into the plant cell’s genome.

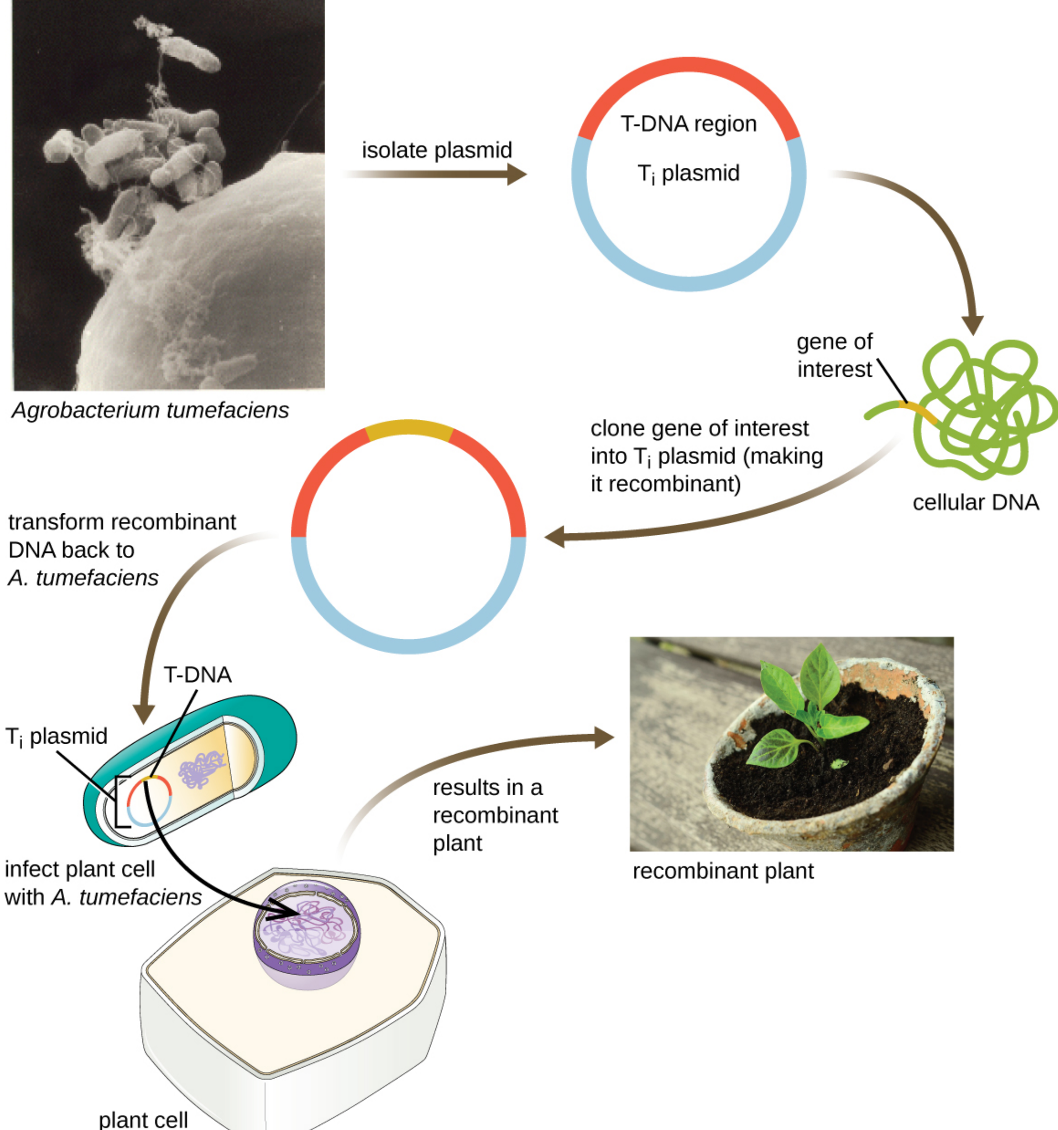


Figure 14.12 The T_i plasmid of *Agrobacterium tumefaciens* is a useful shuttle vector for the uptake of genes of interest into plant cells. The gene of interest is cloned into the T_i plasmid, which is then introduced into plant cells. The gene of interest then recombines into the plant cell's genome, allowing for the production of transgenic plants.

Viral Vectors

Viral vectors can also be used to transfect eukaryotic cells. In fact, this method is often used in gene therapy (see **Gene Therapy**) to introduce healthy genes into human patients suffering from diseases that result from genetic mutations. Viral genes can be deleted and replaced with the gene to be delivered to the patient;[3] the virus then infects the host cell and delivers the foreign DNA into the genome of the targeted cell. Adenoviruses are often used for this purpose because they can be grown to high titer and can infect both nondividing and dividing host cells. However, use of viral vectors for gene therapy can pose some risks for patients, as discussed in **Gene Therapy**.

- What are the methods used to introduce recombinant DNA vectors into animal cells?
- Compare and contrast shuttle vectors and viral vectors.

3. William S.M. Wold and Karoly Toth. "Adenovirus Vectors for Gene Therapy, Vaccination and Cancer Gene Therapy." *Current Gene Therapy 13* no. 6 (2013): 421.

14.2 | Pharmaceutical Applications of Genetic Engineering

Learning Objectives

By the end of this section, you will be able to:

- Explain the uses of genome-wide comparative analyses
- Summarize the advantages of genetically engineered pharmaceutical products

Advances in molecular biology have led to the creation of entirely new fields of science. Among these are fields that study aspects of whole genomes, collectively referred to as whole-genome methods. In this section, we'll provide a brief overview of the whole-genome fields of genomics, transcriptomics, and proteomics.

Genomics, Transcriptomics, and Proteomics

The study and comparison of entire genomes, including the complete set of genes and their nucleotide sequence and organization, is called **genomics**. This field has great potential for future medical advances through the study of the human genome as well as the genomes of infectious organisms. Analysis of microbial genomes has contributed to the development of new antibiotics, diagnostic tools, vaccines, medical treatments, and environmental cleanup techniques.

The field of **transcriptomics** is the science of the entire collection of mRNA molecules produced by cells. Scientists compare gene expression patterns between infected and uninfected host cells, gaining important information about the cellular responses to infectious disease. Additionally, transcriptomics can be used to monitor the gene expression of virulence factors in microorganisms, aiding scientists in better understanding pathogenic processes from this viewpoint.

When genomics and transcriptomics are applied to entire microbial communities, we use the terms **metagenomics** and **metatranscriptomics**, respectively. Metagenomics and metatranscriptomics allow researchers to study genes and gene expression from a collection of multiple species, many of which may not be easily cultured or cultured at all in the laboratory. A DNA microarray (discussed in the previous section) can be used in metagenomics studies.

Another up-and-coming clinical application of genomics and transcriptomics is **pharmacogenomics**, also called **toxicogenomics**, which involves evaluating the effectiveness and safety of drugs on the basis of information from an individual's genomic sequence. Genomic responses to drugs can be studied using experimental animals (such as laboratory rats or mice) or live cells in the laboratory before embarking on studies with humans. Changes in gene expression in the presence of a drug can sometimes be an early indicator of the potential for toxic effects. Personal genome sequence information may someday be used to prescribe medications that will be most effective and least toxic on the basis of the individual patient's genotype.

The study of **proteomics** is an extension of genomics that allows scientists to study the entire complement of proteins in an organism, called the proteome. Even though all cells of a multicellular organism have the same set of genes, cells in various tissues produce different sets of proteins. Thus, the genome is constant, but the proteome varies and is dynamic within an organism. Proteomics may be used to study which proteins are expressed under various conditions within a single cell type or to compare protein expression patterns between different organisms.

The most prominent disease being studied with proteomic approaches is cancer, but this area of study is also being applied to infectious diseases. Research is currently underway to examine the feasibility of using proteomic approaches to diagnose various types of hepatitis, tuberculosis, and HIV infection, which are rather difficult to diagnose using currently available techniques.[4]

A recent and developing proteomic analysis relies on identifying proteins called **biomarkers**, whose expression is affected by the disease process. Biomarkers are currently being used to detect various forms of cancer as well as infections caused by pathogens such as *Yersinia pestis* and *Vaccinia virus*.[5]

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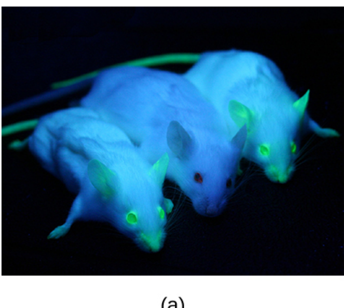
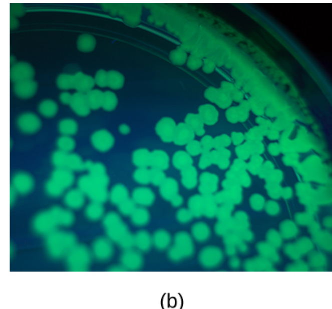
4. E.O. List, D.E. Berryman, B. Bower, L. Sackmann-Sala, E. Gosney, J. Ding, S. Okada, and J.J. Kopchick. "The Use of Proteomics to Study Infectious Diseases." *Infectious Disorders-Drug Targets* (Formerly *Current Drug Targets-Infectious Disorders*) *8* no. 1 (2008): 31–45.

Other “-omic” sciences related to genomics and proteomics include metabolomics, glycomics, and lipidomics, which focus on the complete set of small-molecule metabolites, sugars, and lipids, respectively, found within a cell. Through these various global approaches, scientists continue to collect, compile, and analyze large amounts of genetic information. This emerging field of **bioinformatics** can be used, among many other applications, for clues to treating diseases and understanding the workings of cells.

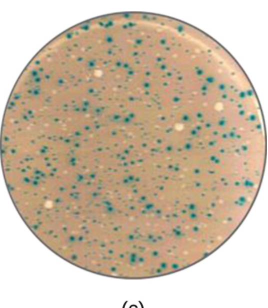
Additionally, researchers can use reverse genetics, a technique related to classic mutational analysis, to determine the function of specific genes. Classic methods of studying gene function involved searching for the genes responsible for a given phenotype. Reverse genetics uses the opposite approach, starting with a specific DNA sequence and attempting to determine what phenotype it produces. Alternatively, scientists can attach known genes (called reporter genes) that encode easily observable characteristics to genes of interest, and the location of expression of such genes of interest can be easily monitored. This gives the researcher important information about what the gene product might be doing or where it is located in the organism. Common reporter genes include bacterial *lacZ*, which encodes beta-galactosidase and whose activity can be monitored by changes in colony color in the presence of X-gal as previously described, and the gene encoding the jellyfish protein green fluorescent protein (GFP) whose activity can be visualized in colonies under ultraviolet light exposure (**Figure 14.13**).



(a)



(b)



(c)

Figure 14.13 (a) The gene encoding green fluorescence protein is a commonly used reporter gene for monitoring gene expression patterns in organisms. Under ultraviolet light, GFP fluoresces. Here, two mice are expressing GFP, while the middle mouse is not. (b) GFP can be used as a reporter gene in bacteria as well. Here, a plate containing bacterial colonies expressing GFP is shown. (c) Blue-white screening in bacteria is accomplished through the use of the *lacZ* reporter gene, followed by plating of bacteria onto medium containing X-gal. Cleavage of X-gal by the LacZ enzyme results in the formation of blue colonies. (credit a: modification of work by Ingrid Moen, Charlotte Jevne, Jian Wang, Karl-Henning Kalland, Martha Chekenya, Lars A Akslen, Linda Sleire, Per Ø Enger, Rolf K Reed, Anne M Øyan, Linda EB Stuhr; credit b: modification of work by “2.5JIGEN.com”/Flickr; credit c: modification of work by American Society for Microbiology)

- How is genomics different from traditional genetics?
- If you wanted to study how two different cells in the body respond to an infection, what –omics field would you apply?
- What are the biomarkers uncovered in proteomics used for?

Resolution

Because Kayla’s symptoms were persistent and serious enough to interfere with daily activities, Kayla’s physician decided to order some laboratory tests. The physician collected samples of Kayla’s blood, cerebrospinal fluid (CSF), and synovial fluid (from one of her swollen knees) and requested PCR analysis on all three samples. The PCR tests on the CSF and synovial fluid came back positive for the presence of *Borrelia burgdorferi*, the bacterium that causes Lyme disease.

Kayla’s physician immediately prescribed a full course of the antibiotic doxycycline. Fortunately, Kayla recovered fully within a few weeks and did not suffer from the long-term symptoms of post-treatment Lyme disease syndrome (PTLDS), which affects 10–20% of Lyme disease patients. To prevent future infections, Kayla’s physician advised her to use insect repellant and wear protective clothing during her outdoor adventures. These measures can limit exposure to Lyme-bearing ticks, which are common in many regions of the United States during the warmer months of the year. Kayla was also advised to make a habit of

5. Mohan Natesan, and Robert G. Ulrich. “Protein Microarrays and Biomarkers of Infectious Disease.” *International Journal of Molecular Sciences 11* no. 12 (2010): 5165–5183.

examining herself for ticks after returning from outdoor activities, as prompt removal of a tick greatly reduces the chances of infection.

Lyme disease is often difficult to diagnose. *B. burgdorferi* is not easily cultured in the laboratory, and the initial symptoms can be very mild and resemble those of many other diseases. But left untreated, the symptoms can become quite severe and debilitating. In addition to two antibody tests, which were inconclusive in Kayla's case, and the PCR test, a Southern blot could be used with *B. burgdorferi*-specific DNA probes to identify DNA from the pathogen. Sequencing of surface protein genes of *Borrelia* species is also being used to identify strains within the species that may be more readily transmitted to humans or cause more severe disease.

*Go back to the **previous** Clinical Focus box.*

Recombinant DNA Technology and Pharmaceutical Production

Genetic engineering has provided a way to create new pharmaceutical products called **recombinant DNA pharmaceuticals**. Such products include antibiotic drugs, vaccines, and hormones used to treat various diseases. **Table 14.1** lists examples of recombinant DNA products and their uses.

For example, the naturally occurring antibiotic synthesis pathways of various *Streptomyces* spp., long known for their antibiotic production capabilities, can be modified to improve yields or to create new antibiotics through the introduction of genes encoding additional enzymes. More than 200 new antibiotics have been generated through the targeted inactivation of genes and the novel combination of antibiotic synthesis genes in antibiotic-producing *Streptomyces* hosts.[6]

Genetic engineering is also used to manufacture subunit vaccines, which are safer than other vaccines because they contain only a single antigenic molecule and lack any part of the genome of the pathogen (see **Vaccines**). For example, a vaccine for hepatitis B is created by inserting a gene encoding a hepatitis B surface protein into a yeast; the yeast then produces this protein, which the human immune system recognizes as an antigen. The hepatitis B antigen is purified from yeast cultures and administered to patients as a vaccine. Even though the vaccine does not contain the hepatitis B virus, the presence of the antigenic protein stimulates the immune system to produce antibodies that will protect the patient against the virus in the event of exposure.[7] [8]

Genetic engineering has also been important in the production of other therapeutic proteins, such as insulin, interferons, and human growth hormone, to treat a variety of human medical conditions. For example, at one time, it was possible to treat diabetes only by giving patients pig insulin, which caused allergic reactions due to small differences between the proteins expressed in human and pig insulin. However, since 1978, recombinant DNA technology has been used to produce large-scale quantities of human insulin using *E. coli* in a relatively inexpensive process that yields a more consistently effective pharmaceutical product. Scientists have also genetically engineered *E. coli* capable of producing human growth hormone (HGH), which is used to treat growth disorders in children and certain other disorders in adults. The HGH gene was cloned from a cDNA library and inserted into *E. coli* cells by cloning it into a bacterial vector. Eventually, genetic engineering will be used to produce DNA vaccines and various gene therapies, as well as customized medicines for fighting cancer and other diseases.

Some Genetically Engineered Pharmaceutical Products and Applications

Recombinant DNA Product	Application
Atrial natriuretic peptide	Treatment of heart disease (e.g., congestive heart failure), kidney disease, high blood pressure
DNase	Treatment of viscous lung secretions in cystic fibrosis

Table 14.1

6. Jose-Luis Adrio and Arnold L. Demain. "Recombinant Organisms for Production of Industrial Products." *Bioengineered Bugs 1* no. 2 (2010): 116–131.
7. U.S. Department of Health and Human Services. "Types of Vaccines." 2013. http://www.vaccines.gov/more_info/types/#subunit. Accessed May 27, 2016.
8. The Internet Drug List. *Recombivax*. 2015. http://www.rxlist.com/recombivax-drug.htm. Accessed May 27, 2016.

Some Genetically Engineered Pharmaceutical Products and Applications

Recombinant DNA Product	Application
Erythropoietin	Treatment of severe anemia with kidney damage
Factor VIII	Treatment of hemophilia
Hepatitis B vaccine	Prevention of hepatitis B infection
Human growth hormone	Treatment of growth hormone deficiency, Turner's syndrome, burns
Human insulin	Treatment of diabetes
Interferons	Treatment of multiple sclerosis, various cancers (e.g., melanoma), viral infections (e.g., Hepatitis B and C)
Tetracenomycins	Used as antibiotics
Tissue plasminogen activator	Treatment of pulmonary embolism in ischemic stroke, myocardial infarction

Table 14.1

- What bacterium has been genetically engineered to produce human insulin for the treatment of diabetes?
- Explain how microorganisms can be engineered to produce vaccines.

RNA Interference Technology

In **Structure and Function of RNA**, we described the function of mRNA, rRNA, and tRNA. In addition to these types of RNA, cells also produce several types of small noncoding RNA molecules that are involved in the regulation of gene expression. These include **antisense RNA** molecules, which are complementary to regions of specific mRNA molecules found in both prokaryotes and eukaryotic cells. Non-coding RNA molecules play a major role in **RNA interference (RNAi)**, a natural regulatory mechanism by which mRNA molecules are prevented from guiding the synthesis of proteins. RNA interference of specific genes results from the base pairing of short, single-stranded antisense RNA molecules to regions within complementary mRNA molecules, preventing protein synthesis. Cells use RNA interference to protect themselves from viral invasion, which may introduce double-stranded RNA molecules as part of the viral replication process (**Figure 14.14**).

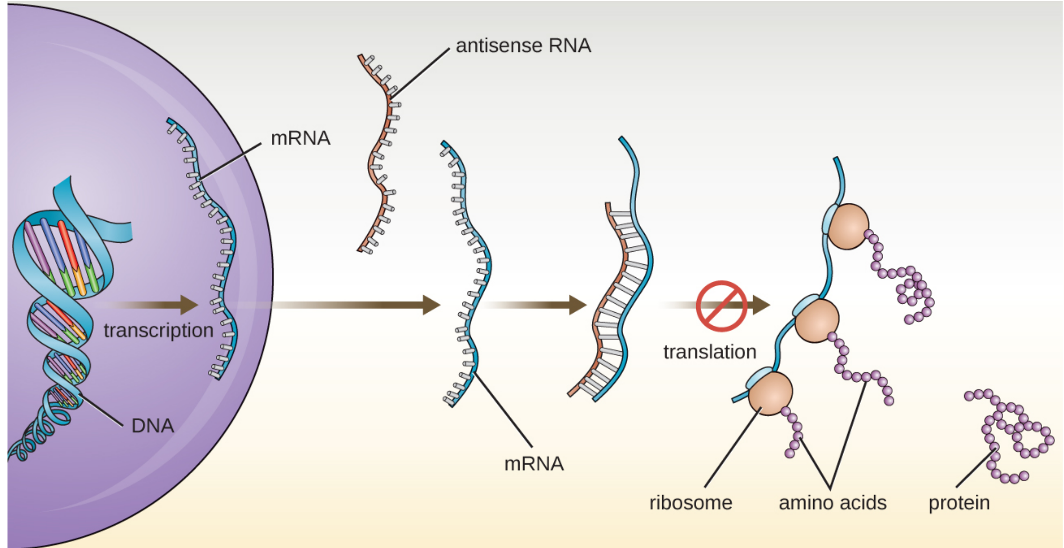


Figure 14.14 Cells like the eukaryotic cell shown in this diagram commonly make small antisense RNA molecules with sequences complementary to specific mRNA molecules. When an antisense RNA molecule is bound to an mRNA molecule, the mRNA can no longer be used to direct protein synthesis. (credit: modification of work by Robinson R)

Researchers are currently developing techniques to mimic the natural process of RNA interference as a way to treat viral infections in eukaryotic cells. RNA interference technology involves using small interfering RNAs (siRNAs) or microRNAs (miRNAs) (**Figure 14.15**). siRNAs are completely complementary to the mRNA transcript of a specific gene of interest while miRNAs are mostly complementary. These double-stranded RNAs are bound to DICER, an endonuclease that cleaves the RNA into short molecules (approximately 20 nucleotides long). The RNAs are then bound to RNA-induced silencing complex (RISC), a ribonucleoprotein. The siRNA-RISC complex binds to mRNA and cleaves it. For miRNA, only one of the two strands binds to RISC. The miRNA-RISC complex then binds to mRNA, inhibiting translation. If the miRNA is completely complementary to the target gene, then the mRNA can be cleaved. Taken together, these mechanisms are known as **gene silencing**.

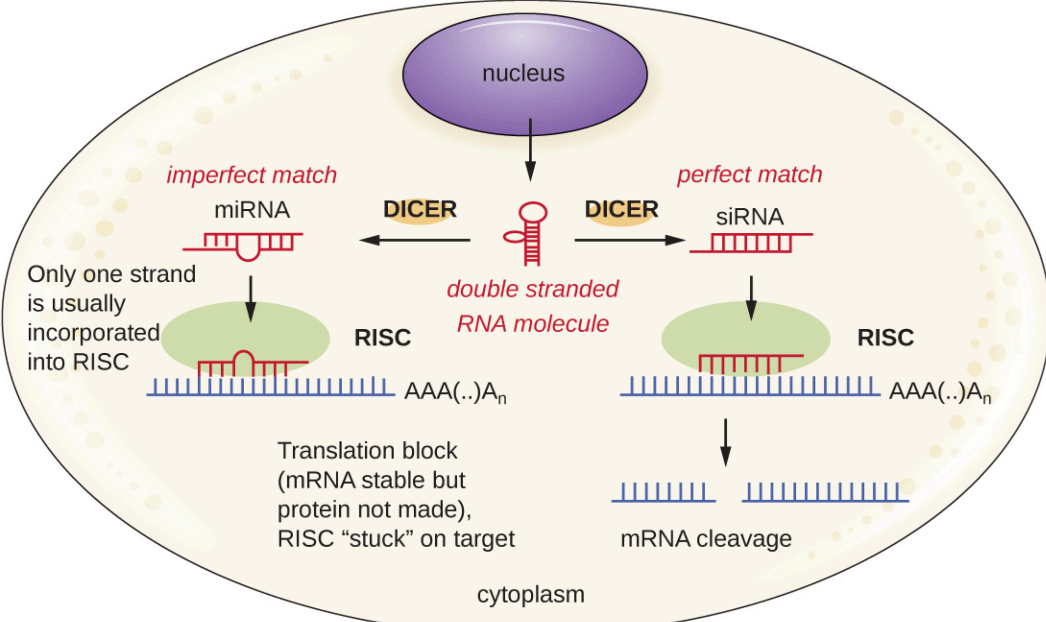


Figure 14.15 This diagram illustrates the process of using siRNA or miRNA in a eukaryotic cell to silence genes involved in the pathogenesis of various diseases. (credit: modification of work by National Center for Biotechnology Information)

14.3 | Cellular Disruption Techniques

The first step of downstream processing development is determining the best method to break open the cells to release the recombinant protein. There are many methods for cell disruption to choose from with the goal of determining the gentlest method that gives the highest yield of functioning protein. If the extraction method is too harsh, it risks denaturing and destroying the target protein. If the extraction method is too mild, the cells will remain intact and may lose precious recombinant protein stuck inside the cell. This module discusses various cell disruption techniques.

CELL DISRUPTION TECHNIQUES

According to an Evaluate Pharma report, the worldwide pharmaceutical industry has now topped a trillion dollars in sales, with most of this growth propelled by new FDA approvals of recombinant protein products such as monoclonal antibody therapeutics, biologics, and biosimilars (generic protein products). In the U.S. alone there were over $250 billion in annual sales and upwards of $150 billion invested in biopharmaceutical R&D (EvaluatePharma, 2016). A critical part of research and development in making recombinant protein products is deciding what organism to use in the manufacture of the recombinant protein and what purification steps to employ in separating the target protein from the cellular contaminants.

Expression vectors

As mentioned previously, a gene of interest may be inserted into a vector, and the recombinant plasmid is placed into a cell where the gene can be expressed. For instance, one might desire to clone the gene coding for human growth hormone or insulin or other medically important proteins and have a bacterium or yeast make large quantities of it very cheaply.

To clone a gene so that it can be expressed, one needs to set up the proper conditions for the human protein to be made in the bacterial cells. This typically involves the use of specially designed plasmids. These plasmids have been engineered to 1) replicate in high numbers; 2) carry markers that allow researchers to identify cells carrying them (antibiotic resistance, for example) and 3) contain sequences (such as a promoter and Shine-Dalgarno sequence) necessary for expression of the desired protein, with convenient sites for insertion of the gene of interest in the appropriate place relative to the control sequences. A plasmid which has all these features is referred to as an expression vector.

In addition to plasmids that can be used for expression in bacterial cells, expression vectors are also available that allow protein expression in a variety of eukaryotic cells. Many sophisticated variations on such vectors have been created that have made it easy to produce and purify large amounts of any protein of interest for which the gene has been cloned. A handy feature in some expression vectors is a sequence encoding an affinity tag either up or downstream of the gene being expressed. This sequence allows a short affinity tag (such as a run of histidine residues) to be fused onto the encoded protein. The tag can be used to readily purify the protein, as described in the section on affinity chromatography.

Recombinant Protein Purification

The optimal protein purification strategy for a biopharmaceutical depends on the properties of the protein, the starting concentration of the extract, and the types of contaminating materials present. Most proteins produced commercially rely on fermentation by microbial or animal cell culture. The process of harvesting and purifying a protein in an industrial setting is referred to as “downstream processing” and includes all steps of production downstream of the fermentation step. Since downstream processing of a protein can often exceed all other costs of production combined, it must be a carefully designed strategy, often requiring extensive development by scientists and engineers.

Downstream Processing

1. Since most proteins are not secreted from cells, the first phase of downstream processing consists of cell disruption, followed by removal of cell debris. Cell disruption can be performed using a relatively mild treatment with chemicals or with a more severe physical disruption by sonication. Clearing the lysate of insoluble debris can be carried out using centrifugation or by filtration.
2. Since processing of large volumes is expensive, the first purification step usually includes concentrating the protein extract to a smaller volume. Protein can be concentrated via precipitation or by ultrafiltration.

3. Once the protein solution volume is reduced to a more manageable size, purification can proceed. Column chromatography offers the highest resolution. Some examples include size exclusion, ion-exchange, and hydrophobic interaction chromatography.
4. For biopharmaceutical products that require higher levels of purity, more sophisticated techniques can be used to polish the purified protein and may include immunoaffinity techniques in combination with high-performance liquid chromatography (HPLC).
5. When the protein is purified sufficiently, it is dried by lyophilization, freeze-dried, or formulated into a solution that stabilizes its activity and integrity.

Cell Disruption Methods

The first step of downstream processing development is determining the best method to break open the cells to release the recombinant protein. There are many methods for cell disruption to choose from with the goal of determining ***the gentlest method that gives the highest yield of functioning protein.*** If the extraction method is too harsh, it risks denaturing and destroying the target protein. If the extraction method is too mild, the cells will remain intact and may lose precious recombinant protein stuck inside the cell.

Part of picking the right method is realizing different types of cells have diverse cellular structures and will, therefore, require appropriate cell disruption methods. Some organisms have cell walls or capsules surrounding the plasma membrane, and others only a plasma membrane. For example, different cell walls require different enzymes to break them; others may need the assistance of sheer force. However, some proteins are susceptible to the shear forces required to break down cell walls; others are sensitive to damage by exposure to air, or heat from the extraction method, or nucleases present in the cellular extract. So how can the target protein stay intact while disrupting as many cells as possible? This is typically done experimentally, through processes of trial and error development. A summary of common cell disruption methods is provided in the table below.

Relative Harshness	Cell Disruption Method	Description
Gentle	Cell lysis	Osmotic shock: rapid immersion in a hypotonic solution
	Enzyme Digest/cell lysis	Digestion of cell wall; contents release following osmotic shock
	Potter-Elvehjem homogenizer	Cells forced through a narrow gap, disruption by shear forces
Moderate	Freeze-thaw	Slow freeze-thaw cycles break cell walls by ice crystal formation
Harsh	Waring blender	Shear forces of rotating blades break cells
	Organic extraction	Mixing with an immiscible organic solvent to dissolve membranes
	Grinding	Abrasive grinding with glass beads or a mortar and pestle
Vigorous	French press cell	Cells forced through a small orifice at high pressure
	Bead mill	Rapid vibrations with glass beads grind cell walls
(most harsh)	Ultra-sonication	High-pressure sound waves cause cell rupture by cavitation

Table 14.2 Summary of common cell disruption techniques

Osmotic shock and enzymes: One way to lyse cells is by lowering the ionic strength of the cellular medium. This can cause cells to swell and burst. Mild surfactants may be used to disrupt membranes. Most bacteria, yeast, and plant tissues are resistant to osmotic shocks, because of the presence of cell walls, and stronger disruption techniques are usually required. Enzymes may be useful in helping to degrade the cell walls. Lysozyme, for example, is very useful for breaking down bacterial walls. Other enzymes commonly employed include cellulase (plants), proteases, mannoses, and others.

Mechanical disruption: Mechanical agitation may be employed in the form of beads that are shaken with a

mixture of cells. In this method, cells are bombarded with tiny, glass beads that break the cells open. **Sonication** (2050 kHz sound waves) provides an alternative type of agitation that can be effective. The method is noisy, however, and generates heat that can be problematic for heat-sensitive compounds.

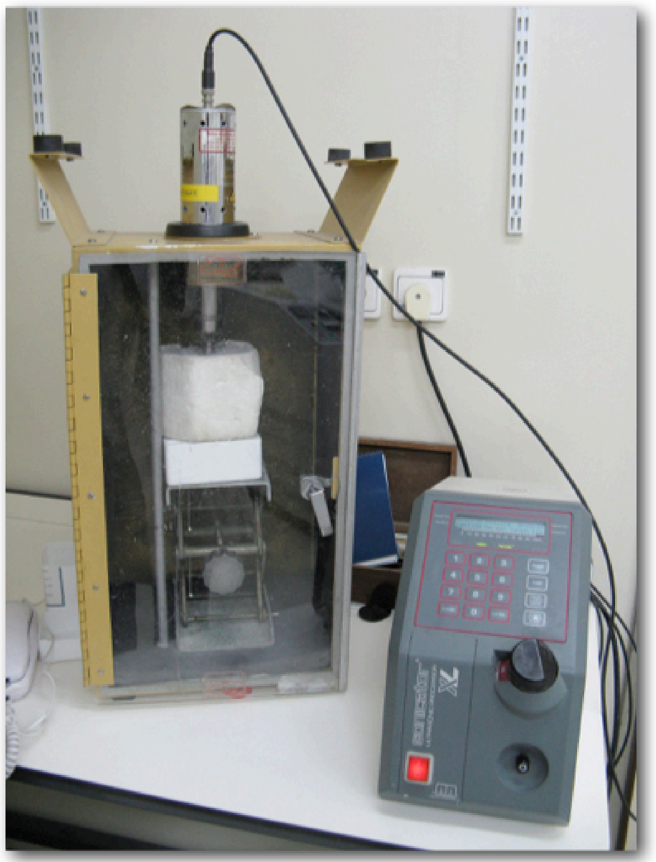


Figure 14.16 A sonication apparatus is set up in a sound containment box. A long metal probe is inserted into a cell sample sitting in ice. Sonication waves are transmitted through a cable attached to the metal probe controlled by an outside controller. Image credit: **Dr. Kevin Ahern (http://biochem.science.oregonstate.edu/content/kevin-ahern)** and **Dr. Indira Rajagopal (http://biochem.science.oregonstate.edu/content/indira-rajagopal)** https://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Pressure disruption: Another means of disrupting cells involves using a “cell bomb.” In this method, cells are placed under very high pressure (up to 25,000 psi) and then the pressure is rapidly released. The rapid pressure change causes dissolved gases in cells to be released as bubbles, which, in turn, break open cells.

Cryogenic Pulverizing: Cryogenic pulverizing, or grinding, is often employed for samples having a tough extracellular matrix, such as connective tissue, seed, and cartilage. In this technique, tissues are frozen using liquid nitrogen and then impact pulverization (typically, grinding, using a mortar and pestle or a powerful electric grinder) is performed. The powder so obtained is then suspended in the appropriate buffer.

Protease Inhibitors:

Proteins must be protected from degradation by proteases during isolation steps by working ***quickly*** and working ***cold*** (keeping all reagents on ice), while also being mindful of ***buffering*** condition. Additionally, protease inhibitors can be employed to reduce proteolytic damage to proteins. For example, PMSF is a broad inhibitor and is successfully used in animal, plant and yeast tissues. Other inhibitors include EDTA, aprotinin, chromostatin, pepstatin, and benzamidine.

Clarification:

Once cells have been disrupted, the cell lysate must be clarified, removing cell debris and unbroken cells either by filtration or by centrifugation. Cellular materials are highly compressible, they rapidly clog pores of filters, so centrifugation is usually the method used to remove particulate matter when the volumes are small.

Fractionation: Using low-speed centrifugation, one can remove cell debris, leaving a supernatant containing the

contents of the cell. By using successively higher centrifugation speeds (and resulting g-forces), it is possible to separate different cellular components, like nuclei, mitochondria from the cytoplasm. These may then be separately lysed to release molecules that are specific to a cellular compartment. The soluble fraction of any lysate can, then, be further separated into its constituents using various methods. The most common for biopharmaceuticals is **column chromatography**.

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Dr. Kevin Ahern (http://biochem.science.oregonstate.edu/content/kevin-ahern) and **Dr. Indira Rajagopal (http://biochem.science.oregonstate.edu/content/indira-rajagopal)**, **https://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy (https://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy)**

Wikipedia: **https://en.wikipedia.org/wiki/Cell_disruption (https://en.wikipedia.org/wiki/Cell_disruption)**

14.4 | Column Chromatography

Even when produced by a recombinant host at high expression levels, recombinant protein extracts are complex mixtures and frequently require multiple purification steps to remove the contaminants. The protein must be separated from hundreds of other proteins with similar properties, as well as all the lipids, nucleic acids, and carbohydrate-containing biomolecules of the cell. One of the most effective and powerful tools of protein purification is column chromatography; a technique used to separate the components of a mixture based on differences in their size, charge or other characteristics.

Column Chromatography

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We will consider several chromatographic approaches in this section. ***Chromatography is used to separate the components of a mixture based on differences in their size, charge or other characteristics.*** During chromatography, the ***mobile phase*** (buffer or another solvent) moves through the ***stationary phase*** (usually a solid matrix) carrying the components of the mixture. Separation of the components is achieved because of the different affinities the components have for the mobile and stationary phases. Molecules in a chromatography method move at different rates, for reasons that vary, depending on the type of chromatography used.

Purification Strategy

There are many types of column chromatography, based on the chemistry of the stationary phase. There is no single best way to purify all proteins. Finding an optimal protein purification strategy requires trial and error because the best method for purification depends on the properties of the protein being isolated as well as the other contaminants in the lysate. In choosing the best types of chromatography to use, there are at least five factors that must be evaluated and include resolution (how well the method removes target away from contaminants), capacity, speed, percent recovery, and cost. In the next two labs, students will separate their target recombinant protein using first a gravity HIC column, then using an ion-exchange FPLC process.

Column Chromatography

We will consider several different kinds of chromatography to illustrate this process. 1. Ion exchange chromatography 2. Gel exclusion chromatography 3. Affinity chromatography 4. HPLC These variations on chromatography are performed with the stationary phase (matrix) held within columns (Figure). Matrix supports are composed of tiny beads suspended in buffer (Figure) and are designed to exploit the chemistry or size differences of the components of the samples and thus provide a means of separation. Columns are “packed” or filled into a column, and a buffer or solvent carries the mixture of compounds to be separated. Molecules in the sample interact differentially with the support and, consequently, travel through it at different speeds, thus enabling separation.

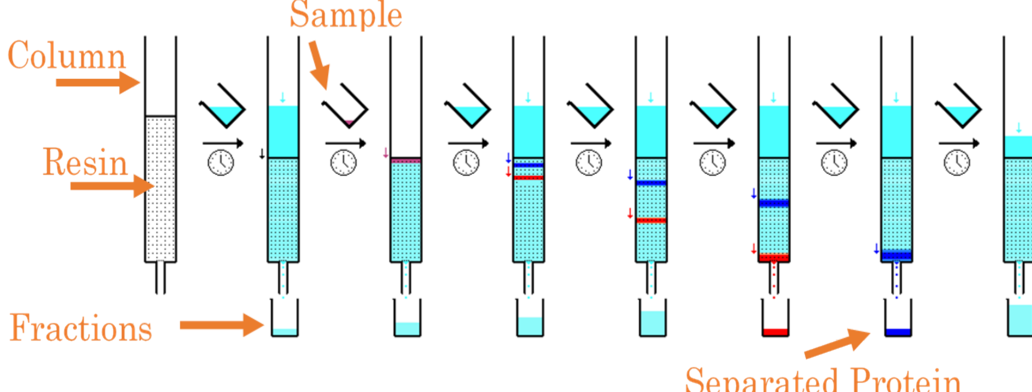


Figure 14.17 Schematic diagram demonstrating the separation of molecules using column chromatography and identifying important elements: column, resin, fraction, sample, and separated molecules. Image Credit: Edited by J. O'Grady, original, by quantockgoblin - Own work, Public Domain, **https://upload.wikimedia.org/wikipedia/commons/3/3f/Column_chromatography_sequence.png (https://upload.wikimedia.org/wikipedia/commons/3/3f/Column_chromatography_sequence.png)**

Size Exclusion Chromatography (SEC)

Size exclusion chromatography (also called molecular exclusion chromatography, gel exclusion chromatography, or gel filtration chromatography) is a low-resolution separation method that employs beads with tiny "tunnels" in them that each has a precise opening. In SEC, separation on the column is a function of size alone: larger molecules are excluded from the pores of the chromatography beads and elute from the column more quickly than smaller molecules (Figure).

In a size exclusion column, the resolving power is a function of the length of the column on which the proteins are separated on, as well as the pore size of the beads, the flow rate of the elution buffer, and the relative sizes of proteins being separated. Since the permeation of the beads by smaller proteins is a diffusion dependent process, the flow rates should be slow enough to allow for this to happen. In general, the resolution of SEC on small columns is not great, but this step can be useful for changing the buffer in which the protein is dissolved in, to the buffer used to elute the proteins from the column. Significant drawbacks to SEC are that the volume of the protein sample applied to the column must not exceed 5% of the column volume, and the protein eluted from the column is diluted by a factor of 10-fold. This means that an SEC step must usually be followed by a step that will concentrate the protein fraction.

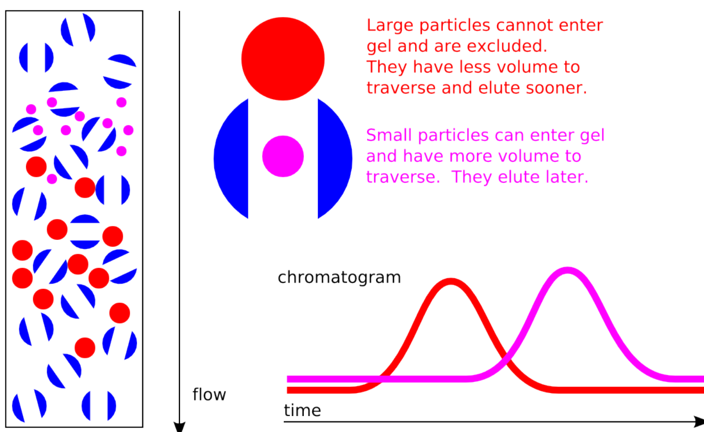


Figure 14.18 Cartoon image is depicting size exclusion chromatography. Left is a cartoon column showing blue beads and large molecules being excluded from them, while pink smaller molecules are entering the beads and have more volume to traverse, delaying their exit from the column. On the right is a chromatograph, showing by time, the larger molecules exit the column first, then the smaller ones. Image credit: By Takometer, **https://en.wikipedia.org/wiki/Size-exclusion_chromatography#/media/File:SizeExChrom.png (https://en.wikipedia.org/wiki/Size-exclusion_chromatography#/media/File:SizeExChrom.png)**

Adsorption Chromatography

The other forms of chromatography listed in Table 8.1 are a form of “adsorption” chromatography, in that the protein that is applied to the column matrix will be adsorbed to the solid phase matrix, allowing separation of proteins that have no affinity to the stationary phase. The proteins left in the column can then be eluted by changing the buffering conditions of the mobile phase. A gradual change in buffering conditions can elute proteins one at a time, allowing their separation from each other.

Adsorption is a surface phenomenon. In the example of column chromatography, protein is adsorbed (collected) on the surface of a solid phase matrix. This is very different from ***absorption***, which describes a substance that diffuses into a liquid or solid to form a solution. For example, in spectrophotometry, we are measuring light being absorbed by a sample.

Adsorption chromatography can be used to concentrate proteins since a large volume of protein can be applied to the column without affecting the amount of protein adsorbed to the column matrix. Careful selection of elution conditions can remove the protein from the column in a much smaller volume. Since adsorption chromatography allows for both the isolation and concentration of a target protein, it is sometimes referred to as the “capture” phase of protein purification.

Ion Exchange Chromatography

In ion exchange chromatography, the support consists of tiny beads to which are attached chemicals possessing a charge. Before use, the beads are equilibrated in a solution containing an appropriate counter-ion to the charged molecule on the bead. Uses Ion exchange resins are useful for separating charged from uncharged, or oppositely charged, biomolecules in solution. The two types of ion chromatography are anion-exchange and cation-exchange.

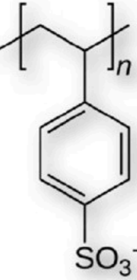
Cation-exchange chromatography is used when the molecule of interest is positively charged. In this type of chromatography, the stationary phase is negatively charged, and positively charged molecules are loaded to be attracted to it.

Anion-exchange chromatography is when the stationary phase is positively charged, and negatively charged molecules are loaded to be attracted to it. The bound molecules then can be eluted and collected using an eluant which contains anions and cations by running higher concentration of ions through the column or changing pH of the column.

To remove the molecules "stuck" to a column, add a high concentration of counter-ions to release them.

An ion exchange column apparatus

Ion exchange beads



Polystyrolsulfonate, a cation exchange resin

Figure 14.19 Top, a photograph of a packed FPLC ion exchange column. Bottom left, golden colored shiny ion exchange beads. The bottom right, chemical figure of a cation exchange negatively charged resin, polystyrosulfonate. Image credit: **Dr. Kevin Ahern (http://biochem.science.oregonstate.edu/content/kevin-ahern)** and **Dr. Indira Rajagopal (http://biochem.science.oregonstate.edu/content/indira-rajagopal)** https://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

The figure shows the repeating unit of polystyrolsulfonate, a compound used as a ***cation exchange resin***. The molecule is negatively charged, and thus the beads would be equilibrated in a buffer containing a positively charged ion, say sodium. Positively charged compounds from a cell lysate passed through the column will exchange with the counter-ions and "stick" to the negatively charged compounds covalently attached to the beads. Molecules in the sample that are neutral in charge or negatively charged will pass through the column. At this point, only positively charged molecules from the original sample would be bound to the column. These may then be washed off, or eluted, by using buffers containing high concentrations of salt. Under these conditions, the interaction between the positively charged molecules and the polystyrosulfonate would be disrupted, allowing the molecules that were bound to the column to be recovered.

Hydrophobic Interaction Chromatography (HIC)

Separation by HIC is based on the reversible interaction between a protein and a hydrophobic surface of chromatographic beads. This interaction is enhanced in the presence of a high ionic strength buffer solution, such as ammonium sulfate. Hydrophobic proteins bind to the column as they are loaded, and proteins with low affinity and other contaminants are washed away. Conditions are then altered so that the bound protein can be eluted differentially, usually by decreasing the salt concentration, or by the addition of chaotropic chemicals, or changes in pH.

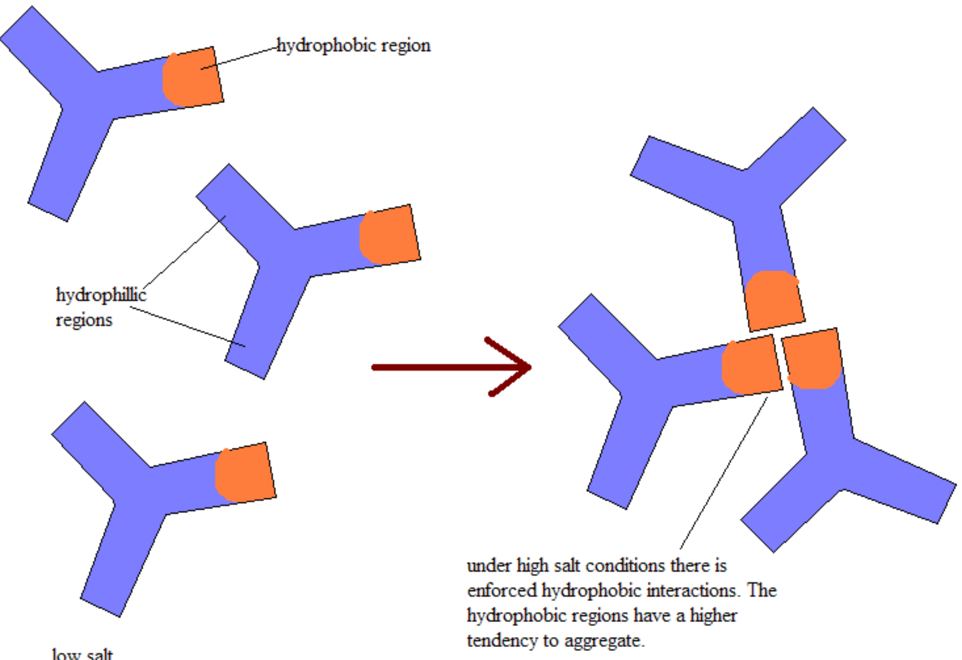


Figure 14.20 A cartoon image depicting hydrophobic interaction. Shown is 3, Y-shaped proteins, two prongs hydrophilic purple, one prong is hydrophobic and orange. On the right is under hydrophobic high salt conditions, the hydrophobic areas bind together. Image credit Daliak **https://commons.wikimedia.org/wiki/File:Hicsalt.png (https://commons.wikimedia.org/wiki/File:Hicsalt.png)**

Affinity chromatography

Affinity chromatography is a very powerful and selective technique that exploits the binding affinities of sample molecules (typically proteins) for molecules covalently linked to the support beads. In contrast to ion-exchange chromatography, where all molecules of a given charge would bind to the column, ***affinity chromatography exploits the specific binding of a protein or proteins to a ligand that is immobilized on the beads in the column.*** There are many high-resolution approaches and may include the use of antibody, nucleic acid, lectin, and other ligands. IMAC ligands are one of the more popular ones and is described below.

Histidine tagging

Histidine tagging (His-tagging) is a special kind of affinity chromatography and is a powerful tool for isolating a recombinant protein from a cell lysate. His-tagging relies on altering the DNA coding region for a protein to add a series of at least six histidine residues to the amino or carboxyl terminal of the encoded protein. This “His-Tag” is useful in purifying the tagged protein because histidine side chains are not found in nature and can readily bind to nickel or cobalt ions.

Separation of His-tagged proteins from a cell lysate is relatively easy (Figure). Passing the crude cell lysate through a column with nickel or cobalt attached to beads allows the His-tagged proteins to “stick,” while the remaining cell proteins all pass quickly through. The His-tagged proteins are then eluted by addition of imidazole to the column. Imidazole, which resembles the side chain of histidine, competes with the His-tagged proteins and displaces them from the column. Surprisingly, many His-tagged proteins appear to function normally despite the added histidine molecules, but if needed, the histidine tags may be cleaved from the purified protein by treatment with a protease that excises the added histidine’s, allowing the recovery of the desired protein with its native sequence.

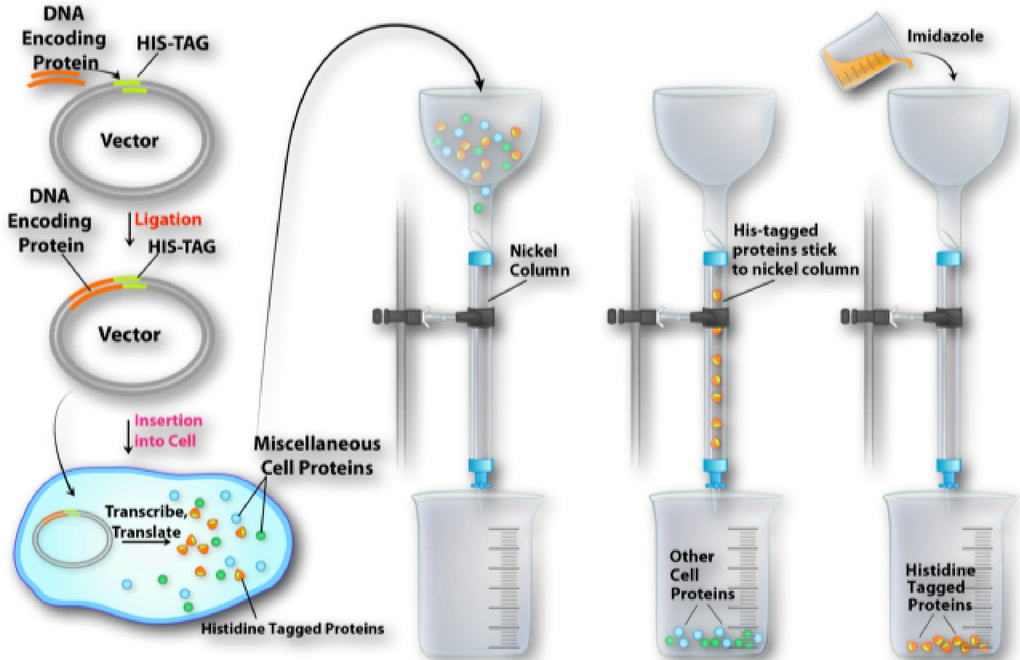


Figure 14.21 Affinity chromatographic purification of a protein by histidine tagging. A cartoon demonstrating how IMAC vector is made and the HIS-tag is used to purify target protein. Left is a vector diagram showing how a gene is inserted beside a HIS-tag. This vector is then inserted into bacteria where it produces recombinant protein with a 6XHIS tag on the end. This is then poured into a column on the right of the figure. The HIS-tag protein sticks to the column, other proteins wash away. The HIS-tag protein is then eluted with imidazole chemical. Image credit: **Dr. Kevin Ahern (http://biochem.science.oregonstate.edu/content/kevin-ahern)** and **Dr. Indira Rajagopal (http://biochem.science.oregonstate.edu/content/indira-rajagopal)** https://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy

Fast protein liquid chromatography (FPLC)

Fast protein liquid chromatography (FPLC), is a form of liquid column chromatography that is often used to analyze or purify mixtures of proteins. Separation of proteins is possible because the components of a protein mixture have different affinities for two materials: a moving mobile phase, and a porous solid stationary phase. ***In FPLC, the mobile phase is a mixture of aqueous buffers that moves through the column via a positive-displacement pump.*** The positive-displacement pump controls the mobile phase buffer flow at a constant rate while adjusting the composition of the buffer by drawing fluids in different proportions from two or more external reservoirs. The stationary phase is a resin composed of beads, usually of cross-linked agarose, packed into a cylindrical glass or plastic column. FPLC resins are available in a wide range of bead sizes and surface ligands depending on the application.

For example, in one type of FPLC strategy, ion exchange, the resin binds the protein of interest via a charge interaction while in buffer A (the running buffer) but become dissociated and return to solution in buffer B (the elution buffer). A mixture containing the target protein of interest is dissolved in 100% buffer A and pumped into the column. The proteins of interest bind to the resin while other contaminants are eluted out. The total flow rate of the buffer is kept constant; however, the proportion of Elution Buffer B is gradually increased from 0% to 100% according to a programmed change in concentration creating a "gradient" flow. The target protein dissociates and elutes; the effluent passes two detectors that measure conductivity and absorption of UV light at 280nm and collected in the fraction collector for further use.

The advantages to FPLC include a better resolution and much faster than gravity, it is automated in both the chromatography method and fraction collection and analysis, and it offers better separation through gradient elution techniques.

High-performance liquid chromatography (HPLC)

HPLC High-performance liquid chromatography (HPLC) is a powerful tool for separating a variety of molecules based on their differential polarities. A more efficient form of column chromatography, it employs columns with tightly packed supports and very tiny beads such that flow of solvents/buffers through the columns requires high

pressures. The supports used may be polar (normal phase separation) or non-polar (reverse phase separation). In normal phase separations, non-polar molecules elute first followed by the more polar compounds. This order is switched in reverse phase chromatography. Of the two, the reverse phase is commonly employed to do more reproducible chromatographic profiles (separations) that it typically produces.

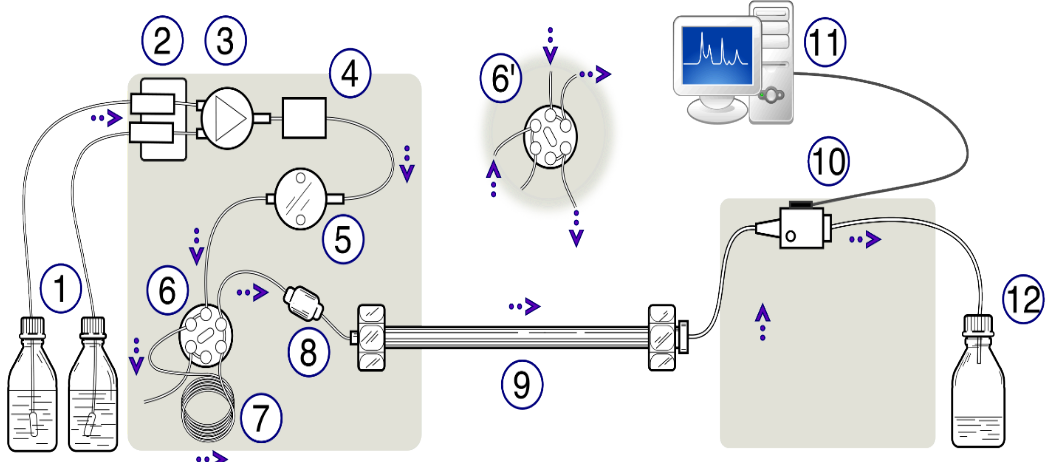


Figure 14.22 Schematic diagram of an HPLC system. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column (guard column), (9) Analytical column, (10) Detector (i.e., IR, UV), (11) Data acquisition, (12) Waste or fraction collector. Image credit: **https://en.wikipedia.org/wiki/High-performance_liquid_chromatography#/media/File:HPLC_apparatus.svg (https://en.wikipedia.org/wiki/High-performance_liquid_chromatography#/media/File:HPLC_apparatus.svg)**

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https://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy (https://biochem.science.oregonstate.edu/content/biochemistry-free-and-easy)

Wikipedia **https://en.wikipedia.org/wiki/Ion_chromatography (https://en.wikipedia.org/wiki/Ion_chromatography)**

14.5 | Advanced Chromatography Applications

Learning Objectives

By the end of this section, you will be able to:

- Describe examples of biosynthesis products within a cell that can be detected to identify bacteria

Accurate identification of bacterial isolates is essential in a clinical microbiology laboratory because the results often inform decisions about treatment that directly affect patient outcomes. For example, cases of food poisoning require accurate identification of the causative agent so that physicians can prescribe appropriate treatment. Likewise, it is important to accurately identify the causative pathogen during an outbreak of disease so that appropriate strategies can be employed to contain the epidemic.

There are many ways to detect, characterize, and identify microorganisms. Some methods rely on phenotypic biochemical characteristics, while others use genotypic identification. The biochemical characteristics of a bacterium provide many traits that are useful for classification and identification. Analyzing the nutritional and metabolic capabilities of the bacterial isolate is a common approach for determining the genus and the species of the bacterium. Some of the most important metabolic pathways that bacteria use to survive will be discussed in **Microbial Metabolism (https://legacy.cnx.org/content/m58818/latest/)** . In this section, we will discuss a few methods that use biochemical characteristics to identify microorganisms.

Some microorganisms store certain compounds as granules within their cytoplasm, and the contents of these granules can be used for identification purposes. For example, poly-β-hydroxybutyrate (PHB) is a carbon- and energy-storage compound found in some nonfluorescent bacteria of the genus *Pseudomonas*. Different species within this genus can be classified by the presence or the absence of PHB and fluorescent pigments. The human pathogen *P. aeruginosa* and the plant pathogen *P. syringae* are two examples of fluorescent *Pseudomonas* species that do not accumulate PHB granules.

Other systems rely on biochemical characteristics to identify microorganisms by their biochemical reactions, such as carbon utilization and other metabolic tests. In small laboratory settings or in teaching laboratories, those assays are carried out using a limited number of test tubes. However, more modern systems, such as the one developed by Biolog, Inc., are based on panels of biochemical reactions performed simultaneously and analyzed by software. Biolog's system identifies cells based on their ability to metabolize certain biochemicals and on their physiological properties, including pH and chemical sensitivity. It uses all major classes of biochemicals in its analysis. Identifications can be performed manually or with the semi- or fully automated instruments.

Another automated system identifies microorganisms by determining the specimen's mass spectrum and then comparing it to a database that contains known mass spectra for thousands of microorganisms. This method is based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) and uses disposable MALDI plates on which the microorganism is mixed with a specialized matrix reagent (**Figure 14.23**). The sample/reagent mixture is irradiated with a high-intensity pulsed ultraviolet laser, resulting in the ejection of gaseous ions generated from the various chemical constituents of the microorganism. These gaseous ions are collected and accelerated through the mass spectrometer, with ions traveling at a velocity determined by their mass-to-charge ratio (m/z), thus, reaching the detector at different times. A plot of detector signal versus m/z yields a mass spectrum for the organism that is uniquely related to its biochemical composition. Comparison of the mass spectrum to a library of reference spectra obtained from identical analyses of known microorganisms permits identification of the unknown microbe.

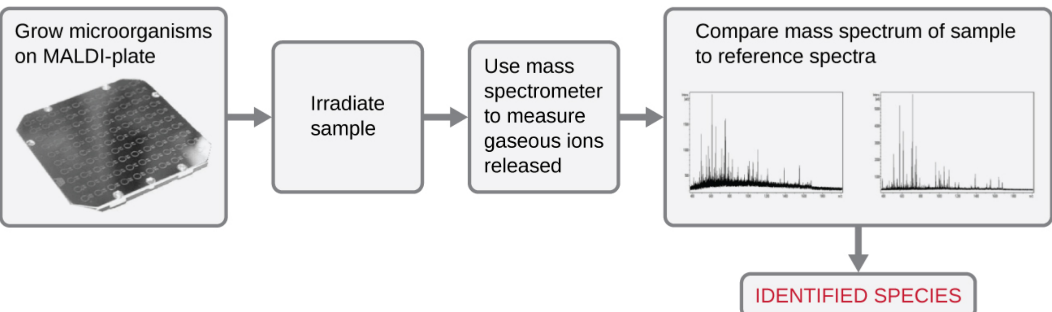


Figure 14.23 MALDI-TOF methods are now routinely used for diagnostic procedures in clinical microbiology laboratories. This technology is able to rapidly identify some microorganisms that cannot be readily identified by more traditional methods. (credit "MALDI plate photo": modification of work by Chen Q, Liu T, Chen G; credit "graphs": modification of work by Bailes J, Vidal L, Ivanov DA, Soloviev M)

Microbes can also be identified by measuring their unique lipid profiles. As we have learned, fatty acids of lipids can vary in chain length, presence or absence of double bonds, and number of double bonds, hydroxyl groups, branches, and rings. To identify a microbe by its lipid composition, the fatty acids present in their membranes are analyzed. A common biochemical analysis used for this purpose is a technique used in clinical, public health, and food laboratories. It relies on detecting unique differences in fatty acids and is called **fatty acid methyl ester (FAME) analysis**. In a FAME analysis, fatty acids are extracted from the membranes of microorganisms, chemically altered to form volatile methyl esters, and analyzed by gas chromatography (GC). The resulting GC chromatogram is compared with reference chromatograms in a database containing data for thousands of bacterial isolates to identify the unknown microorganism (**Figure 14.24**).

This OpenStax book is available for free at https://legacy.cnx.org/content/col26095/1.5

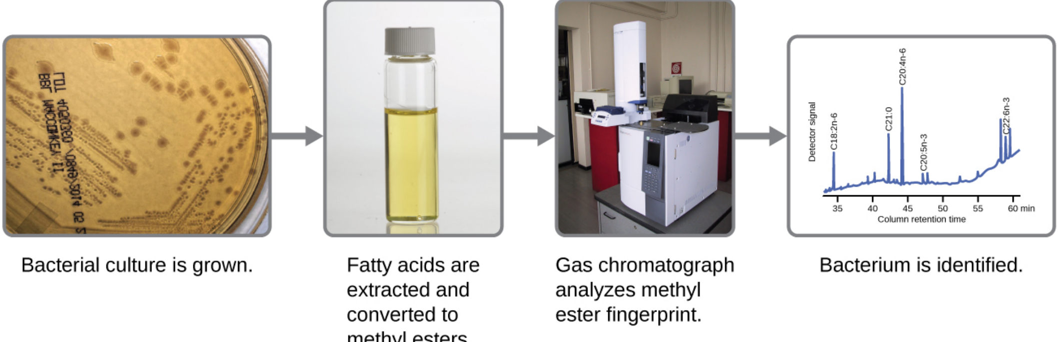


Figure 14.24 Fatty acid methyl ester (FAME) analysis in bacterial identification results in a chromatogram unique to each bacterium. Each peak in the gas chromatogram corresponds to a particular fatty acid methyl ester and its height is proportional to the amount present in the cell. (credit “culture”: modification of work by the Centers for Disease Control and Prevention; credit “graph”: modification of work by Zhang P. and Liu P.)

A related method for microorganism identification is called **phospholipid-derived fatty acids (PLFA) analysis**. Membranes are mostly composed of phospholipids, which can be saponified (hydrolyzed with alkali) to release the fatty acids. The resulting fatty acid mixture is then subjected to FAME analysis, and the measured lipid profiles can be compared with those of known microorganisms to identify the unknown microorganism.

Bacterial identification can also be based on the proteins produced under specific growth conditions within the human body. These types of identification procedures are called **proteomic analysis**. To perform proteomic analysis, proteins from the pathogen are first separated by high-pressure liquid chromatography (HPLC), and the collected fractions are then digested to yield smaller peptide fragments. These peptides are identified by mass spectrometry and compared with those of known microorganisms to identify the unknown microorganism in the original specimen.

Microorganisms can also be identified by the carbohydrates attached to proteins (glycoproteins) in the plasma membrane or cell wall. Antibodies and other carbohydrate-binding proteins can attach to specific carbohydrates on cell surfaces, causing the cells to clump together. Serological tests (e.g., the Lancefield groups tests, which are used for identification of *Streptococcus* species) are performed to detect the unique carbohydrates located on the surface of the cell.

Resolution

Penny stopped using her new sunscreen and applied the corticosteroid cream to her rash as directed. However, after several days, her rash had not improved and actually seemed to be getting worse. She made a follow-up appointment with her doctor, who observed a bumpy red rash and pus-filled blisters around hair follicles (**Figure 14.25**). The rash was especially concentrated in areas that would have been covered by a swimsuit. After some questioning, Penny told the physician that she had recently attended a pool party and spent some time in a hot tub. In light of this new information, the doctor suspected a case of hot tub rash, an infection frequently caused by the bacterium *Pseudomonas aeruginosa*, an opportunistic pathogen that can thrive in hot tubs and swimming pools, especially when the water is not sufficiently chlorinated. *P. aeruginosa* is the same bacterium that is associated with infections in the lungs of patients with cystic fibrosis.

The doctor collected a specimen from Penny’s rash to be sent to the clinical microbiology lab. Confirmatory tests were carried out to distinguish *P. aeruginosa* from enteric pathogens that can also be present in pool and hot-tub water. The test included the production of the blue-green pigment pyocyanin on cetrimide agar and growth at 42 °C. Cetrimide is a selective agent that inhibits the growth of other species of microbial flora and also enhances the production of *P. aeruginosa* pigments pyocyanin and fluorescein, which are a characteristic blue-green and yellow-green, respectively.

Tests confirmed the presence of *P. aeruginosa* in Penny’s skin sample, but the doctor decided not to prescribe an antibiotic. Even though *P. aeruginosa* is a bacterium, *Pseudomonas* species are generally resistant to many antibiotics. Luckily, skin infections like Penny’s are usually self-limiting; the rash typically lasts about 2 weeks and resolves on its own, with or without medical treatment. The doctor advised Penny to wait it out and keep using the corticosteroid cream. The cream will not kill the *P. aeruginosa* on Penny’s skin, but it should calm her rash and minimize the itching by suppressing her body’s inflammatory response to the bacteria.

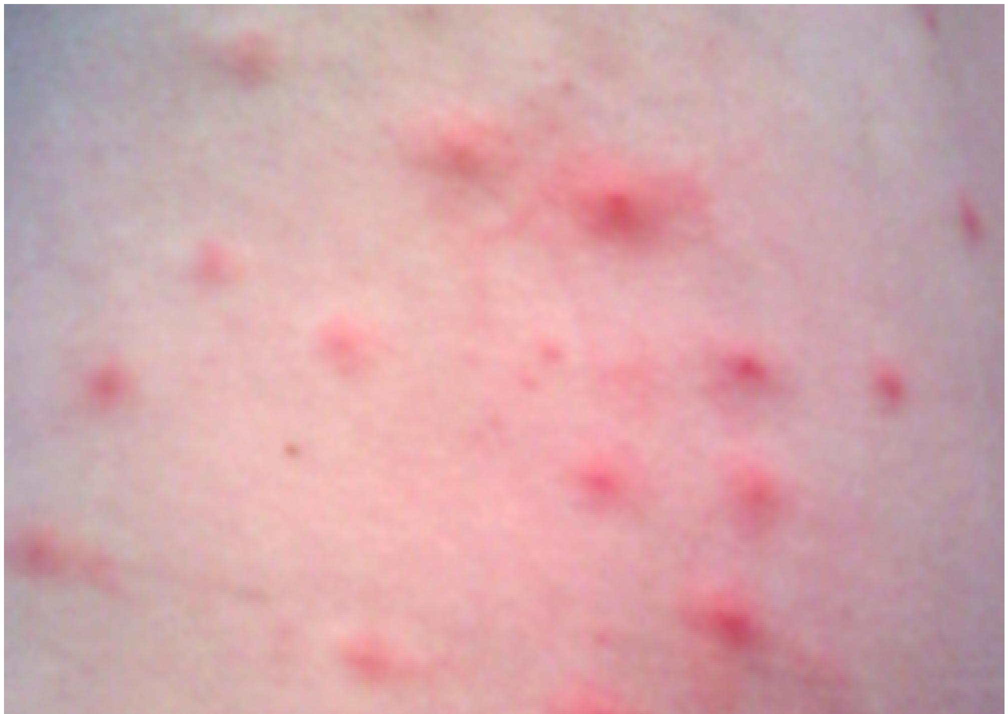


Figure 14.25 Exposure to *Pseudomonas aeruginosa* in the water of a pool or hot tub can sometimes cause a skin infection that manifests as “hot tub rash.” (credit: modification of work by “Lsupellmel”/Wikimedia Commons)

Go back to the ***previous (https://legacy.cnx.org/content/m58815/latest/#fs-id1167662559579)*** *Clinical Focus box.*

14.6 | Visualizing and Characterizing DNA, RNA, and Protein

Learning Objectives

By the end of this section, you will be able to:

- Explain the use of nucleic acid probes to visualize specific DNA sequences
- Explain the use of gel electrophoresis to separate DNA fragments
- Explain the principle of restriction fragment length polymorphism analysis and its uses
- Compare and contrast Southern and northern blots
- Explain the principles and uses of microarray analysis
- Describe the methods uses to separate and visualize protein variants
- Explain the method and uses of polymerase chain reaction and DNA sequencing

The sequence of a DNA molecule can help us identify an organism when compared to known sequences housed in a database. The sequence can also tell us something about the function of a particular part of the DNA, such as whether it encodes a particular protein. Comparing **protein signatures**—the expression levels of specific arrays of proteins—between samples is an important method for evaluating cellular responses to a multitude of environmental factors and stresses. Analysis of protein signatures can reveal the identity of an organism or how a cell is responding during disease.

The DNA and proteins of interest are microscopic and typically mixed in with many other molecules including

DNA or proteins irrelevant to our interests. Many techniques have been developed to isolate and characterize molecules of interest. These methods were originally developed for research purposes, but in many cases they have been simplified to the point that routine clinical use is possible. For example, many pathogens, such as the bacterium *Helicobacter pylori*, which causes stomach ulcers, can be detected using protein-based tests. In addition, an increasing number of highly specific and accurate DNA amplification-based identification assays can now detect pathogens such as antibiotic-resistant enteric bacteria, herpes simplex virus, varicella-zoster virus, and many others.

Molecular Analysis of DNA

In this subsection, we will outline some of the basic methods used for separating and visualizing specific fragments of DNA that are of interest to a scientist. Some of these methods do not require knowledge of the complete sequence of the DNA molecule. Before the advent of rapid DNA sequencing, these methods were the only ones available to work with DNA, but they still form the basic arsenal of tools used by molecular geneticists to study the body's responses to microbial and other diseases.

Nucleic Acid Probing

DNA molecules are small, and the information contained in their sequence is invisible. How does a researcher isolate a particular stretch of DNA, or having isolated it, determine what organism it is from, what its sequence is, or what its function is? One method to identify the presence of a certain DNA sequence uses artificially constructed pieces of DNA called probes. Probes can be used to identify different bacterial species in the environment and many DNA probes are now available to detect pathogens clinically. For example, DNA probes are used to detect the vaginal pathogens *Candida albicans*, *Gardnerella vaginalis*, and *Trichomonas vaginalis*.

To screen a genomic library for a particular gene or sequence of interest, researchers must know something about that gene. If researchers have a portion of the sequence of DNA for the gene of interest, they can design a **DNA probe**, a single-stranded DNA fragment that is complementary to part of the gene of interest and different from other DNA sequences in the sample. The DNA probe may be synthesized chemically by commercial laboratories, or it may be created by cloning, isolating, and denaturing a DNA fragment from a living organism. In either case, the DNA probe must be labeled with a molecular tag or beacon, such as a radioactive phosphorus atom (as is used for **autoradiography**) or a fluorescent dye (as is used in fluorescent *in situ* hybridization, or FISH), so that the probe and the DNA it binds to can be seen (Figure 14.26). The DNA sample being probed must also be denatured to make it single-stranded so that the single-stranded DNA probe can anneal to the single-stranded DNA sample at locations where their sequences are complementary. While these techniques are valuable for diagnosis, their direct use on sputum and other bodily samples may be problematic due to the complex nature of these samples. DNA often must first be isolated from bodily samples through chemical extraction methods before a DNA probe can be used to identify pathogens.

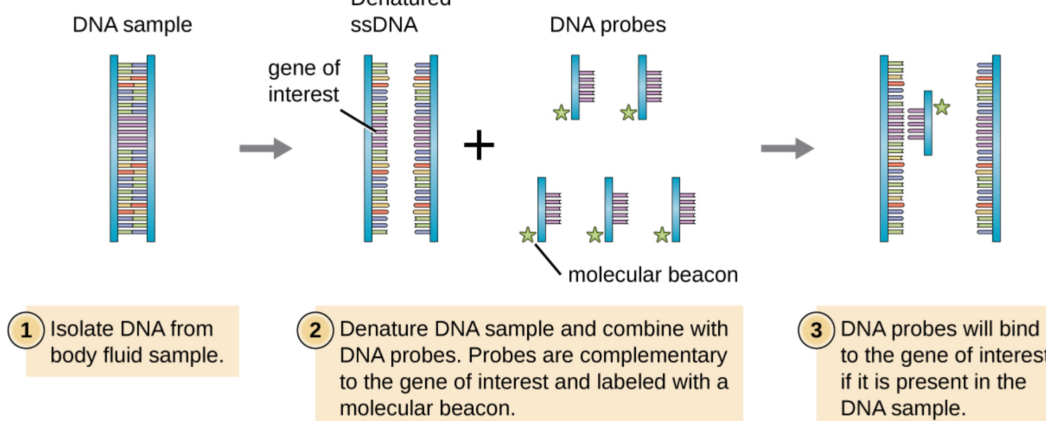


Figure 14.26 DNA probes can be used to confirm the presence of a suspected pathogen in patient samples. This diagram illustrates how a DNA probe can be used to search for a gene of interest associated with the suspected pathogen.

Part 2

The mild, flu-like symptoms that Kayla is experiencing could be caused by any number of infectious agents. In addition, several non-infectious autoimmune conditions, such as multiple sclerosis, systemic lupus

erythematosus (SLE), and amyotrophic lateral sclerosis (ALS), also have symptoms that are consistent with Kayla's early symptoms. However, over the course of several weeks, Kayla's symptoms worsened. She began to experience joint pain in her knees, heart palpitations, and a strange limpness in her facial muscles. In addition, she suffered from a stiff neck and painful headaches. Reluctantly, she decided it was time to seek medical attention.

- Do Kayla's new symptoms provide any clues as to what type of infection or other medical condition she may have?
- What tests or tools might a health-care provider use to pinpoint the pathogen causing Kayla's symptoms?

*Jump to the **next** Clinical Focus box. Go back to the **previous** Clinical Focus box.*

Agarose Gel Electrophoresis

There are a number of situations in which a researcher might want to physically separate a collection of DNA fragments of different sizes. A researcher may also digest a DNA sample with a restriction enzyme to form fragments. The resulting size and fragment distribution pattern can often yield useful information about the sequence of DNA bases that can be used, much like a bar-code scan, to identify the individual or species to which the DNA belongs.

Gel electrophoresis is a technique commonly used to separate biological molecules based on size and biochemical characteristics, such as charge and polarity. **Agarose gel electrophoresis** is widely used to separate DNA (or RNA) of varying sizes that may be generated by restriction enzyme digestion or by other means, such as the PCR (**Figure 14.27**).

Due to its negatively charged backbone, DNA is strongly attracted to a positive electrode. In agarose gel electrophoresis, the gel is oriented horizontally in a buffer solution. Samples are loaded into sample wells on the side of the gel closest to the negative electrode, then drawn through the molecular sieve of the agarose matrix toward the positive electrode. The agarose matrix impedes the movement of larger molecules through the gel, whereas smaller molecules pass through more readily. Thus, the distance of migration is inversely correlated to the size of the DNA fragment, with smaller fragments traveling a longer distance through the gel. Sizes of DNA fragments within a sample can be estimated by comparison to fragments of known size in a DNA ladder also run on the same gel. To separate very large DNA fragments, such as chromosomes or viral genomes, agarose gel electrophoresis can be modified by periodically alternating the orientation of the electric field during pulsed-field gel electrophoresis (PFGE). In PFGE, smaller fragments can reorient themselves and migrate slightly faster than larger fragments and this technique can thus serve to separate very large fragments that would otherwise travel together during standard agarose gel electrophoresis. In any of these electrophoresis techniques, the locations of the DNA or RNA fragments in the gel can be detected by various methods. One common method is adding ethidium bromide, a stain that inserts into the nucleic acids at non-specific locations and can be visualized when exposed to ultraviolet light. Other stains that are safer than ethidium bromide, a potential carcinogen, are now available.

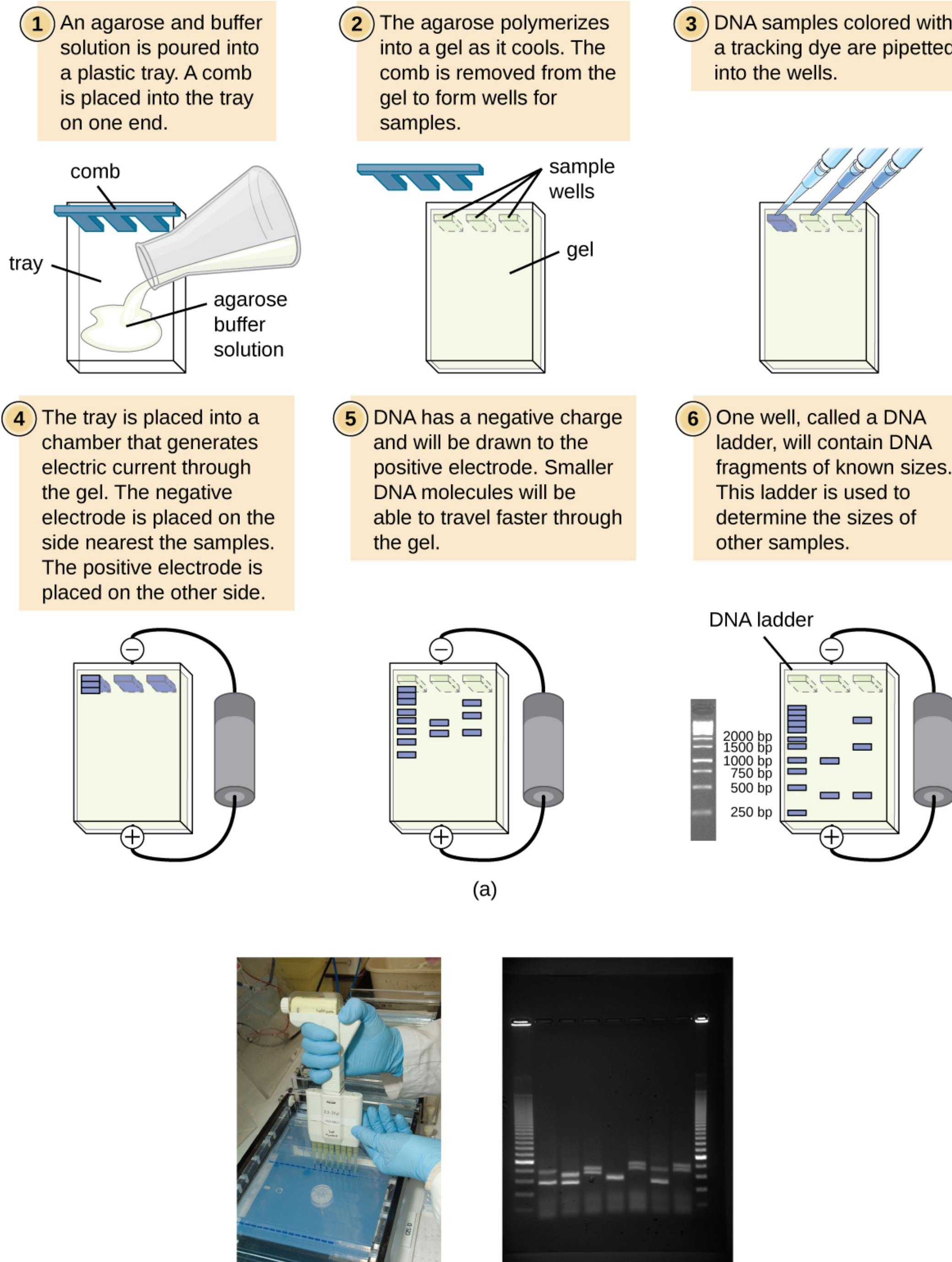


Figure 14.27 (a) The process of agarose gel electrophoresis. (b) A researcher loading samples into a gel. (c) This photograph shows a completed electrophoresis run on an agarose gel. The DNA ladder is located in lanes 1 and 9. Seven samples are located in lanes 2 through 8. The gel was stained with ethidium bromide and photographed under ultraviolet light. (credit a: modification of work by Magnus Manske; credit b: modification of work by U.S. Department of Agriculture; credit c: modification of work by James Jacob)

Restriction Fragment Length Polymorphism (RFLP) Analysis

Restriction enzyme recognition sites are short (only a few nucleotides long), sequence-specific palindromes, and may be found throughout the genome. Thus, differences in DNA sequences in the genomes of individuals will lead to differences in distribution of restriction-enzyme recognition sites that can be visualized as distinct banding patterns on a gel after agarose gel electrophoresis. **Restriction fragment length polymorphism (RFLP)** analysis compares DNA banding patterns of different DNA samples after restriction digestion (Figure

14.28).

RFLP analysis has many practical applications in both medicine and forensic science. For example, epidemiologists use RFLP analysis to track and identify the source of specific microorganisms implicated in outbreaks of food poisoning or certain infectious diseases. RFLP analysis can also be used on human DNA to determine inheritance patterns of chromosomes with variant genes, including those associated with heritable diseases or to establish paternity.

Forensic scientists use RFLP analysis as a form of DNA fingerprinting, which is useful for analyzing DNA obtained from crime scenes, suspects, and victims. DNA samples are collected, the numbers of copies of the sample DNA molecules are increased using PCR, and then subjected to restriction enzyme digestion and agarose gel electrophoresis to generate specific banding patterns. By comparing the banding patterns of samples collected from the crime scene against those collected from suspects or victims, investigators can definitively determine whether DNA evidence collected at the scene was left behind by suspects or victims.

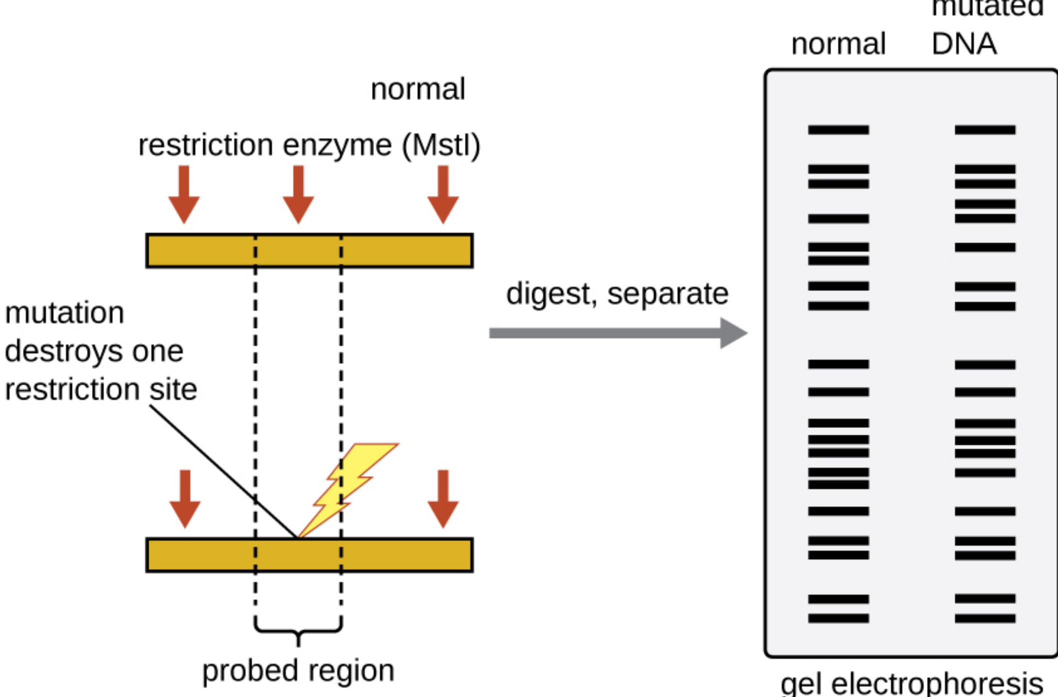


Figure 14.28 RFLP analysis can be used to differentiate DNA sequences. In this example, a normal chromosome is digested into two fragments, whereas digestion of a mutated chromosome produces only one fragment. The small red arrows pointing to the two different chromosome segments show the locations of the restriction enzyme recognition sites. After digestion and agarose gel electrophoresis, the banding patterns reflect the change by showing the loss of two shorter bands and the gain of a longer band. (credit: modification of work by National Center for Biotechnology Information)

Southern Blots and Modifications

Several molecular techniques capitalize on sequence complementarity and hybridization between nucleic acids of a sample and DNA probes. Typically, probing nucleic-acid samples within a gel is unsuccessful because as the DNA probe soaks into a gel, the sample nucleic acids within the gel diffuse out. Thus, blotting techniques are commonly used to transfer nucleic acids to a thin, positively charged membrane made of nitrocellulose or nylon. In the **Southern blot** technique, developed by Sir Edwin Southern in 1975, DNA fragments within a sample are first separated by agarose gel electrophoresis and then transferred to a membrane through capillary action (Figure 14.29). The DNA fragments that bind to the surface of the membrane are then exposed to a specific single-stranded DNA probe labeled with a radioactive or fluorescent molecular beacon to aid in detection. Southern blots may be used to detect the presence of certain DNA sequences in a given DNA sample. Once the target DNA within the membrane is visualized, researchers can cut out the portion of the membrane containing the fragment to recover the DNA fragment of interest.

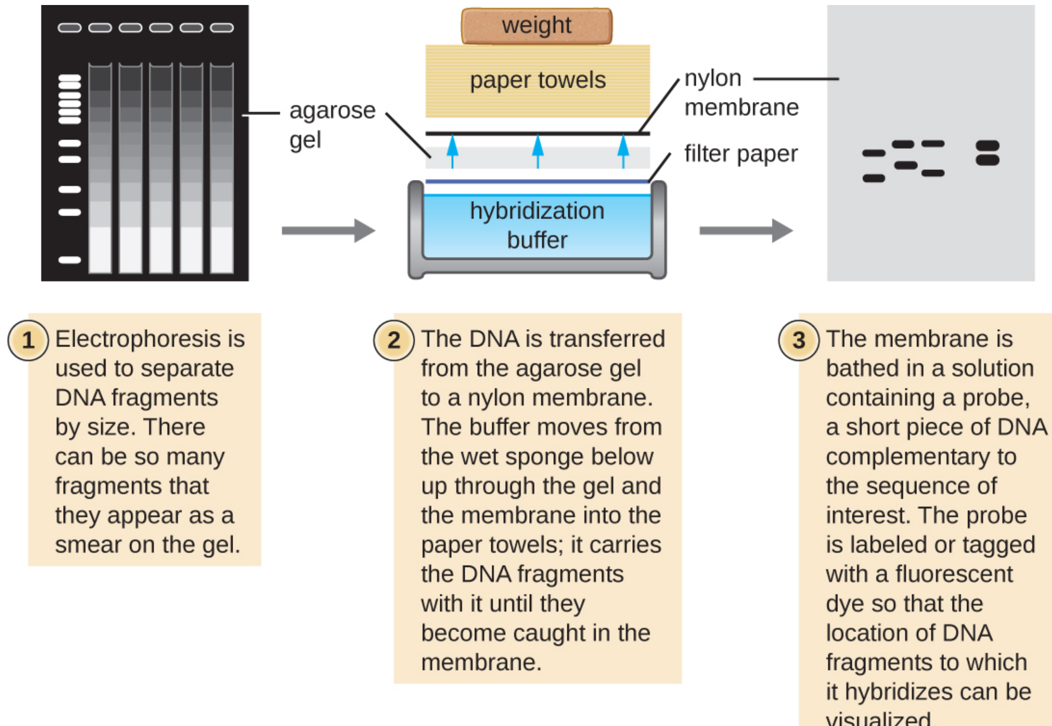


Figure 14.29 In the Southern blot technique, DNA fragments are first separated by agarose gel electrophoresis, then transferred by capillary action to a nylon membrane, which is then soaked with a DNA probe tagged with a molecular beacon for easy visualization.

Variations of the Southern blot—the dot blot, slot blot, and the spot blot—do not involve electrophoresis, but instead concentrate DNA from a sample into a small location on a membrane. After hybridization with a DNA probe, the signal intensity detected is measured, allowing the researcher to estimate the amount of target DNA present within the sample.

A colony blot is another variation of the Southern blot in which colonies representing different clones in a genomic library are transferred to a membrane by pressing the membrane onto the culture plate. The cells on the membrane are lysed and the membrane can then be probed to determine which colonies within a genomic library harbor the target gene. Because the colonies on the plate are still growing, the cells of interest can be isolated from the plate.

In the **northern blot**, another variation of the Southern blot, RNA (not DNA) is immobilized on the membrane and probed. Northern blots are typically used to detect the amount of mRNA made through gene expression within a tissue or organism sample.

Microarray Analysis

Another technique that capitalizes on the hybridization between complementary nucleic acid sequences is called **microarray analysis**. Microarray analysis is useful for the comparison of gene-expression patterns between different cell types—for example, cells infected with a virus versus uninfected cells, or cancerous cells versus healthy cells (**Figure 14.30**).

Typically, DNA or cDNA from an experimental sample is deposited on a glass slide alongside known DNA sequences. Each slide can hold more than 30,000 different DNA fragment types. Distinct DNA fragments (encompassing an organism's entire genomic library) or cDNA fragments (corresponding to an organism's full complement of expressed genes) can be individually spotted on a glass slide.

Once deposited on the slide, genomic DNA or mRNA can be isolated from the two samples for comparison. If mRNA is isolated, it is reverse-transcribed to cDNA using reverse transcriptase. Then the two samples of genomic DNA or cDNA are labeled with different fluorescent dyes (typically red and green). The labeled genomic DNA samples are then combined in equal amounts, added to the microarray chip, and allowed to hybridize to complementary spots on the microarray.

Hybridization of sample genomic DNA molecules can be monitored by measuring the intensity of fluorescence

at particular spots on the microarray. Differences in the amount of hybridization between the samples can be readily observed. If only one sample's nucleic acids hybridize to a particular spot on the microarray, then that spot will appear either green or red. However, if both samples' nucleic acids hybridize, then the spot will appear yellow due to the combination of the red and green dyes.

Although microarray technology allows for a holistic comparison between two samples in a short time, it requires sophisticated (and expensive) detection equipment and analysis software. Because of the expense, this technology is typically limited to research settings. Researchers have used microarray analysis to study how gene expression is affected in organisms that are infected by bacteria or viruses or subjected to certain chemical treatments.

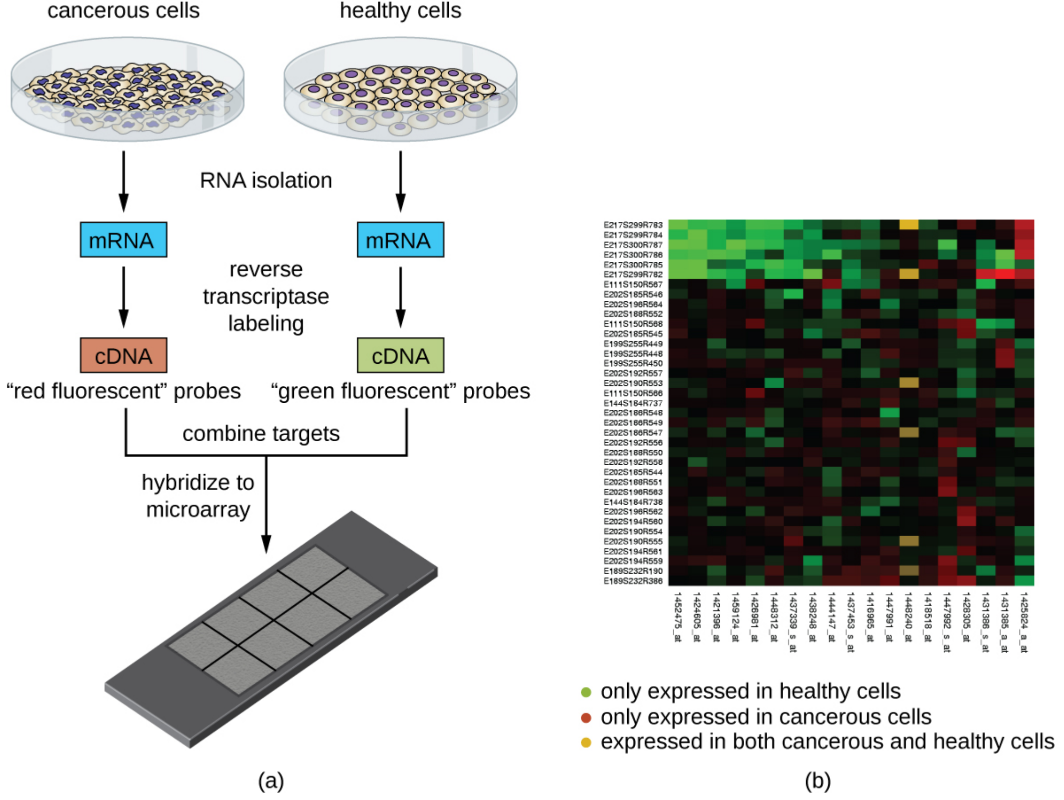


Figure 14.30 (a) The steps in microarray analysis are illustrated. Here, gene expression patterns are compared between cancerous cells and healthy cells. (b) Microarray information can be expressed as a heat map. Genes are shown on the left side; different samples are shown across the bottom. Genes expressed only in cancer cells are shown in varying shades of red; genes expressed only in normal cells are shown in varying shades of green. Genes that are expressed in both cancerous and normal cells are shown in yellow.

Explore **microchip technology (https://openstax.org/l/22intwebmictec)** at this interactive website.

- What does a DNA probe consist of?
- Why is a Southern blot used after gel electrophoresis of a DNA digest?

Molecular Analysis of Proteins

In many cases it may not be desirable or possible to study DNA or RNA directly. Proteins can provide species-specific information for identification as well as important information about how and whether a cell or tissue is responding to the presence of a pathogenic microorganism. Various proteins require different methods for isolation and characterization.

Polyacrylamide Gel Electrophoresis

A variation of gel electrophoresis, called **polyacrylamide gel electrophoresis (PAGE)**, is commonly used

for separating proteins. In PAGE, the gel matrix is finer and composed of polyacrylamide instead of agarose. Additionally, PAGE is typically performed using a vertical gel apparatus (**Figure 14.31**). Because of the varying charges associated with amino acid side chains, PAGE can be used to separate intact proteins based on their net charges. Alternatively, proteins can be denatured and coated with a negatively charged detergent called sodium dodecyl sulfate (SDS), masking the native charges and allowing separation based on size only. PAGE can be further modified to separate proteins based on two characteristics, such as their charges at various pHs as well as their size, through the use of two-dimensional PAGE. In any of these cases, following electrophoresis, proteins are visualized through staining, commonly with either Coomassie blue or a silver stain.

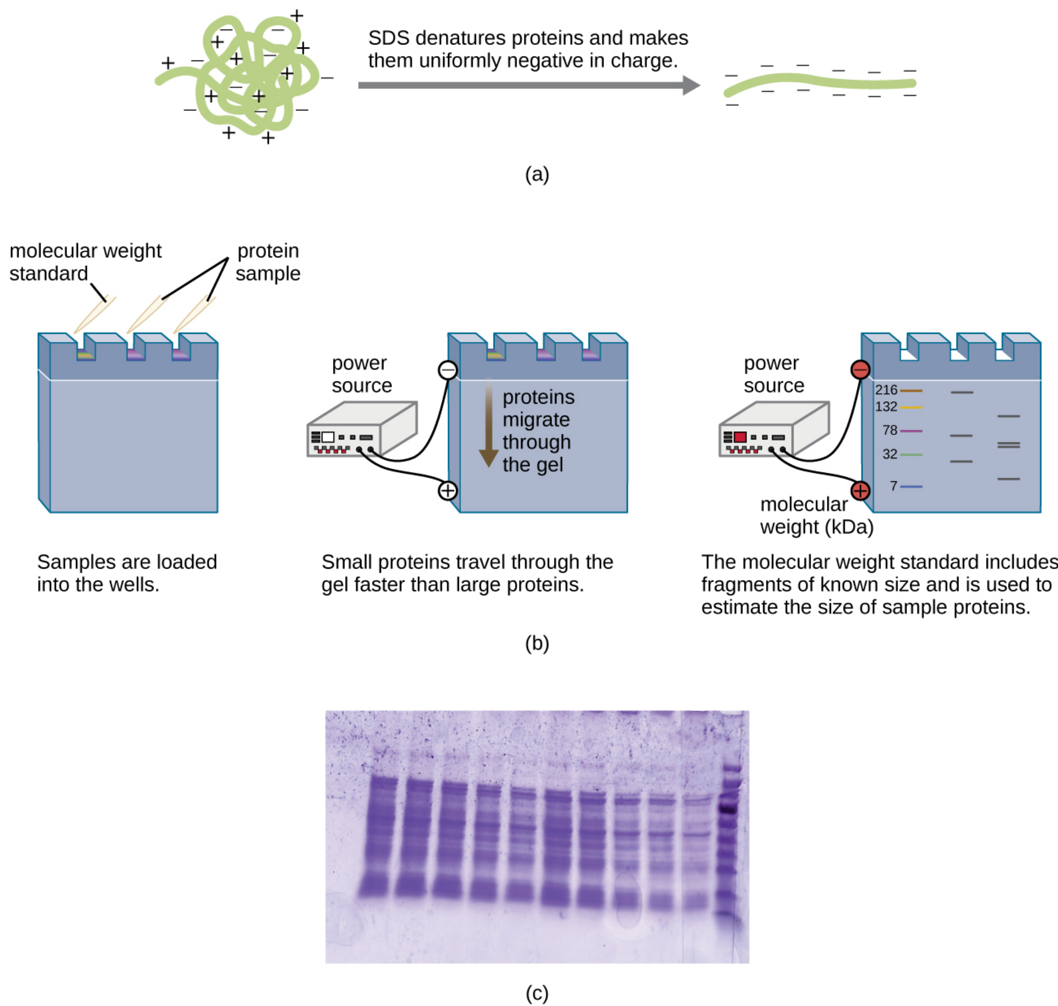


Figure 14.31 (a) SDS is a detergent that denatures proteins and masks their native charges, making them uniformly negatively charged. (b) The process of SDS-PAGE is illustrated in these steps. (c) A photograph of an SDS-PAGE gel shows Coomassie stained bands where proteins of different size have migrated along the gel in response to the applied voltage. A size standard lane is visible on the right side of the gel. (credit b: modification of work by “GeneEd”/YouTube)

- On what basis are proteins separated in SDS-PAGE?

Part 3

When Kayla described her symptoms, her physician at first suspected bacterial meningitis, which is consistent with her headaches and stiff neck. However, she soon ruled this out as a possibility because meningitis typically progresses more quickly than what Kayla was experiencing. Many of her symptoms still paralleled those of amyotrophic lateral sclerosis (ALS) and systemic lupus erythematosus (SLE), and the physician also considered Lyme disease a possibility given how much time Kayla spends in the woods. Kayla did not recall any recent tick bites (the typical means by which Lyme disease is transmitted) and she did not have the typical bull’s-eye rash associated with Lyme disease (**Figure 14.32**). However, 20–30% of

patients with Lyme disease never develop this rash, so the physician did not want to rule it out.

Kayla's doctor ordered an MRI of her brain, a complete blood count to test for anemia, blood tests assessing liver and kidney function, and additional tests to confirm or rule out SLE or Lyme disease. Her test results were inconsistent with both SLE and ALS, and the result of the test looking for Lyme disease antibodies was "equivocal," meaning inconclusive. Having ruled out ALS and SLE, Kayla's doctor decided to run additional tests for Lyme disease.

- Why would Kayla's doctor still suspect Lyme disease even if the test results did not detect Lyme antibodies in the blood?
- What type of molecular test might be used for the detection of blood antibodies to Lyme disease?

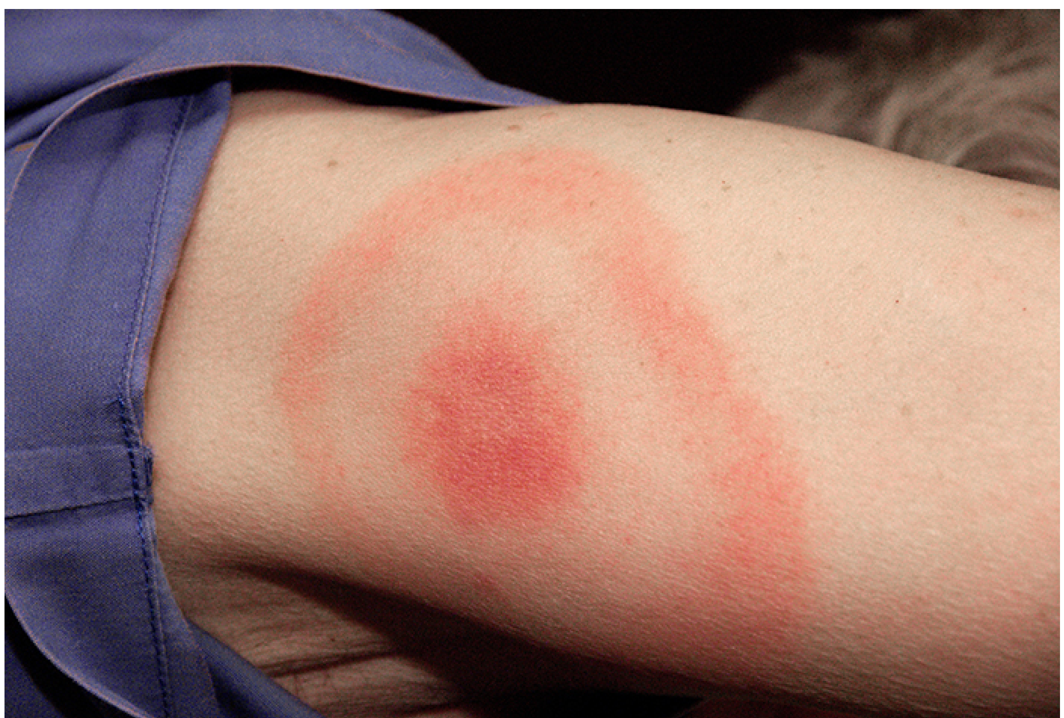


Figure 14.32 A bulls-eye rash is one of the common symptoms of Lyme diseases, but up to 30% of infected individuals never develop a rash. (credit: Centers for Disease Control and Prevention)

*Jump to the **next** Clinical Focus box. Go back to the **previous** Clinical Focus box.*

Amplification-Based DNA Analysis Methods

Various methods can be used for obtaining sequences of DNA, which are useful for studying disease-causing organisms. With the advent of rapid sequencing technology, our knowledge base of the entire genomes of pathogenic organisms has grown phenomenally. We start with a description of the polymerase chain reaction, which is not a sequencing method but has allowed researchers and clinicians to obtain the large quantities of DNA needed for sequencing and other studies. The polymerase chain reaction eliminates the dependence we once had on cells to make multiple copies of DNA, achieving the same result through relatively simple reactions outside the cell.

Polymerase Chain Reaction (PCR)

Most methods of DNA analysis, such as restriction enzyme digestion and agarose gel electrophoresis, or DNA sequencing require large amounts of a specific DNA fragment. In the past, large amounts of DNA were produced by growing the host cells of a genomic library. However, libraries take time and effort to prepare and DNA samples of interest often come in minute quantities. The **polymerase chain reaction (PCR)** permits rapid amplification in the number of copies of specific DNA sequences for further analysis (**Figure 14.33**). One of the most powerful techniques in molecular biology, PCR was developed in 1983 by Kary Mullis while at Cetus Corporation. PCR has specific applications in research, forensic, and clinical laboratories, including:

- determining the sequence of nucleotides in a specific region of DNA
- amplifying a target region of DNA for cloning into a plasmid vector
- identifying the source of a DNA sample left at a crime scene
- analyzing samples to determine paternity
- comparing samples of ancient DNA with modern organisms
- determining the presence of difficult to culture, or unculturable, microorganisms in humans or environmental samples

PCR is an *in vitro* laboratory technique that takes advantage of the natural process of DNA replication. The heat-stable DNA polymerase enzymes used in PCR are derived from hyperthermophilic prokaryotes. *Taq DNA polymerase*, commonly used in PCR, is derived from the *Thermus aquaticus* bacterium isolated from a hot spring in Yellowstone National Park. DNA replication requires the use of primers for the initiation of replication to have free 3′-hydroxyl groups available for the addition of nucleotides by DNA polymerase. However, while primers composed of RNA are normally used in cells, DNA primers are used for PCR. **DNA primers** are preferable due to their stability, and DNA primers with known sequences targeting a specific DNA region can be chemically synthesized commercially. These DNA primers are functionally similar to the DNA probes used for the various hybridization techniques described earlier, binding to specific targets due to complementarity between the target DNA sequence and the primer.

PCR occurs over multiple cycles, each containing three steps: denaturation, annealing, and extension. Machines called thermal cyclers are used for PCR; these machines can be programmed to automatically cycle through the temperatures required at each step (**Figure 14.1**). First, double-stranded template DNA containing the target sequence is denatured at approximately 95 °C. The high temperature required to physically (rather than enzymatically) separate the DNA strands is the reason the heat-stable DNA polymerase is required. Next, the temperature is lowered to approximately 50 °C. This allows the DNA primers complementary to the ends of the target sequence to anneal (stick) to the template strands, with one primer annealing to each strand. Finally, the temperature is raised to 72 °C, the optimal temperature for the activity of the heat-stable DNA polymerase, allowing for the addition of nucleotides to the primer using the single-stranded target as a template. Each cycle doubles the number of double-stranded target DNA copies. Typically, PCR protocols include 25–40 cycles, allowing for the amplification of a single target sequence by tens of millions to over a trillion.

Natural DNA replication is designed to copy the entire genome, and initiates at one or more origin sites. Primers are constructed during replication, not before, and do not consist of a few specific sequences. PCR targets specific regions of a DNA sample using sequence-specific primers. In recent years, a variety of isothermal PCR amplification methods that circumvent the need for thermal cycling have been developed, taking advantage of accessory proteins that aid in the DNA replication process. As the development of these methods continues and their use becomes more widespread in research, forensic, and clinical labs, thermal cyclers may become obsolete.

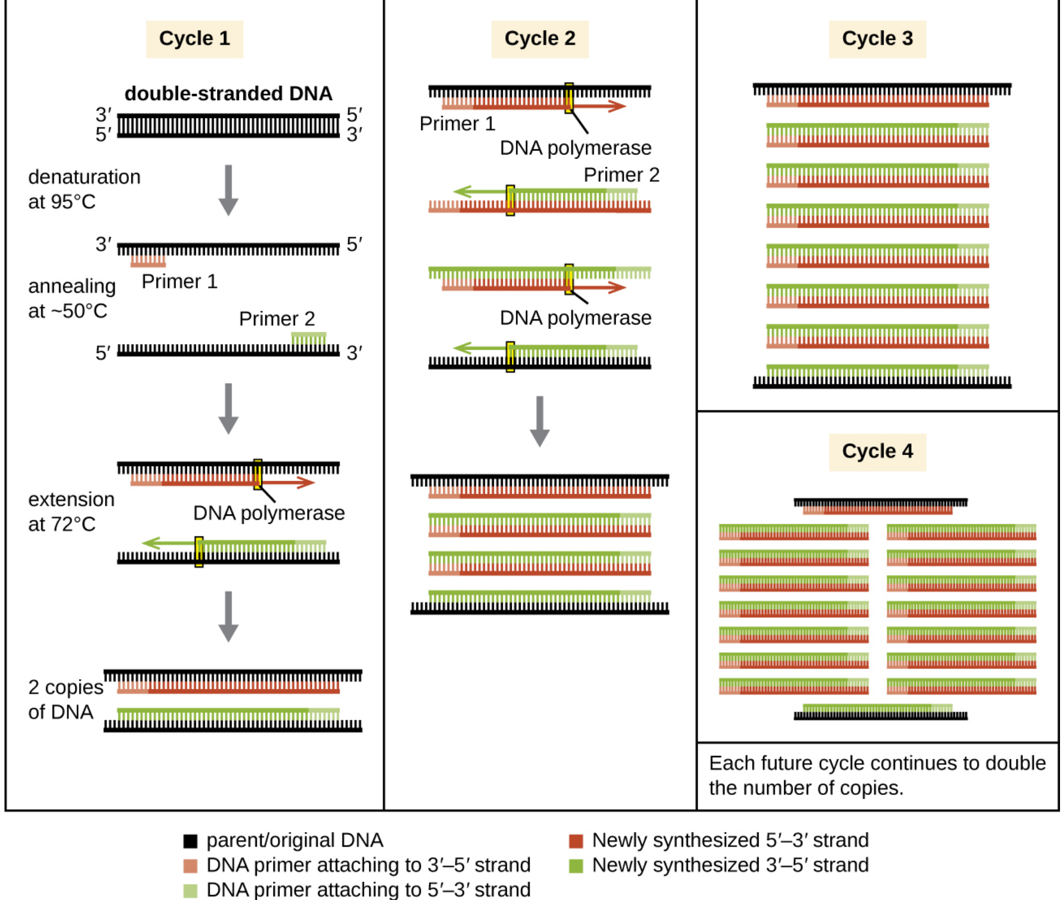


Figure 14.33 The polymerase chain reaction (PCR) is used to produce many copies of a specific sequence of DNA.

Deepen your understanding of the polymerase chain reaction by viewing this **animation (https://openstax.org/l/22polychareami)** and working through an **interactive (https://openstax.org/l/22intexerpolchr)** exercise.

PCR Variations

Several later modifications to PCR further increase the utility of this technique. **Reverse transcriptase PCR (RT-PCR)** is used for obtaining DNA copies of a specific mRNA molecule. RT-PCR begins with the use of the reverse transcriptase enzyme to convert mRNA molecules into cDNA. That cDNA is then used as a template for traditional PCR amplification. RT-PCR can detect whether a specific gene has been expressed in a sample. Another recent application of PCR is **real-time PCR**, also known as **quantitative PCR (qPCR)**. Standard PCR and RT-PCR protocols are not quantitative because any one of the reagents may become limiting before all of the cycles within the protocol are complete, and samples are only analyzed at the end. Because it is not possible to determine when in the PCR or RT-PCR protocol a given reagent has become limiting, it is not possible to know how many cycles were completed prior to this point, and thus it is not possible to determine how many original template molecules were present in the sample at the start of PCR. In qPCR, however, the use of fluorescence allows one to monitor the increase in a double-stranded template during a PCR reaction as it occurs. These kinetics data can then be used to quantify the amount of the original target sequence. The use of qPCR in recent years has further expanded the capabilities of PCR, allowing researchers to determine the number of DNA copies, and sometimes organisms, present in a sample. In clinical settings, qRT-PCR is used to determine viral load in HIV-positive patients to evaluate the effectiveness of their therapy.

DNA Sequencing

A basic sequencing technique is the **chain termination method**, also known as the **dideoxy method** or the **Sanger DNA sequencing method**, developed by Frederick Sanger in 1972. The chain termination method involves DNA replication of a single-stranded template with the use of a DNA primer to initiate synthesis of

a complementary strand, DNA polymerase, a mix of the four regular deoxynucleotide (dNTP) monomers, and a small proportion of dideoxynucleotides (ddNTPs), each labeled with a molecular beacon. The ddNTPs are monomers missing a hydroxyl group (–OH) at the site at which another nucleotide usually attaches to form a chain (**Figure 14.34**). Every time a ddNTP is randomly incorporated into the growing complementary strand, it terminates the process of DNA replication for that particular strand. This results in multiple short strands of replicated DNA that are each terminated at a different point during replication. When the reaction mixture is subjected to gel electrophoresis, the multiple newly replicated DNA strands form a ladder of differing sizes. Because the ddNTPs are labeled, each band on the gel reflects the size of the DNA strand when the ddNTP terminated the reaction.

In Sanger's day, four reactions were set up for each DNA molecule being sequenced, each reaction containing only one of the four possible ddNTPs. Each ddNTP was labeled with a radioactive phosphorus molecule. The products of the four reactions were then run in separate lanes side by side on long, narrow PAGE gels, and the bands of varying lengths were detected by autoradiography. Today, this process has been simplified with the use of ddNTPs, each labeled with a different colored fluorescent dye or fluorochrome (**Figure 14.35**), in one sequencing reaction containing all four possible ddNTPs for each DNA molecule being sequenced (**Figure 14.36**). These fluorochromes are detected by fluorescence spectroscopy. Determining the fluorescence color of each band as it passes by the detector produces the nucleotide sequence of the template strand.

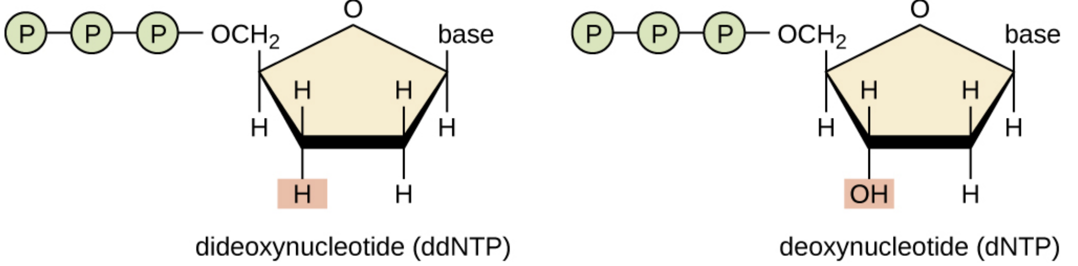


Figure 14.34 A dideoxynucleotide is similar in structure to a deoxynucleotide, but is missing the 3′ hydroxyl group (indicated by the shaded box). When a dideoxynucleotide is incorporated into a DNA strand, DNA synthesis stops.

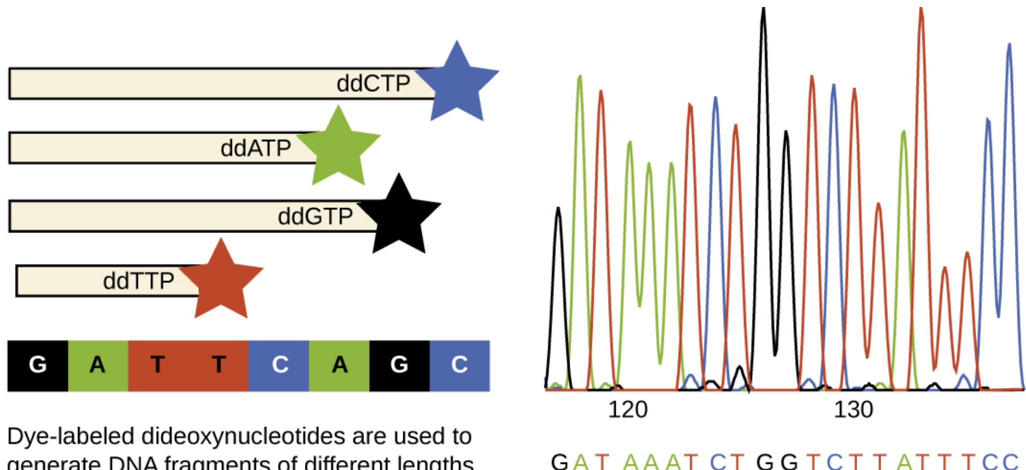


Figure 14.35 Frederick Sanger's dideoxy chain termination method is illustrated, using ddNTPs tagged with fluorochromes. Using ddNTPs, a mixture of DNA fragments of every possible size, varying in length by only one nucleotide, can be generated. The DNA is separated on the basis of size and each band can be detected with a fluorescence detector.

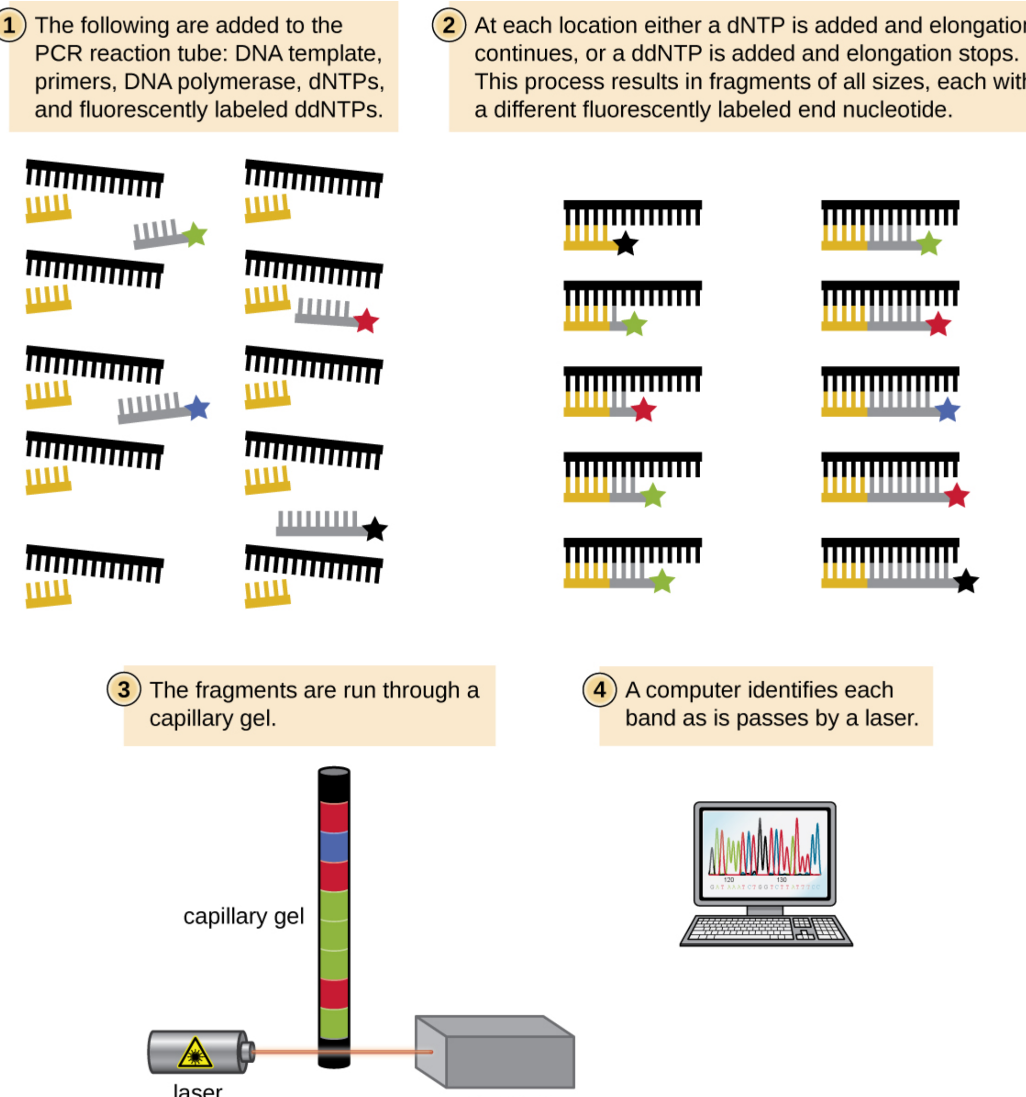


Figure 14.36 This diagram summarizes the Sanger sequencing method using fluorochrome-labeled ddNTPs and capillary gel electrophoresis.

Since 2005, automated sequencing techniques used by laboratories fall under the umbrella of **next generation sequencing**, which is a group of automated techniques used for rapid DNA sequencing. These methods have revolutionized the field of molecular genetics because the low-cost sequencers can generate sequences of hundreds of thousands or millions of short fragments (25 to 600 base pairs) just in one day. Although several variants of next generation sequencing technologies are made by different companies (for example, 454 Life Sciences' pyrosequencing and Illumina's Solexa technology), they all allow millions of bases to be sequenced quickly, making the sequencing of entire genomes relatively easy, inexpensive, and commonplace. In **454 sequencing (pyrosequencing)**, for example, a DNA sample is fragmented into 400–600-bp single-strand fragments, modified with the addition of DNA adapters to both ends of each fragment. Each DNA fragment is then immobilized on a bead and amplified by PCR, using primers designed to anneal to the adapters, creating a bead containing many copies of that DNA fragment. Each bead is then put into a separate well containing sequencing enzymes. To the well, each of the four nucleotides is added one after the other; when each one is incorporated, pyrophosphate is released as a byproduct of polymerization, emitting a small flash of light that is recorded by a detector. This provides the order of nucleotides incorporated as a new strand of DNA is made and is an example of synthesis sequencing. Next generation sequencers use sophisticated software to get through the cumbersome process of putting all the fragments in order. Overall, these technologies continue to advance rapidly, decreasing the cost of sequencing and increasing the availability of sequence data from a wide variety of organisms quickly.

The National Center for Biotechnology Information houses a widely used genetic sequence database called GenBank where researchers deposit genetic information for public use. Upon publication of sequence data, researchers upload it to GenBank, giving other researchers access to the information. The collaboration allows researchers to compare newly discovered or unknown sample sequence information with the vast array of sequence data that already exists.

View an **animation (https://openstax.org/l/22454seqanim)** about 454 sequencing to deepen your understanding of this method.

Using a NAAT to Diagnose a *C. difficile* Infection

Javier, an 80-year-old patient with a history of heart disease, recently returned home from the hospital after undergoing an angioplasty procedure to insert a stent into a cardiac artery. To minimize the possibility of infection, Javier was administered intravenous broad-spectrum antibiotics during and shortly after his procedure. He was released four days after the procedure, but a week later, he began to experience mild abdominal cramping and watery diarrhea several times a day. He lost his appetite, became severely dehydrated, and developed a fever. He also noticed blood in his stool. Javier's wife called the physician, who instructed her to take him to the emergency room immediately.

The hospital staff ran several tests and found that Javier's kidney creatinine levels were elevated compared with the levels in his blood, indicating that his kidneys were not functioning well. Javier's symptoms suggested a possible infection with *Clostridium difficile*, a bacterium that is resistant to many antibiotics. The hospital collected and cultured a stool sample to look for the production of toxins A and B by *C. difficile*, but the results came back negative. However, the negative results were not enough to rule out a *C. difficile* infection because culturing of *C. difficile* and detection of its characteristic toxins can be difficult, particularly in some types of samples. To be safe, they proceeded with a diagnostic nucleic acid amplification test (NAAT). Currently NAATs are the clinical diagnostician's gold standard for detecting the genetic material of a pathogen. In Javier's case, qPCR was used to look for the gene encoding *C. difficile* toxin B (*tcdB*). When the qPCR analysis came back positive, the attending physician concluded that Javier was indeed suffering from a *C. difficile* infection and immediately prescribed the antibiotic vancomycin, to be administered intravenously. The antibiotic cleared the infection and Javier made a full recovery.

Because infections with *C. difficile* were becoming widespread in Javier's community, his sample was further analyzed to see whether the specific strain of *C. difficile* could be identified. Javier's stool sample was subjected to ribotyping and repetitive sequence-based PCR (rep-PCR) analysis. In ribotyping, a short sequence of DNA between the 16S rRNA and 23S rRNA genes is amplified and subjected to restriction digestion (**Figure 14.37**). This sequence varies between strains of *C. difficile*, so restriction enzymes will cut in different places. In rep-PCR, DNA primers designed to bind to short sequences commonly found repeated within the *C. difficile* genome were used for PCR. Following restriction digestion, agarose gel electrophoresis was performed in both types of analysis to examine the banding patterns that resulted from each procedure (**Figure 14.38**). Rep-PCR can be used to further subtype various ribotypes, increasing resolution for detecting differences between strains. The ribotype of the strain infecting Javier was found to be ribotype 27, a strain known for its increased virulence, resistance to antibiotics, and increased prevalence in the United States, Canada, Japan, and Europe.[9]

- How do banding patterns differ between strains of *C. difficile*?
- Why do you think laboratory tests were unable to detect toxin production directly?

9. Patrizia Spigaglia, Fabrizio Barbanti, Anna Maria Dionisi, and Paola Mastrantonio. "*Clostridium difficile* Isolates Resistant to Fluoroquinolones in Italy: Emergence of PCR Ribotype 018." *Journal of Clinical Microbiology 48* no. 8 (2010): 2892–2896.

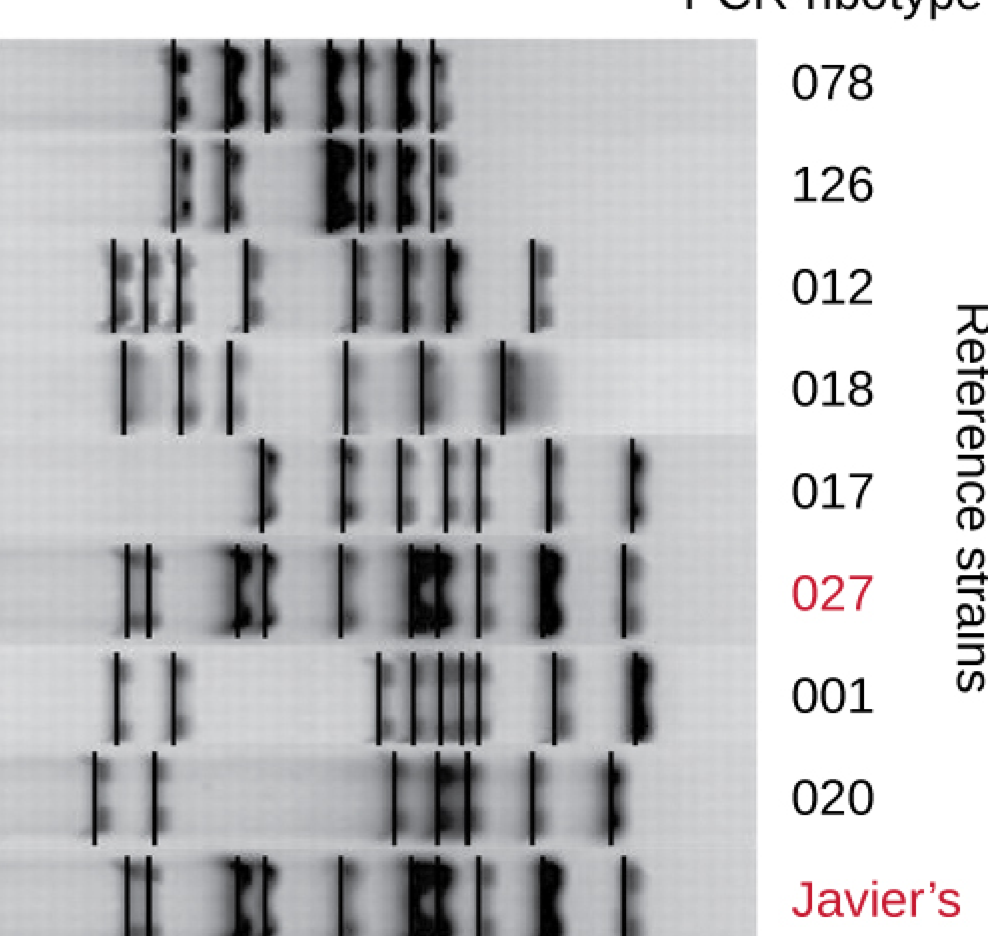


Figure 14.37 A gel showing PCR products of various *Clostridium difficile* strains. Javier's sample is shown at the bottom; note that it matches ribotype 27 in the reference set. (credit: modification of work by American Society for Microbiology)

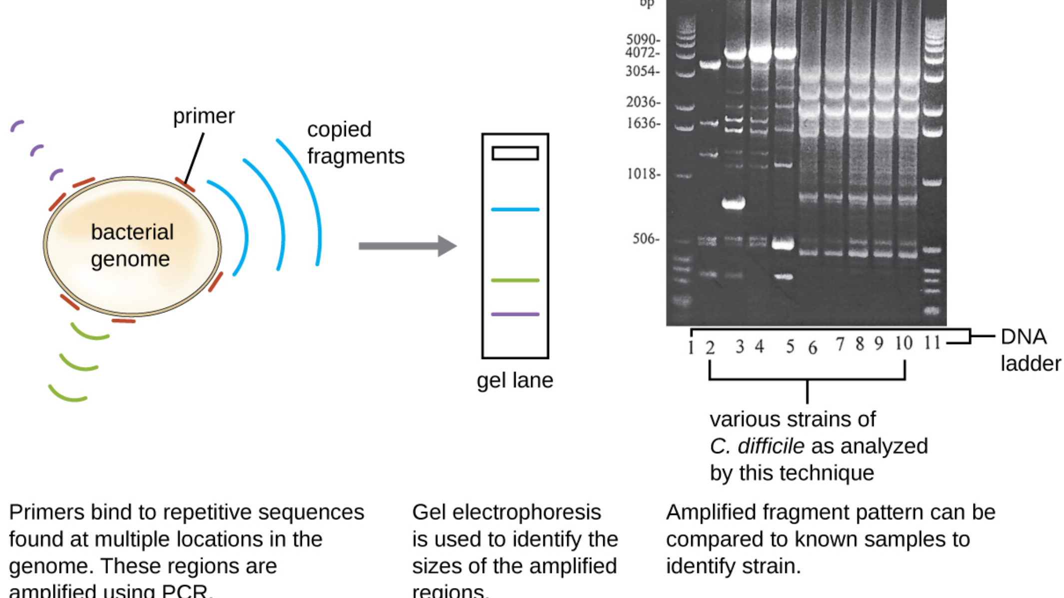


Figure 14.38 Strains of infectious bacteria, such as *C. difficile,* can be identified by molecular analysis. PCR ribotyping is commonly used to identify particular *C. difficile* strains. Rep-PCR is an alternate molecular technique that is also used to identify particular *C. difficile* strains. (credit b: modification of work by American Society for Microbiology)

- How is PCR similar to the natural DNA replication process in cells? How is it different?
- Compare RT-PCR and qPCR in terms of their respective purposes.
- In chain-termination sequencing, how is the identity of each nucleotide in a sequence determined?

14.7 | Genomics and Proteomics

By the end of this section, you will be able to:

- Define genomics and proteomics
- Define whole genome sequencing
- Explain different applications of genomics and proteomics

The study of nucleic acids began with the discovery of DNA, progressed to the study of genes and small fragments, and has now exploded to the field of **genomics**. Genomics is the study of entire genomes, including the complete set of genes, their nucleotide sequence and organization, and their interactions within a species and with other species. The advances in genomics have been made possible by DNA sequencing technology. Just as information technology has led to Google Maps that enable us to get detailed information about locations around the globe, genomic information is used to create similar maps of the DNA of different organisms.

Mapping Genomes

Genome mapping is the process of finding the location of genes on each chromosome. The maps that are created are comparable to the maps that we use to navigate streets. A **genetic map** is an illustration that lists genes and their location on a chromosome. Genetic maps provide the big picture (similar to a map of interstate highways) and use genetic markers (similar to landmarks). A genetic marker is a gene or sequence on a chromosome that shows genetic linkage with a trait of interest. The genetic marker tends to be inherited with the gene of interest, and one measure of distance between them is the recombination frequency during meiosis. Early geneticists called this linkage analysis.

Physical maps get into the intimate details of smaller regions of the chromosomes (similar to a detailed road map) (**Figure 14.39**). A physical map is a representation of the physical distance, in nucleotides, between genes or genetic markers. Both genetic linkage maps and physical maps are required to build a complete picture of the genome. Having a complete map of the genome makes it easier for researchers to study individual genes. Human genome maps help researchers in their efforts to identify human disease-causing genes related to illnesses such as cancer, heart disease, and cystic fibrosis, to name a few. In addition, genome mapping can be used to help identify organisms with beneficial traits, such as microbes with the ability to clean up pollutants or even prevent pollution. Research involving plant genome mapping may lead to methods that produce higher crop yields or to the development of plants that adapt better to climate change.

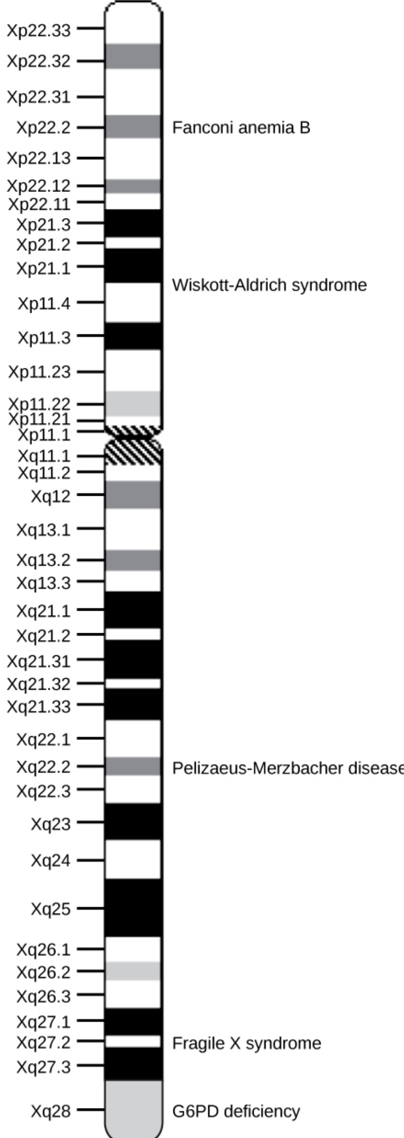


Figure 14.39 This is a physical map of the human X chromosome. (credit: modification of work by NCBI, NIH)

Genetic maps provide the outline, and physical maps provide the details. It is easy to understand why both types of genome-mapping techniques are important to show the big picture. Information obtained from each technique is used in combination to study the genome. Genomic mapping is used with different model organisms that are used for research. Genome mapping is still an ongoing process, and as more advanced techniques are developed, more advances are expected. Genome mapping is similar to completing a complicated puzzle using every piece of available data. Mapping information generated in laboratories all over the world is entered into central databases, such as the National Center for Biotechnology Information (NCBI). Efforts are made to make the information more easily accessible to researchers and the general public. Just as we use global positioning systems instead of paper maps to navigate through roadways, NCBI allows us to use a genome viewer tool to

simplify the data mining process.

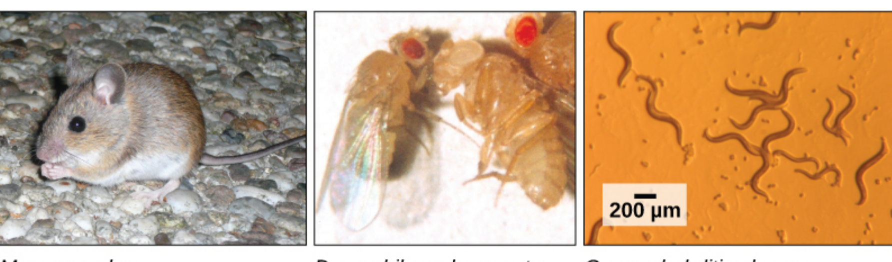
Online Mendelian Inheritance in Man (OMIM) (http://openstax.org/l/OMIM2) is a searchable online catalog of human genes and genetic disorders. This website shows genome mapping, and also details the history and research of each trait and disorder. Click the link to search for traits (such as handedness) and genetic disorders (such as diabetes).

Whole Genome Sequencing

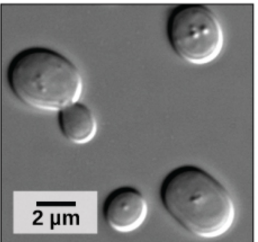
Although there have been significant advances in the medical sciences in recent years, doctors are still confounded by many diseases and researchers are using whole genome sequencing to get to the bottom of the problem. **Whole genome sequencing** is a process that determines the DNA sequence of an entire genome. Whole genome sequencing is a brute-force approach to problem solving when there is a genetic basis at the core of a disease. Several laboratories now provide services to sequence, analyze, and interpret entire genomes.

In 2010, whole genome sequencing was used to save a young boy whose intestines had multiple mysterious abscesses. The child had several colon operations with no relief. Finally, a whole genome sequence revealed a defect in a pathway that controls apoptosis (programmed cell death). A bone marrow transplant was used to overcome this genetic disorder, leading to a cure for the boy. He was the first person to be successfully diagnosed using whole genome sequencing.

The first genomes to be sequenced, such as those belonging to viruses, bacteria, and yeast, were smaller in terms of the number of nucleotides than the genomes of multicellular organisms. The genomes of other model organisms, such as the mouse (*Mus musculus*), the fruit fly (*Drosophila melanogaster*), and the nematode (*Caenorhabditis elegans*) are now known. A great deal of basic research is performed in **model organisms** because the information can be applied to other organisms. A model organism is a species that is studied as a model to understand the biological processes in other species that can be represented by the model organism. For example, fruit flies are able to metabolize alcohol like humans, so the genes affecting sensitivity to alcohol have been studied in fruit flies in an effort to understand the variation in sensitivity to alcohol in humans. Having entire genomes sequenced helps with the research efforts in these model organisms (**Figure 14.40**).



Mus musculus *Drosophila melanogaster* *Caernorhabditis elegans*



Saccharomyces cerevisiae *Arabidopsis thaliana*

Figure 14.40 Much basic research is done with model organisms, such as the mouse, *Mus musculus*; the fruit fly, *Drosophila melanogaster*; the nematode *Caenorhabditis elegans*; the yeast *Saccharomyces cerevisiae*; and the common weed, *Arabidopsis thaliana*. (credit "mouse": modification of work by Florean Fortescue; credit "nematodes": modification of work by "snickclunk"/Flickr; credit "common weed": modification of work by Peggy Greb, USDA; scale-bar data from Matt Russell)

The first human genome sequence was published in 2003. The number of whole genomes that have been sequenced steadily increases and now includes hundreds of species and thousands of individual human genomes.

Applying Genomics

The introduction of DNA sequencing and whole genome sequencing projects, particularly the Human Genome Project, has expanded the applicability of DNA sequence information. Genomics is now being used in a wide variety of fields, such as metagenomics, pharmacogenomics, and mitochondrial genomics. The most commonly known application of genomics is to understand and find cures for diseases.

Predicting Disease Risk at the Individual Level

Predicting the risk of disease involves screening and identifying currently healthy individuals by genome analysis at the individual level. Intervention with lifestyle changes and drugs can be recommended before disease onset. However, this approach is most applicable when the problem arises from a single gene mutation. Such defects only account for about 5 percent of diseases found in developed countries. Most of the common diseases, such as heart disease, are multifactorial or polygenic, which refers to a phenotypic characteristic that is determined by two or more genes, and also environmental factors such as diet. In April 2010, scientists at Stanford University published the genome analysis of a healthy individual (Stephen Quake, a scientist at Stanford University, who had his genome sequenced); the analysis predicted his propensity to acquire various diseases. A risk assessment was done to analyze Quake's percentage of risk for 55 different medical conditions. A rare genetic mutation was found that showed him to be at risk for sudden heart attack. He was also predicted to have a 23 percent risk of developing prostate cancer and a 1.4 percent risk of developing Alzheimer's disease. The scientists used databases and several publications to analyze the genomic data. Even though genomic sequencing is becoming more affordable and analytical tools are becoming more reliable, ethical issues surrounding genomic analysis at a population level remain to be addressed. For example, could such data be legitimately used to charge more or less for insurance or to affect credit ratings?

Genome-wide Association Studies

Since 2005, it has been possible to conduct a type of study called a genome-wide association study, or GWAS. A GWAS is a method that identifies differences between individuals in single nucleotide polymorphisms (SNPs) that may be involved in causing diseases. The method is particularly suited to diseases that may be affected by one or many genetic changes throughout the genome. It is very difficult to identify the genes involved in such a disease using family history information. The GWAS method relies on a genetic database that has been in

development since 2002 called the International HapMap Project. The HapMap Project sequenced the genomes of several hundred individuals from around the world and identified groups of SNPs. The groups include SNPs that are located near to each other on chromosomes so they tend to stay together through recombination. The fact that the group stays together means that identifying one marker SNP is all that is needed to identify all the SNPs in the group. There are several million SNPs identified, but identifying them in other individuals who have not had their complete genome sequenced is much easier because only the marker SNPs need to be identified.

In a common design for a GWAS, two groups of individuals are chosen; one group has the disease, and the other group does not. The individuals in each group are matched in other characteristics to reduce the effect of confounding variables causing differences between the two groups. For example, the genotypes may differ because the two groups are mostly taken from different parts of the world. Once the individuals are chosen, and typically their numbers are a thousand or more for the study to work, samples of their DNA are obtained. The DNA is analyzed using automated systems to identify large differences in the percentage of particular SNPs between the two groups. Often the study examines a million or more SNPs in the DNA. The results of GWAS can be used in two ways: the genetic differences may be used as markers for susceptibility to the disease in undiagnosed individuals, and the particular genes identified can be targets for research into the molecular pathway of the disease and potential therapies. An offshoot of the discovery of gene associations with disease has been the formation of companies that provide so-called “personal genomics” that will identify risk levels for various diseases based on an individual’s SNP complement. The science behind these services is controversial.

Because GWAS looks for associations between genes and disease, these studies provide data for other research into causes, rather than answering specific questions themselves. An association between a gene difference and a disease does not necessarily mean there is a cause-and-effect relationship. However, some studies have provided useful information about the genetic causes of diseases. For example, three different studies in 2005 identified a gene for a protein involved in regulating inflammation in the body that is associated with a disease-causing blindness called age-related macular degeneration. This opened up new possibilities for research into the cause of this disease. A large number of genes have been identified to be associated with Crohn’s disease using GWAS, and some of these have suggested new hypothetical mechanisms for the cause of the disease.

Pharmacogenomics

Pharmacogenomics involves evaluating the effectiveness and safety of drugs on the basis of information from an individual's genomic sequence. Personal genome sequence information can be used to prescribe medications that will be most effective and least toxic on the basis of the individual patient’s genotype. Studying changes in gene expression could provide information about the gene transcription profile in the presence of the drug, which can be used as an early indicator of the potential for toxic effects. For example, genes involved in cellular growth and controlled cell death, when disturbed, could lead to the growth of cancerous cells. Genome-wide studies can also help to find new genes involved in drug toxicity. The gene signatures may not be completely accurate, but can be tested further before pathologic symptoms arise.

Metagenomics

Traditionally, microbiology has been taught with the view that microorganisms are best studied under pure culture conditions, which involves isolating a single type of cell and culturing it in the laboratory. Because microorganisms can go through several generations in a matter of hours, their gene expression profiles adapt to the new laboratory environment very quickly. On the other hand, many species resist being cultured in isolation. Most microorganisms do not live as isolated entities, but in microbial communities known as biofilms. For all of these reasons, pure culture is not always the best way to study microorganisms. **Metagenomics** is the study of the collective genomes of multiple species that grow and interact in an environmental niche. Metagenomics can be used to identify new species more rapidly and to analyze the effect of pollutants on the environment (**Figure 14.41**). Metagenomics techniques can now also be applied to communities of higher eukaryotes, such as fish.

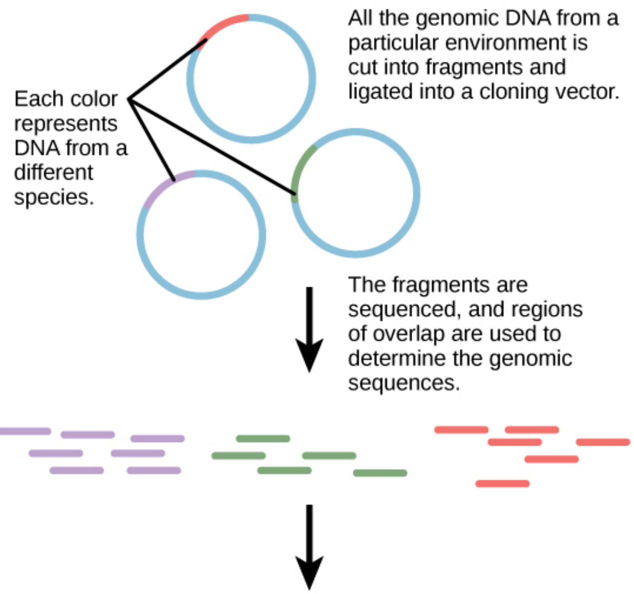


Figure 14.41 Metagenomics involves isolating DNA from multiple species within an environmental niche. The DNA is cut up and sequenced, allowing entire genome sequences of multiple species to be reconstructed from the sequences of overlapping pieces.

Creation of New Biofuels

Knowledge of the genomics of microorganisms is being used to find better ways to harness biofuels from algae and cyanobacteria. The primary sources of fuel today are coal, oil, wood, and other plant products such as ethanol. Although plants are renewable resources, there is still a need to find more alternative renewable sources of energy to meet our population's energy demands. The microbial world is one of the largest resources for genes that encode new enzymes and produce new organic compounds, and it remains largely untapped. This vast genetic resource holds the potential to provide new sources of biofuels (**Figure 14.42**).

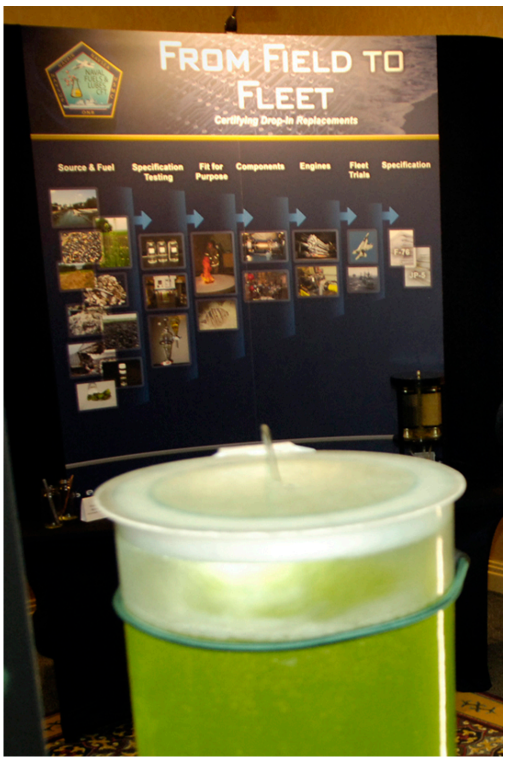


Figure 14.42 Renewable fuels were tested in Navy ships and aircraft at the first Naval Energy Forum. (credit: modification of work by John F. Williams, US Navy)

Mitochondrial Genomics

Mitochondria are intracellular organelles that contain their own DNA. Mitochondrial DNA mutates at a rapid rate and is often used to study evolutionary relationships. Another feature that makes studying the mitochondrial genome interesting is that in most multicellular organisms, the mitochondrial DNA is passed on from the mother during the process of fertilization. For this reason, mitochondrial genomics is often used to trace genealogy.

Genomics in Forensic Analysis

Information and clues obtained from DNA samples found at crime scenes have been used as evidence in court cases, and genetic markers have been used in forensic analysis. Genomic analysis has also become useful in this field. In 2001, the first use of genomics in forensics was published. It was a collaborative effort between academic research institutions and the FBI to solve the mysterious cases of anthrax (**Figure 14.43**) that was transported by the US Postal Service. Anthrax bacteria were made into an infectious powder and mailed to news media and two U.S. Senators. The powder infected the administrative staff and postal workers who opened or handled the letters. Five people died, and 17 were sickened from the bacteria. Using microbial genomics, researchers determined that a specific strain of anthrax was used in all the mailings; eventually, the source was traced to a scientist at a national biodefense laboratory in Maryland.

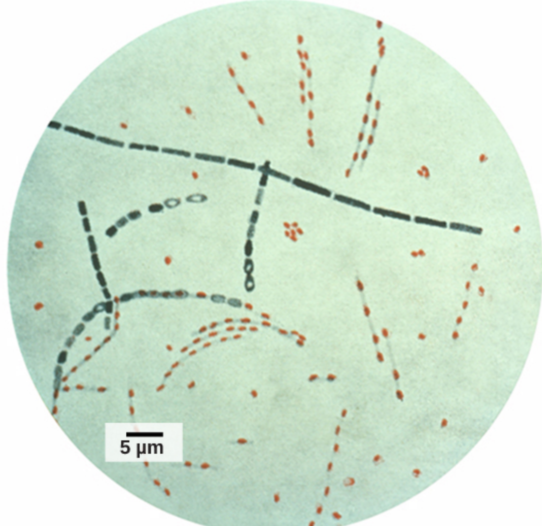


Figure 14.43 *Bacillus anthracis* is the organism that causes anthrax. (credit: modification of work by CDC; scale-bar data from Matt Russell)

Genomics in Agriculture

Genomics can reduce the trials and failures involved in scientific research to a certain extent, which could improve the quality and quantity of crop yields in agriculture (**Figure 14.44**). Linking traits to genes or gene signatures helps to improve crop breeding to generate hybrids with the most desirable qualities. Scientists use genomic data to identify desirable traits, and then transfer those traits to a different organism to create a new genetically modified organism, as described in the previous module. Scientists are discovering how genomics can improve the quality and quantity of agricultural production. For example, scientists could use desirable traits to create a useful product or enhance an existing product, such as making a drought-sensitive crop more tolerant of the dry season.

Figure 14.44 Transgenic agricultural plants can be made to resist disease. These transgenic plums are resistant to the plum pox virus. (credit: Scott Bauer, USDA ARS)

Proteomics

Proteins are the final products of genes that perform the function encoded by the gene. Proteins are composed of amino acids and play important roles in the cell. All enzymes (except ribozymes) are proteins and act as catalysts that affect the rate of reactions. Proteins are also regulatory molecules, and some are hormones. Transport proteins, such as hemoglobin, help transport oxygen to various organs. Antibodies that defend against foreign particles are also proteins. In the diseased state, protein function can be impaired because of changes at the genetic level or because of direct impact on a specific protein.

A proteome is the entire set of proteins produced by a cell type. Proteomes can be studied using the knowledge of genomes because genes code for mRNAs, and the mRNAs encode proteins. The study of the function of proteomes is called **proteomics**. Proteomics complements genomics and is useful when scientists want to test their hypotheses that were based on genes. Even though all cells in a multicellular organism have the same set of genes, the set of proteins produced in different tissues is different and dependent on gene expression. Thus, the genome is constant, but the proteome varies and is dynamic within an organism. In addition, RNAs can be alternatively spliced (cut and pasted to create novel combinations and novel proteins), and many proteins are modified after translation. Although the genome provides a blueprint, the final architecture depends on several factors that can change the progression of events that generate the proteome.

Genomes and proteomes of patients suffering from specific diseases are being studied to understand the genetic basis of the disease. The most prominent disease being studied with proteomic approaches is cancer (**Figure 14.45**). Proteomic approaches are being used to improve the screening and early detection of cancer; this is achieved by identifying proteins whose expression is affected by the disease process. An individual protein is called a **biomarker**, whereas a set of proteins with altered expression levels is called a **protein signature**. For a biomarker or protein signature to be useful as a candidate for early screening and detection of a cancer, it must be secreted in body fluids such as sweat, blood, or urine, so that large-scale screenings can be performed in a noninvasive fashion. The current problem with using biomarkers for the early detection of cancer is the high rate of false-negative results. A false-negative result is a negative test result that should have been positive. In other words, many cases of cancer go undetected, which makes biomarkers unreliable. Some examples of protein biomarkers used in cancer detection are CA-125 for ovarian cancer and PSA for prostate cancer. Protein signatures may be more reliable than biomarkers to detect cancer cells. Proteomics is also being used to develop individualized treatment plans, which involves the prediction of whether or not an individual will respond to specific drugs and the side effects that the individual may have. Proteomics is also being used to predict the possibility of disease recurrence.

Figure 14.45 This machine is preparing to do a proteomic pattern analysis to identify specific cancers so that an accurate cancer prognosis can be made. (credit: Dorie Hightower, NCI, NIH)

The National Cancer Institute has developed programs to improve the detection and treatment of cancer. The Clinical Proteomic Technologies for Cancer and the Early Detection Research Network are efforts to identify protein signatures specific to different types of cancers. The Biomedical Proteomics Program is designed to identify protein signatures and design effective therapies for cancer patients.

14.8 | Gene Therapy

Learning Objectives

By the end of this section, you will be able to:

- Summarize the mechanisms, risks, and potential benefits of gene therapy
- Identify ethical issues involving gene therapy and the regulatory agencies that provide oversight for clinical trials
- Compare somatic-cell and germ-line gene therapy

Many types of genetic engineering have yielded clear benefits with few apparent risks. Few would question, for example, the value of our now abundant supply of human insulin produced by genetically engineered bacteria. However, many emerging applications of genetic engineering are much more controversial, often because their potential benefits are pitted against significant risks, real or perceived. This is certainly the case for **gene therapy**, a clinical application of genetic engineering that may one day provide a cure for many diseases but is still largely an experimental approach to treatment.

Mechanisms and Risks of Gene Therapy

Human diseases that result from genetic mutations are often difficult to treat with drugs or other traditional forms of therapy because the signs and symptoms of disease result from abnormalities in a patient's genome. For example, a patient may have a genetic mutation that prevents the expression of a specific protein required for the normal function of a particular cell type. This is the case in patients with Severe Combined Immunodeficiency (SCID), a genetic disease that impairs the function of certain white blood cells essential to the immune system.

Gene therapy attempts to correct genetic abnormalities by introducing a nonmutated, functional gene into the patient's genome. The nonmutated gene encodes a functional protein that the patient would otherwise be unable to produce. Viral vectors such as adenovirus are sometimes used to introduce the functional gene; part of the viral genome is removed and replaced with the desired gene (Figure 14.46). More advanced forms of gene therapy attempt to correct the mutation at the original site in the genome, such as is the case with treatment of SCID.

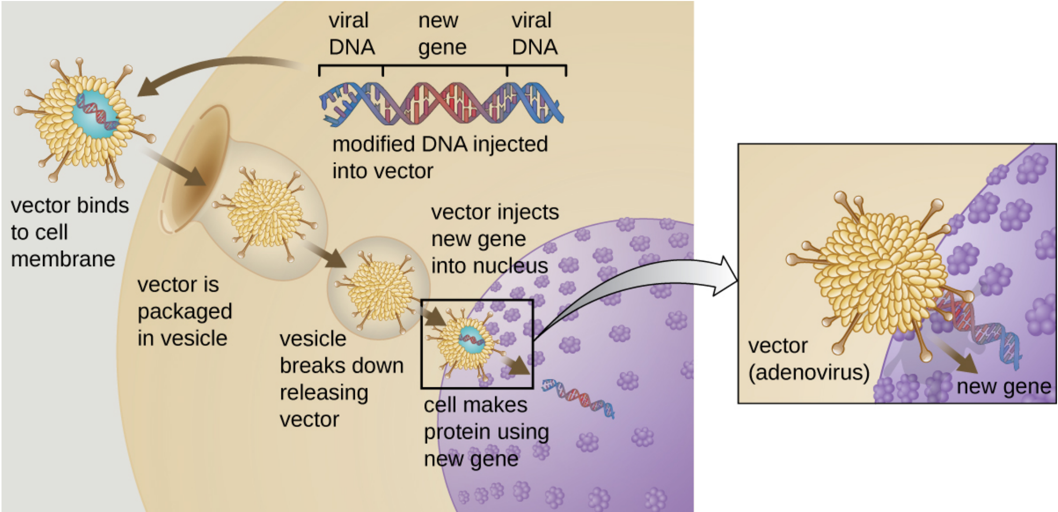


Figure 14.46 Gene therapy using an adenovirus vector can be used to treat or cure certain genetic diseases in which a patient has a defective gene. (credit: modification of work by National Institutes of Health)

So far, gene therapies have proven relatively ineffective, with the possible exceptions of treatments for cystic fibrosis and adenosine deaminase deficiency, a type of SCID. Other trials have shown the clear hazards of attempting genetic manipulation in complex multicellular organisms like humans. In some patients, the use of an adenovirus vector can trigger an unanticipated inflammatory response from the immune system, which may lead to organ failure. Moreover, because viruses can often target multiple cell types, the virus vector may infect cells not targeted for the therapy, damaging these other cells and possibly leading to illnesses such as cancer. Another potential risk is that the modified virus could revert to being infectious and cause disease in the patient. Lastly, there is a risk that the inserted gene could unintentionally inactivate another important gene in the patient's genome, disrupting normal cell cycling and possibly leading to tumor formation and cancer. Because gene therapy involves so many risks, candidates for gene therapy need to be fully informed of these risks before providing informed consent to undergo the therapy.

Gene Therapy Gone Wrong

The risks of gene therapy were realized in the 1999 case of Jesse Gelsinger, an 18-year-old patient who received gene therapy as part of a clinical trial at the University of Pennsylvania. Jesse received gene therapy for a condition called ornithine transcarbamylase (OTC) deficiency, which leads to ammonia accumulation in the blood due to deficient ammonia processing. Four days after the treatment, Jesse died after a massive immune response to the adenovirus vector.[10]

Until that point, researchers had not really considered an immune response to the vector to be a legitimate risk, but on investigation, it appears that the researchers had some evidence suggesting that this was a possible outcome. Prior to Jesse's treatment, several other human patients had suffered side effects of the treatment, and three monkeys used in a trial had died as a result of inflammation and clotting disorders. Despite this information, it appears that neither Jesse nor his family were made aware of these outcomes when they consented to the therapy. Jesse's death was the first patient death due to a gene therapy treatment and resulted in the immediate halting of the clinical trial in which he was involved, the subsequent halting of all other gene therapy trials at the University of Pennsylvania, and the investigation of all other gene therapy trials in the United States. As a result, the regulation and oversight of gene therapy overall was reexamined, resulting in new regulatory protocols that are still in place today.

- Explain how gene therapy works in theory.
- Identify some risks of gene therapy.

10. Barbara Sibbald. "Death but One Unintended Consequence of Gene-Therapy Trial." *Canadian Medical Association Journal 164* no. 11 (2001): 1612–1612.

Oversight of Gene Therapy

Presently, there is significant oversight of gene therapy clinical trials. At the federal level, three agencies regulate gene therapy in parallel: the Food and Drug Administration (FDA), the Office of Human Research Protection (OHRP), and the Recombinant DNA Advisory Committee (RAC) at the National Institutes of Health (NIH). Along with several local agencies, these federal agencies interact with the institutional review board to ensure that protocols are in place to protect patient safety during clinical trials. Compliance with these protocols is enforced mostly on the local level in cooperation with the federal agencies. Gene therapies are currently under the most extensive federal and local review compared to other types of therapies, which are more typically only under the review of the FDA. Some researchers believe that these extensive regulations actually inhibit progress in gene therapy research. In 2013, the Institute of Medicine (now the National Academy of Medicine) called upon the NIH to relax its review of gene therapy trials in most cases.[11] However, ensuring patient safety continues to be of utmost concern.

Ethical Concerns

Beyond the health risks of gene therapy, the ability to genetically modify humans poses a number of ethical issues related to the limits of such "therapy." While current research is focused on gene therapy for genetic diseases, scientists might one day apply these methods to manipulate other genetic traits not perceived as desirable. This raises questions such as:

- Which genetic traits are worthy of being "corrected"?
- Should gene therapy be used for cosmetic reasons or to enhance human abilities?
- Should genetic manipulation be used to impart desirable traits to the unborn?
- Is everyone entitled to gene therapy, or could the cost of gene therapy create new forms of social inequality?
- Who should be responsible for regulating and policing inappropriate use of gene therapies?

The ability to alter reproductive cells using gene therapy could also generate new ethical dilemmas. To date, the various types of gene therapies have been targeted to somatic cells, the non-reproductive cells within the body. Because somatic cell traits are not inherited, any genetic changes accomplished by somatic-cell gene therapy would not be passed on to offspring. However, should scientists successfully introduce new genes to germ cells (eggs or sperm), the resulting traits could be passed on to offspring. This approach, called germ-line gene therapy, could potentially be used to combat heritable diseases, but it could also lead to unintended consequences for future generations. Moreover, there is the question of informed consent, because those impacted by germ-line gene therapy are unborn and therefore unable to choose whether they receive the therapy. For these reasons, the U.S. government does not currently fund research projects investigating germ-line gene therapies in humans.

Risky Gene Therapies

While there are currently no gene therapies on the market in the United States, many are in the pipeline and it is likely that some will eventually be approved. With recent advances in gene therapies targeting p53, a gene whose somatic cell mutations have been implicated in over 50% of human cancers,[12] cancer treatments through gene therapies could become much more widespread once they reach the commercial market.

Bringing any new therapy to market poses ethical questions that pit the expected benefits against the risks. How quickly should new therapies be brought to the market? How can we ensure that new therapies have been sufficiently tested for safety and effectiveness before they are marketed to the public? The process by which new therapies are developed and approved complicates such questions, as those involved in the approval process are often under significant pressure to get a new therapy approved even in the face of significant risks.

To receive FDA approval for a new therapy, researchers must collect significant laboratory data from animal trials and submit an Investigational New Drug (IND) application to the FDA's Center for Drug Evaluation and Research (CDER). Following a 30-day waiting period during which the FDA reviews the IND, clinical trials involving human subjects may begin. If the FDA perceives a problem prior to or during the clinical trial, the FDA can order a "clinical hold" until any problems are addressed. During clinical trials, researchers

11. Kerry Grens. "Report: Ease Gene Therapy Reviews." *The Scientist*, December 9, 2013. http://www.the-scientist.com/?articles.view/articleNo/38577/title/Report--Ease-Gene-Therapy-Reviews/. Accessed May 27, 2016.
12. Zhen Wang and Yi Sun. "Targeting p53 for Novel Anticancer Therapy." *Translational Oncology 3*, no. 1 (2010): 1–12.

collect and analyze data on the therapy's effectiveness and safety, including any side effects observed. Once the therapy meets FDA standards for effectiveness and safety, the developers can submit a New Drug Application (NDA) that details how the therapy will be manufactured, packaged, monitored, and administered.

Because new gene therapies are frequently the result of many years (even decades) of laboratory and clinical research, they require a significant financial investment. By the time a therapy has reached the clinical trials stage, the financial stakes are high for pharmaceutical companies and their shareholders. This creates potential conflicts of interest that can sometimes affect the objective judgment of researchers, their funders, and even trial participants. The Jesse Gelsinger case (see **Case in Point: Gene Therapy Gone Wrong**) is a classic example. Faced with a life-threatening disease and no reasonable treatments available, it is easy to see why a patient might be eager to participate in a clinical trial no matter the risks. It is also easy to see how a researcher might view the short-term risks for a small group of study participants as a small price to pay for the potential benefits of a game-changing new treatment.

Gelsinger's death led to increased scrutiny of gene therapy, and subsequent negative outcomes of gene therapy have resulted in the temporary halting of clinical trials pending further investigation. For example, when children in France treated with gene therapy for SCID began to develop leukemia several years after treatment, the FDA temporarily stopped clinical trials of similar types of gene therapy occurring in the United States.[13] Cases like these highlight the need for researchers and health professionals not only to value human well-being and patients' rights over profitability, but also to maintain scientific objectivity when evaluating the risks and benefits of new therapies.

- Why is gene therapy research so tightly regulated?
- What is the main ethical concern associated with germ-line gene therapy?

13. Erika Check. "Gene Therapy: A Tragic Setback." *Nature 420* no. 6912 (2002): 116–118.

KEY TERMS

biomarker an individual protein that is uniquely produced in a diseased state

genetic map an outline of genes and their location on a chromosome that is based on recombination frequencies between markers

genomics the study of entire genomes, including the complete set of genes, their nucleotide sequence and organization, and their interactions within a species and with other species

metagenomics the study of the collective genomes of multiple species that grow and interact in an environmental niche

model organism a species that is studied and used as a model to understand the biological processes in other species represented by the model organism

pharmacogenomics the study of drug interactions with the genome or proteome; also called toxicogenomics

physical map a representation of the physical distance between genes or genetic markers

protein signature a set of over- or under-expressed proteins characteristic of cells in a particular diseased tissue

proteomics study of the function of proteomes

whole genome sequencing a process that determines the nucleotide sequence of an entire genome

CHAPTER SUMMARY

14.1 Tools of Genetic Engineering

- **Biotechology** is the science of utilizing living systems to benefit humankind. In recent years, the ability to directly alter an organism's genome through **genetic engineering** has been made possible due to advances in **recombinant DNA technology,** which allows researchers to create **recombinant DNA molecules** with new combinations of genetic material.
- **Molecular cloning** involves methods used to construct recombinant DNA and facilitate their replication in host organisms. These methods include the use of **restriction enzymes** (to cut both foreign DNA and **plasmid vectors)**, **ligation** (to paste fragments of DNA together), and the introduction of recombinant DNA into a host organism (often bacteria).
- **Blue-white screening** allows selection of bacterial transformants that contain recombinant plasmids using the phenotype of a **reporter gene** that is disabled by insertion of the DNA fragment.
- **Genomic libraries** can be made by cloning genomic fragments from one organism into plasmid vectors or into bacteriophage.
- **cDNA libraries** can be generated to represent the mRNA molecules expressed in a cell at a given point.
- **Transfection** of eukaryotic hosts can be achieved through various methods using **electroporation**, **gene guns**, **microinjection**, **shuttle vectors**, and **viral vectors**.

14.2 Pharmaceutical Applications of Genetic Engineering

- The science of **genomics** allows researchers to study organisms on a holistic level and has many applications of medical relevance.
- **Transcriptomics** and **proteomics** allow researchers to compare gene expression patterns between different cells and shows great promise in better understanding global responses to various conditions.
- The various –omics technologies complement each other and together provide a more complete picture of an organism's or microbial community's (**metagenomics**) state.

- The analysis required for large data sets produced through genomics, transcriptomics, and **proteomics** has led to the emergence of **bioinformatics**.
- **Reporter genes** encoding easily observable characteristics are commonly used to track gene expression patterns of genes of unknown function.
- The use of recombinant DNA technology has revolutionized the pharmaceutical industry, allowing for the rapid production of high-quality **recombinant DNA pharmaceuticals** used to treat a wide variety of human conditions.
- **RNA interference** technology has great promise as a method of treating viral infections by silencing the expression of specific genes

14.5 Advanced Chromatography Applications

- Accurate identification of bacteria is essential in a clinical laboratory for diagnostic and management of epidemics, pandemics, and food poisoning caused by bacterial outbreaks.
- The phenotypic identification of microorganisms involves using observable traits, including profiles of structural components such as lipids, biosynthetic products such as sugars or amino acids, or storage compounds such as poly-β-hydroxybutyrate.
- An unknown microbe may be identified from the unique mass spectrum produced when it is analyzed by **matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF)**.
- Microbes can be identified by determining their lipid compositions, using **fatty acid methyl esters** (**FAME**) or **phospholipid-derived fatty acids** (**PLFA**) **analysis**.
- **Proteomic analysis**, the study of all accumulated proteins of an organism; can also be used for bacterial identification.
- Glycoproteins in the plasma membrane or cell wall structures can bind to lectins or antibodies and can be used for identification.

14.6 Visualizing and Characterizing DNA, RNA, and Protein

- Finding a gene of interest within a sample requires the use of a single-stranded **DNA probe** labeled with a molecular beacon (typically radioactivity or fluorescence) that can hybridize with a complementary single-stranded nucleic acid in the sample.
- **Agarose gel electrophoresis** allows for the separation of DNA molecules based on size.
- **Restriction fragment length polymorphism (RFLP)** analysis allows for the visualization by agarose gel electrophoresis of distinct variants of a DNA sequence caused by differences in restriction sites.
- **Southern blot** analysis allows researchers to find a particular DNA sequence within a sample whereas **northern blot** analysis allows researchers to detect a particular mRNA sequence expressed in a sample.
- **Microarray technology** is a nucleic acid hybridization technique that allows for the examination of many thousands of genes at once to find differences in genes or gene expression patterns between two samples of genomic DNA or cDNA,
- **Polyacrylamide gel electrophoresis (PAGE)** allows for the separation of proteins by size, especially if native protein charges are masked through pretreatment with SDS.
- **Polymerase chain reaction** allows for the rapid amplification of a specific DNA sequence. Variations of PCR can be used to detect mRNA expression (**reverse transcriptase PCR**) or to quantify a particular sequence in the original sample **(real-time PCR**).
- Although the development of **Sanger DNA sequencing** was revolutionary, advances in **next generation sequencing** allow for the rapid and inexpensive sequencing of the genomes of many organisms, accelerating the volume of new sequence data.

14.7 Genomics and Proteomics

Genome mapping is similar to solving a big, complicated puzzle with pieces of information coming from laboratories all over the world. Genetic maps provide an outline for the location of genes within a genome, and they estimate the distance between genes and genetic markers on the basis of the recombination frequency during meiosis. Physical maps provide detailed information about the physical distance between the genes. The most detailed information is available through sequence mapping. Information from all mapping and sequencing sources is combined to study an entire genome.

Whole genome sequencing is the latest available resource to treat genetic diseases. Some doctors are using whole genome sequencing to save lives. Genomics has many industrial applications, including biofuel development, agriculture, pharmaceuticals, and pollution control.

Imagination is the only barrier to the applicability of genomics. Genomics is being applied to most fields of biology; it can be used for personalized medicine, prediction of disease risks at an individual level, the study of drug interactions before the conduction of clinical trials, and the study of microorganisms in the environment as opposed to the laboratory. It is also being applied to the generation of new biofuels, genealogical assessment using mitochondria, advances in forensic science, and improvements in agriculture.

Proteomics is the study of the entire set of proteins expressed by a given type of cell under certain environmental conditions. In a multicellular organism, different cell types will have different proteomes, and these will vary with changes in the environment. Unlike a genome, a proteome is dynamic and under constant flux, which makes it more complicated and more useful than the knowledge of genomes alone.

14.8 Gene Therapy

- While gene therapy shows great promise for the treatment of genetic diseases, there are also significant risks involved.
- There is considerable federal and local regulation of the development of gene therapies by pharmaceutical companies for use in humans.
- Before gene therapy use can increase dramatically, there are many ethical issues that need to be addressed by the medical and research communities, politicians, and society at large.

REVIEW QUESTIONS

1. Which of the following is required for repairing the phosphodiester backbone of DNA during molecular cloning?

a. cDNA
b. reverse transcriptase
c. restriction enzymes
d. DNA ligase

2. All of the following are processes used to introduce DNA molecules into bacterial cells *except*:

a. transformation
b. transduction
c. transcription
d. conjugation

3. The enzyme that uses RNA as a template to produce a DNA copy is called:

a. a restriction enzyme
b. DNA ligase
c. reverse transcriptase
d. DNA polymerase

4. In blue-white screening, what do blue colonies represent?

a. cells that have not taken up the plasmid vector
b. cells with recombinant plasmids containing a new insert
c. cells containing empty plasmid vectors
d. cells with a non-functional *lacZ* gene

5. The T_i plasmid is used for introducing genes into:

a. animal cells
b. plant cells
c. bacteriophages
d. *E. coli* cells

6. The science of studying the entire collection of mRNA molecules produced by cells, allowing scientists to monitor differences in gene expression patterns between cells, is called:

a. genomics
b. transcriptomics
c. proteomics
d. pharmacogenomics

7. The science of studying genomic fragments from microbial communities, allowing researchers to study genes from a collection of multiple species, is called:

a. pharmacogenomics
b. transcriptomics
c. metagenomics
d. proteomics

8. The insulin produced by recombinant DNA technology is
a. a combination of *E. coli* and human insulin.
b. identical to human insulin produced in the pancreas.
c. cheaper but less effective than pig insulin for treating diabetes.
d. engineered to be more effective than human insulin.

9. Which of the following characteristics/compounds is not considered to be a phenotypic biochemical characteristic used of microbial identification?

A. poly-β-hydroxybutyrate
B. small-subunit (16S) rRNA gene
C. carbon utilization
D. lipid composition

10. Proteomic analysis is a methodology that deals with which of the following?
A. the analysis of proteins functioning as enzymes within the cell
B. analysis of transport proteins in the cell
C. the analysis of integral proteins of the cell membrane
D. the study of all accumulated proteins of an organism

11. Which method involves the generation of gas phase ions from intact microorganisms?
A. FAME
B. PLFA
C. MALDI-TOF
D. Lancefield group testing

12. Which method involves the analysis of membrane-bound carbohydrates?
A. FAME
B. PLFA
C. MALDI-TOF
D. Lancefield group testing

13. Which method involves conversion of a microbe's lipids to volatile compounds for analysis by gas chromatography?
A. FAME
B. proteomic analysis
C. MALDI-TOF
D. Lancefield group testing

14. Which technique is used to separate protein fragments based on size?
a. polyacrylamide gel electrophoresis
b. Southern blot
c. agarose gel electrophoresis
d. polymerase chain reaction

15. Which technique uses restriction enzyme digestion followed by agarose gel electrophoresis to generate a banding pattern for comparison to another sample processed in the same way?
a. qPCR
b. RT-PCR
c. RFLP
d. 454 sequencing

16. All of the following techniques involve hybridization between single-stranded nucleic acid molecules *except*:
a. Southern blot analysis
b. RFLP analysis
c. northern blot analysis
d. microarray analysis

17. What is the most challenging issue facing genome sequencing?
a. the inability to develop fast and accurate sequencing techniques
b. the ethics of using information from genomes at the individual level
c. the availability and stability of DNA
d. all of the above

18. Genomics can be used in agriculture to:
a. generate new hybrid strains
b. improve disease resistance
c. improve yield
d. all of the above

19. What kind of diseases are studied using genome-wide association studies?
a. viral diseases
b. single-gene inherited diseases
c. diseases caused by multiple genes
d. diseases caused by environmental factors

20. At what point can the FDA halt the development or use of gene therapy?
a. on submission of an IND application
b. during clinical trials
c. after manufacturing and marketing of the approved therapy
d. all of the answers are correct

TRUE/FALSE

14.1 Tools of Genetic Engineering

21. Recombination is a process not usually observed in nature.

22. It is generally easier to introduce recombinant DNA into prokaryotic cells than into eukaryotic cells.

14.2 Pharmaceutical Applications of Genetic Engineering

23. RNA interference does not influence the

sequence of genomic DNA.

14.5 Advanced Chromatography Applications

24. MALDI-TOF relies on obtaining a unique mass spectrum for the bacteria tested and then checking the acquired mass spectrum against the spectrum databases registered in the analysis software to identify the microorganism.

25. Lancefield group tests can identify microbes using antibodies that specifically bind cell-surface proteins.

14.6 Visualizing and Characterizing DNA, RNA, and Protein

26. In agarose gel electrophoresis, DNA will be attracted to the negative electrode.

FILL IN THE BLANK

14.1 Tools of Genetic Engineering

27. The process of introducing DNA molecules into eukaryotic cells is called ________.

14.2 Pharmaceutical Applications of Genetic Engineering

28. The application of genomics to evaluate the effectiveness and safety of drugs on the basis of information from an individual's genomic sequence is called ____________.

29. A gene whose expression can be easily visualized and monitored is called a ________.

14.5 Advanced Chromatography Applications

30. A FAME analysis involves the conversion of _______ to more volatile _____ for analysis using ____________.

14.6 Visualizing and Characterizing DNA, RNA, and Protein

31. The __________ blot technique is used to find an RNA fragment within a sample that is complementary to a DNA probe.

32. The PCR step during which the double-stranded template molecule becomes single-stranded is called _____________.

33. The sequencing method involving the incorporation of ddNTPs is called __________.

14.8 Gene Therapy

34. _____________ is a common viral vector used in gene therapy for introducing a new gene into a specifically targeted cell type.

SHORT ANSWER

14.1 Tools of Genetic Engineering

35. Name three elements incorporated into a plasmid vector for efficient cloning.

36. When would a scientist want to generate a cDNA library instead of a genomic library?

37. What is one advantage of generating a genomic library using phages instead of plasmids?

14.2 Pharmaceutical Applications of Genetic Engineering

38. If all cellular proteins are encoded by the cell's genes, what information does proteomics provide that genomics cannot?

14.5 Advanced Chromatography Applications

39. Compare MALDI-TOF, FAME, and PLFA, and explain how each technique would be used to identify pathogens.

14.6 Visualizing and Characterizing DNA, RNA, and Protein

40. Why is it important that a DNA probe be labeled with a molecular beacon?

41. When separating proteins strictly by size, why is exposure to SDS first required?

42. Why must the DNA polymerase used during PCR be heat-stable?

14.8 Gene Therapy

43. Briefly describe the risks associated with somatic cell gene therapy.

CRITICAL THINKING

44. Is biotechnology always associated with genetic engineering? Explain your answer.

45. Which is more efficient: blunt-end cloning or sticky-end cloning? Why?

46. What are some advantages of cloning human genes into bacteria to treat human diseases caused by specific protein deficiencies?

47. Suppose you are working in a molecular biology laboratory and are having difficulty performing the PCR successfully. You decide to double-check the PCR protocol programmed into the thermal cycler and discover that the annealing temperature was

programmed to be 65 °C instead of 50 °C, as you had intended. What effects would this mistake have on the PCR reaction? Refer to **Figure 14.33**.

48. What is the advantage of microarray analysis over northern blot analysis in monitoring changes in gene expression?

49. What is the difference between reverse transcriptase PCR (RT-PCR) and real-time quantitative PCR (qPCR)?

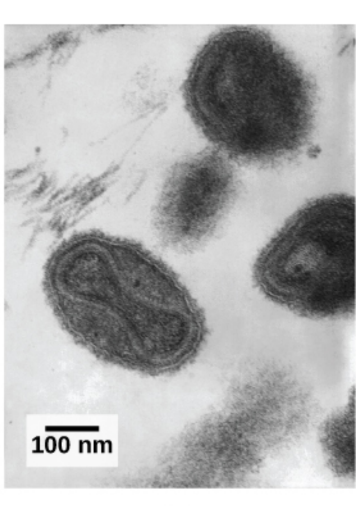
50. Describe two of the applications for genome mapping.

51. Identify a possible advantage and a possible disadvantage of a genetic test that would identify genes in individuals that increase their probability of having Alzheimer's disease later in life.

52. Compare the ethical issues involved in the use of somatic cell gene therapy and germ-line gene therapy.

15 | VIRUSES, VACCINES, AND THE IMMUNE SYSTEM

(a)



(b)

Figure 15.1 (a) This smallpox (variola) vaccine is derived from calves exposed to cowpox virus. Vaccines provoke a reaction in the immune system that prepares it for a subsequent infection by smallpox. (b) Viewed under a transmission electron microscope, you can see the variola's dumbbell-shaped structure that contains the viral DNA. (credit a: modification of work by James Gathany, CDC; credit b: modification of work by Dr. Fred Murphy; Sylvia Whitfield, CDC; scale-bar data from Matt Russell)

Chapter Outline

15.1: Viruses
15.2: Innate Immunity
15.3: Adaptive Immunity
15.4: Isolation, Culture, and Identification of Viruses
15.5: Vaccines

Introduction

Organisms have a wide array of adaptations for preventing attacks of parasites and diseases. The vertebrate defense systems, including those of humans, are complex and multilayered, with defenses unique to vertebrates. These unique vertebrate defenses interact with other defense systems inherited from ancestral lineages, and include complex and specific pathogen recognition and memory mechanisms. Research continues to unravel the complexities and vulnerabilities of the immune system.

Despite a poor understanding of the workings of the body in the early 18th century in Europe, the practice of inoculation as a method to prevent the often-deadly effects of smallpox was introduced from the courts of the Ottoman Empire. The method involved causing limited infection with the smallpox virus by introducing the pus of an affected individual to a scratch in an uninfected person. The resulting infection was milder than if it had been caught naturally and mortality rates were shown to be about two percent rather than 30 percent from natural infections. Moreover, the inoculation gave the individual immunity to the disease. It was from these early

experiences with inoculation that the methods of vaccination were developed, in which a weakened or relatively harmless (killed) derivative of a pathogen is introduced into the individual. The vaccination induces immunity to the disease with few of the risks of being infected. A modern understanding of the causes of the infectious disease and the mechanisms of the immune system began in the late 19th century and continues to grow today.

15.1 | Viruses

By the end of this section, you will be able to:

- Describe how viruses were first discovered and how they are detected
- Explain the detailed steps of viral replication
- Describe how vaccines are used in prevention and treatment of viral diseases

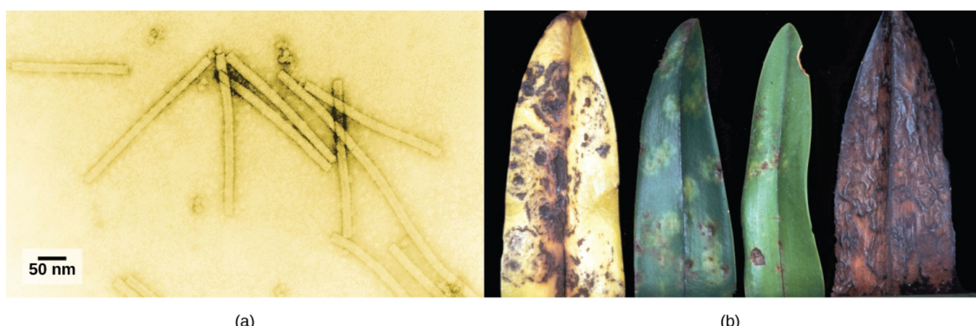


Figure 15.2 (a) The tobacco mosaic virus, seen by transmission electron microscopy, was the first virus to be discovered. (b) The leaves of an infected plant are shown. (credit a: scale-bar data from Matt Russell; credit b: modification of work by USDA, Department of Plant Pathology Archive, North Carolina State University)

No one knows exactly when viruses emerged or from where they came, since viruses do not leave historical footprints such as fossils. Modern viruses are thought to be a mosaic of bits and pieces of nucleic acids picked up from various sources along their respective evolutionary paths. Viruses are **acellular**, parasitic entities that are not classified within any domain because they are not considered alive. They have no plasma membrane, internal organelles, or metabolic processes, and they do not divide. Instead, they infect a host cell and use the host's replication processes to produce progeny virus particles. Viruses infect all forms of organisms including bacteria, archaea, fungi, plants, and animals. Living things grow, metabolize, and reproduce. Viruses replicate, but to do so, they are entirely dependent on their host cells. They do not metabolize or grow, but are assembled in their mature form.

Viruses are diverse. They vary in their structure, their replication methods, and in their target hosts or even host cells. While most biological diversity can be understood through evolutionary history, such as how species have adapted to conditions and environments, much about virus origins and evolution remains unknown.

How Viruses Replicate

Viruses were first discovered after the development of a porcelain filter, called the Chamberland-Pasteur filter, which could remove all bacteria visible under the microscope from any liquid sample. In 1886, Adolph Meyer demonstrated that a disease of tobacco plants, tobacco mosaic disease, could be transferred from a diseased plant to a healthy one through liquid plant extracts. In 1892, Dmitri Ivanowski showed that this disease could be transmitted in this way even after the Chamberland-Pasteur filter had removed all viable bacteria from the extract. Still, it was many years before it was proven that these "filterable" infectious agents were not simply very small bacteria but were a new type of tiny, disease-causing particle.

Virions, single virus particles, are very small, about 20–250 nanometers (1 nanometer = 1/1,000,000 mm). These individual virus particles are the infectious form of a virus outside the host cell. Unlike bacteria (which are about 100 times larger), we cannot see viruses with a light microscope, with the exception of some large virions of the poxvirus family (Figure 15.3).

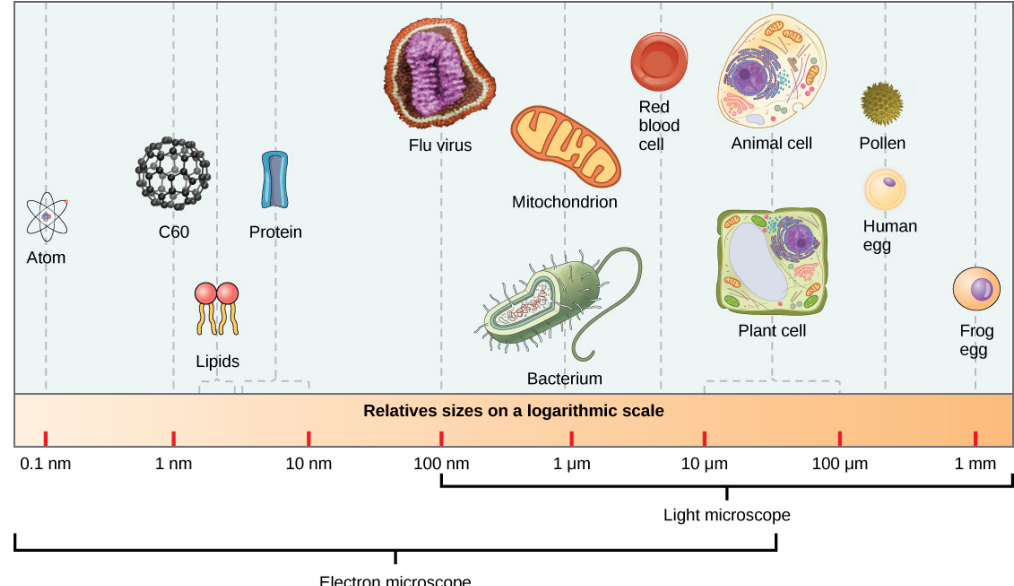


Figure 15.3 The size of a virus is very small relative to the size of cells and organelles.

It was not until the development of the electron microscope in the 1940s that scientists got their first good view of the structure of the tobacco mosaic virus (**Figure 15.2**) and others. The surface structure of virions can be observed by both scanning and transmission electron microscopy, whereas the internal structures of the virus can only be observed in images from a transmission electron microscope (**Figure 15.4**).

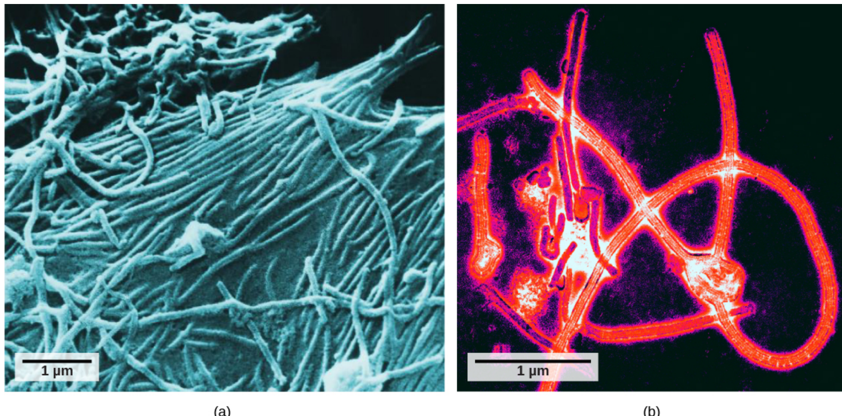


Figure 15.4 The ebola virus is shown here as visualized through (a) a scanning electron micrograph and (b) a transmission electron micrograph. (credit a: modification of work by Cynthia Goldsmith, CDC; credit b: modification of work by Thomas W. Geisbert, Boston University School of Medicine; scale-bar data from Matt Russell)

The use of this technology has allowed for the discovery of many viruses of all types of living organisms. They were initially grouped by shared morphology, meaning their size, shape, and distinguishing structures. Later, groups of viruses were classified by the type of nucleic acid they contained, DNA or RNA, and whether their nucleic acid was single- or double-stranded. More recently, molecular analysis of viral replication cycles has further refined their classification.

A **virion** consists of a nucleic-acid core, an outer protein coating, and sometimes an outer envelope made of protein and phospholipid membranes derived from the host cell. The most visible difference between members of viral families is their morphology, which is quite diverse. An interesting feature of viral complexity is that the complexity of the host does not correlate to the complexity of the virion. Some of the most complex virion structures are observed in bacteriophages, viruses that infect the simplest living organisms, bacteria.

Viruses come in many shapes and sizes, but these are consistent and distinct for each viral family (**Figure 15.5**). All virions have a nucleic-acid genome covered by a protective layer of protein, called a **capsid**. The capsid is made of protein subunits called capsomeres. Some viral capsids are simple polyhedral "spheres," whereas others are quite complex in structure. The outer structure surrounding the capsid of some viruses is called the

viral envelope. All viruses use some sort of **glycoprotein** to attach to their host cells at molecules on the cell called viral receptors. The virus exploits these cell-surface molecules, which the cell uses for some other purpose, as a way to recognize and infect specific cell types. For example, the measles virus uses a cell-surface glycoprotein in humans that normally functions in immune reactions and possibly in the sperm-egg interaction at fertilization. Attachment is a requirement for viruses to later penetrate the cell membrane, inject the viral genome, and complete their replication inside the cell.

The T4 bacteriophage, which infects the *E. coli* bacterium, is among the most complex virion known; T4 has a protein tail structure that the virus uses to attach to the host cell and a head structure that houses its DNA.

Adenovirus, a nonenveloped animal virus that causes respiratory illnesses in humans, uses protein spikes protruding from its capsomeres to attach to the host cell. Nonenveloped viruses also include those that cause polio (poliovirus), plantar warts (papillomavirus), and hepatitis A (hepatitis A virus). Nonenveloped viruses tend to be more robust and more likely to survive under harsh conditions, such as the gut.

Enveloped virions like HIV (human immunodeficiency virus), the causative agent in AIDS (acquired immune deficiency syndrome), consist of nucleic acid (RNA in the case of HIV) and capsid proteins surrounded by a phospholipid bilayer envelope and its associated proteins (Figure 15.5). Chicken pox, influenza, and mumps are examples of diseases caused by viruses with envelopes. Because of the fragility of the envelope, nonenveloped viruses are more resistant to changes in temperature, pH, and some disinfectants than enveloped viruses.

Overall, the shape of the virion and the presence or absence of an envelope tells us little about what diseases the viruses may cause or what species they might infect, but is still a useful means to begin viral classification.

Visual Connection

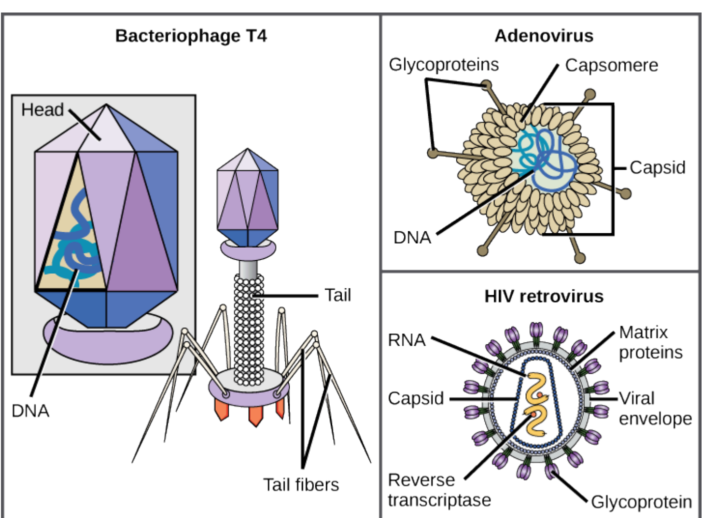


Figure 15.5 Viruses can be complex in shape or relatively simple. This figure shows three relatively complex virions: the bacteriophage T4, with its DNA-containing head group and tail fibers that attach to host cells; adenovirus, which uses spikes from its capsid to bind to the host cells; and HIV, which uses glycoproteins embedded in its envelope to do so. Notice that HIV has proteins called matrix proteins, internal to the envelope, which help stabilize virion shape. HIV is a retrovirus, which means it reverse transcribes its RNA genome into DNA, which is then spliced into the host's DNA. (credit "bacteriophage, adenovirus": modification of work by NCBI, NIH; credit "HIV retrovirus": modification of work by NIAID, NIH)

Which of the following statements about virus structure is true?

a. All viruses are encased in a viral membrane.
b. The capsomere is made up of small protein subunits called capsids.
c. DNA is the genetic material in all viruses.
d. Glycoproteins help the virus attach to the host cell.

Unlike all living organisms that use DNA as their genetic material, viruses may use either DNA or RNA as theirs. The virus core contains the genome or total genetic content of the virus. Viral genomes tend to be small compared to bacteria or eukaryotes, containing only those genes that code for proteins the virus cannot get

from the host cell. This genetic material may be single-stranded or double-stranded. It may also be linear or circular. While most viruses contain a single segment of nucleic acid, others have genomes that consist of several segments.

DNA viruses have a DNA core. The viral DNA directs the host cell's replication proteins to synthesize new copies of the viral genome and to transcribe and translate that genome into viral proteins. DNA viruses cause human diseases such as chickenpox, hepatitis B, and some venereal diseases like herpes and genital warts.

RNA viruses contain only RNA in their cores. To replicate their genomes in the host cell, the genomes of RNA viruses encode enzymes not found in host cells. RNA polymerase enzymes are not as stable as DNA polymerases and often make mistakes during transcription. For this reason, mutations, changes in the nucleotide sequence, in RNA viruses occur more frequently than in DNA viruses. This leads to more rapid evolution and change in RNA viruses. For example, the fact that influenza is an RNA virus is one reason a new flu vaccine is needed every year. Human diseases caused by RNA viruses include hepatitis C, measles, and rabies.

Viruses can be seen as obligate intracellular parasites. The virus must attach to a living cell, be taken inside, manufacture its proteins and copy its genome, and find a way to escape the cell so the virus can infect other cells and ultimately other individuals. Viruses can infect only certain species of hosts and only certain cells within that host. The molecular basis for this specificity is that a particular surface molecule, known as the viral receptor, must be found on the host cell surface for the virus to attach. Also, metabolic differences seen in different cell types based on differential gene expression are a likely factor in which cells a virus may use to replicate. The cell must be making the substances the virus needs, such as enzymes the virus genome itself does not have genes for, or the virus will not be able to replicate using that cell.

Steps of Virus Infections

A virus must "take over" a cell to replicate. The viral replication cycle can produce dramatic biochemical and structural changes in the host cell, which may cause cell damage. These changes, called **cytopathic** effects, can change cell functions or even destroy the cell. Some infected cells, such as those infected by the common cold virus (rhinovirus), die through lysis (bursting) or **apoptosis** (programmed cell death or "cell suicide"), releasing all the progeny virions at once. The symptoms of viral diseases result from the immune response to the virus, which attempts to control and eliminate the virus from the body, and from cell damage caused by the virus. Many animal viruses, such as HIV (human immunodeficiency virus), leave the infected cells of the immune system by a process known as budding, where virions leave the cell individually. During the budding process, the cell does not undergo lysis and is not immediately killed. However, the damage to the cells that HIV infects may make it impossible for the cells to function as mediators of immunity, even though the cells remain alive for a period of time. Most productive viral infections follow similar steps in the virus replication cycle: attachment, penetration, uncoating, replication, assembly, and release.

A virus attaches to a specific receptor site on the host-cell membrane through attachment proteins in the capsid or proteins embedded in its envelope. The attachment is specific, and typically a virus will only attach to cells of one or a few species and only certain cell types within those species with the appropriate receptors.

View this **video (http://openstax.org/l/influenza2)** for a visual explanation of how influenza attacks the body.

Unlike animal viruses, the nucleic acid of bacteriophages is injected into the host cell naked, leaving the capsid outside the cell. Plant and animal viruses can enter their cells through endocytosis, in which the cell membrane surrounds and engulfs the entire virus. Some enveloped viruses enter the cell when the viral envelope fuses directly with the cell membrane. Once inside the cell, the viral capsid is degraded and the viral nucleic acid is released, which then becomes available for replication and transcription.

The replication mechanism depends on the viral genome. DNA viruses usually use host cell proteins and enzymes to make additional DNA that is used to copy the genome or be transcribed to messenger RNA (mRNA), which is then used in protein synthesis. RNA viruses, such as the influenza virus, usually use the RNA core as a template for synthesis of viral genomic RNA and mRNA. The viral mRNA is translated into viral enzymes and capsid proteins to assemble new virions (**Figure 15.6**). Of course, there are exceptions to

this pattern. If a host cell does not provide the enzymes necessary for viral replication, viral genes supply the information to direct synthesis of the missing proteins. Retroviruses, such as HIV, have an RNA genome that must be reverse transcribed to make DNA, which then is inserted into the host's DNA. To convert RNA into DNA, retroviruses contain genes that encode the virus-specific enzyme reverse transcriptase that transcribes an RNA template to DNA. The fact that HIV produces some of its own enzymes, which are not found in the host, has allowed researchers to develop drugs that inhibit these enzymes. These drugs, including the reverse transcriptase inhibitor AZT, inhibit HIV replication by reducing the activity of the enzyme without affecting the host's metabolism.

The last stage of viral replication is the release of the new virions into the host organism, where they are able to infect adjacent cells and repeat the replication cycle. Some viruses are released when the host cell dies and other viruses can leave infected cells by budding through the membrane without directly killing the cell.

Visual Connection

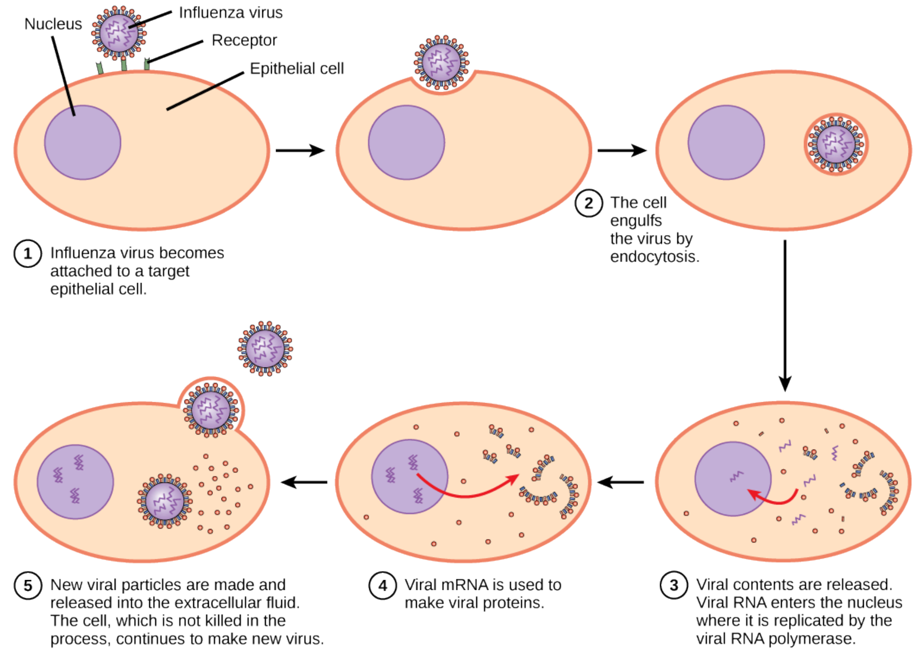


Figure 15.6 In influenza virus infection, glycoproteins attach to a host epithelial cell. As a result, the virus is engulfed. RNA and proteins are made and assembled into new virions.

Influenza virus is packaged in a viral envelope, which fuses with the plasma membrane. This way, the virus can exit the host cell without killing it. What advantage does the virus gain by keeping the host cell alive?

Click through this **tutorial (http://openstax.org/l/viruses2)** on viruses to identify structures, modes of transmission, replication, and more.

Viruses and Disease

Viruses cause a variety of diseases in animals, including humans, ranging from the common cold to potentially fatal illnesses like meningitis (**Figure 15.7**). These diseases can be treated by antiviral drugs or by vaccines, but some viruses, such as HIV, are capable of avoiding the immune response and mutating so as to become resistant to antiviral drugs.

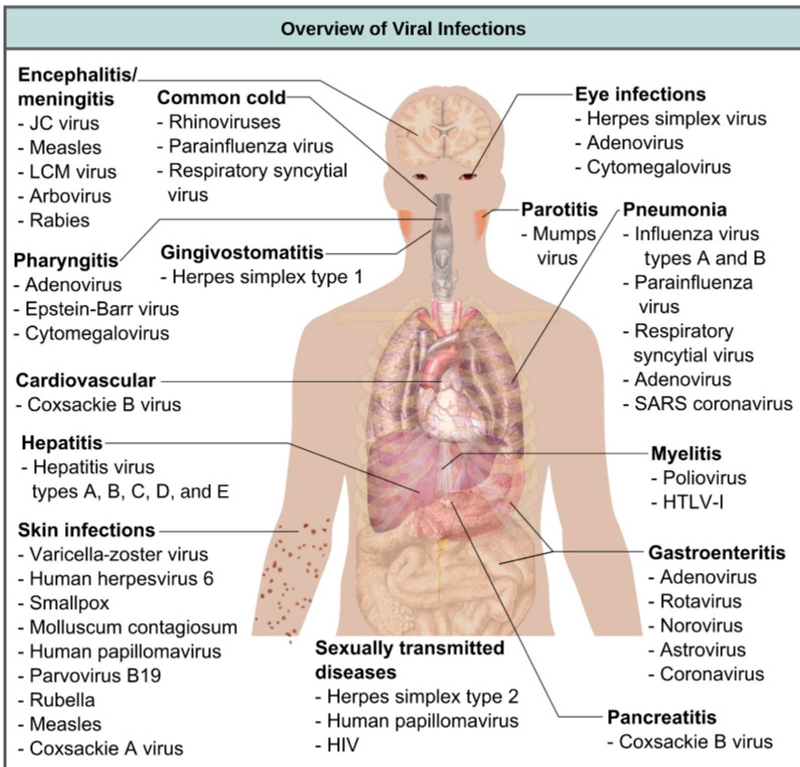


Figure 15.7 Viruses are the cause of dozens of ailments in humans, ranging from mild illnesses to serious diseases. (credit: modification of work by Mikael Häggström)

Vaccines for Prevention

While we do have limited numbers of effective antiviral drugs, such as those used to treat HIV and influenza, the primary method of controlling viral disease is by vaccination, which is intended to prevent outbreaks by building immunity to a virus or virus family. A **vaccine** may be prepared using weakened live viruses, killed viruses, or molecular subunits of the virus. In general, live viruses lead to better immunity, but have the possibility of causing disease at some low frequency. Killed viral vaccine and the subunit viruses are both incapable of causing disease, but in general lead to less effective or long-lasting immunity.

Weakened live viral vaccines are designed in the laboratory to cause few symptoms in recipients while giving them immunity against future infections. Polio was one disease that represented a milestone in the use of vaccines. Mass immunization campaigns in the U.S. in the 1950s (killed vaccine) and 1960s (live vaccine) essentially eradicated the disease, which caused muscle paralysis in children and generated fear in the general population when regional epidemics occurred. The success of the polio vaccine paved the way for the routine dispensation of childhood vaccines against measles, mumps, rubella, chickenpox, and other diseases.

Live vaccines are usually made by **attenuation** (weakening) of the “wild-type” (disease-causing) virus by growing it in the laboratory in tissues or at temperatures different from what the virus is accustomed to in the host. For example, the virus may be grown in cells in a test tube, in bird embryos, or in live animals. The adaptation to these new cells or temperature induces mutations in the virus’ genomes, allowing them to grow better in the laboratory while inhibiting their ability to cause disease when reintroduced into the conditions found in the host. These attenuated viruses thus still cause an infection, but they do not grow very well, allowing the immune response to develop in time to prevent major disease. The danger of using live vaccines, which are usually more effective than killed vaccines, is the low but significant risk that these viruses will revert back to their disease-causing form by back mutations. Back mutations occur when the vaccine undergoes mutations in the host such that it readapts to the host and can again cause disease, which can then be spread to other humans in an epidemic. This happened as recently as 2007 in Nigeria where mutations in a polio vaccine led to an epidemic of polio in that country.

Some vaccines are in continuous development because certain viruses, such as influenza and HIV, have a high mutation rate compared to other viruses or host cells. With influenza, mutation in genes for the surface molecules helps the virus evade the protective immunity that may have been obtained in a previous influenza season, making it necessary for individuals to get vaccinated every year. Other viruses, such as those that cause the

childhood diseases measles, mumps, and rubella, mutate so little that the same vaccine is used year after year.

Vaccines and Antiviral Drugs for Treatment

In some cases, vaccines can be used to treat an active viral infection. In the case of rabies, a fatal neurological disease transmitted in the saliva of rabies virus-infected animals, the progression of the disease from the time of the animal bite to the time it enters the central nervous system may be two weeks or longer. This is enough time to vaccinate an individual who suspects being bitten by a rabid animal, and the boosted immune response from the vaccination is enough to prevent the virus from entering nervous tissue. Thus, the fatal neurological consequences of the disease are averted and the individual only has to recover from the infected bite. This approach is also being used for the treatment of Ebola, one of the fastest and most deadly viruses affecting humans, though usually infecting limited populations. Ebola is also a leading cause of death in gorillas. Transmitted by bats and great apes, this virus can cause death in 70–90 percent of the infected within two weeks. Using newly developed vaccines that boost the immune response, there is hope that immune systems of affected individuals will be better able to control the virus, potentially reducing mortality rates.

Another way of treating viral infections is the use of antiviral drugs. These drugs often have limited ability to cure viral disease but have been used to control and reduce symptoms for a wide variety of viral diseases. For most viruses, these drugs inhibit the virus by blocking the actions of one or more of its proteins. It is important that the targeted proteins be encoded for by viral genes and that these molecules are not present in a healthy host cell. In this way, viral growth is inhibited without damaging the host. There are large numbers of antiviral drugs available to treat infections, some specific for a particular virus and others that can affect multiple viruses.

Antivirals have been developed to treat genital herpes (herpes simplex II) and influenza. For genital herpes, drugs such as acyclovir can reduce the number and duration of the episodes of active viral disease during which patients develop viral lesions in their skins cells. As the virus remains latent in nervous tissue of the body for life, this drug is not a cure but can make the symptoms of the disease more manageable. For influenza, drugs like Tamiflu can reduce the duration of “flu” symptoms by one or two days, but the drug does not prevent symptoms entirely. Other antiviral drugs, such as Ribavirin, have been used to treat a variety of viral infections.

By far the most successful use of antivirals has been in the treatment of the retrovirus HIV, which causes a disease that, if untreated, is usually fatal within 10–12 years after being infected. Anti-HIV drugs have been able to control viral replication to the point that individuals receiving these drugs survive for a significantly longer time than the untreated.

Anti-HIV drugs inhibit viral replication at many different phases of the HIV replicative cycle. Drugs have been developed that inhibit the fusion of the HIV viral envelope with the plasma membrane of the host cell (fusion inhibitors), the conversion of its RNA genome to double-stranded DNA (reverse transcriptase inhibitors), the integration of the viral DNA into the host genome (integrase inhibitors), and the processing of viral proteins (protease inhibitors).

When any of these drugs are used individually, the virus’ high mutation rate allows the virus to rapidly evolve resistance to the drug. The breakthrough in the treatment of HIV was the development of highly active anti-retroviral therapy (HAART), which involves a mixture of different drugs, sometimes called a drug “cocktail.” By attacking the virus at different stages of its replication cycle, it is difficult for the virus to develop resistance to multiple drugs at the same time. Still, even with the use of combination HAART therapy, there is concern that, over time, the virus will evolve resistance to this therapy. Thus, new anti-HIV drugs are constantly being developed with the hope of continuing the battle against this highly fatal virus.

15.2 | Innate Immunity

By the end of this section, you will be able to:

- Describe the body’s innate physical and chemical defenses
- Explain the inflammatory response
- Describe the complement system

The vertebrate, including human, immune system is a complex multilayered system for defending against external and internal threats to the integrity of the body. The system can be divided into two types of defense systems: the innate immune system, which is nonspecific toward a particular kind of pathogen, and the adaptive

immune system, which is specific (Figure 15.8). **Innate immunity** is not caused by an infection or vaccination and depends initially on physical and chemical barriers that work on all pathogens, sometimes called the first line of defense. The second line of defense of the innate system includes chemical signals that produce inflammation and fever responses as well as mobilizing protective cells and other chemical defenses. The adaptive immune system mounts a highly specific response to substances and organisms that do not belong in the body. The adaptive system takes longer to respond and has a memory system that allows it to respond with greater intensity should the body reencounter a pathogen even years later.

Vertebrate Immunity		
Innate Immune System		Adaptive Immune System
Physical Barriers	Internal Defenses	
• Skin, hair, cilia • Mucus membranes • Mucus and chemical secretions • Digestive enzymes in mouth • Stomach acid	• Inflammatory response • Complement proteins • Phagocytic cells • Natural killer (NK) cells	• Antibodies and the humoral immune response • Cell-mediated immune response • Memory response

Figure 15.8 There are two main parts to the vertebrate immune system. The innate immune system, which is made up of physical barriers and internal defenses, responds to all pathogens. The adaptive immune system is highly specific.

External and Chemical Barriers

The body has significant physical barriers to potential pathogens. The skin contains the protein keratin, which resists physical entry into cells. Other body surfaces, particularly those associated with body openings, are protected by the mucous membranes. The sticky mucus provides a physical trap for pathogens, preventing their movement deeper into the body. The openings of the body, such as the nose and ears, are protected by hairs that catch pathogens, and the mucous membranes of the upper respiratory tract have cilia that constantly move pathogens trapped in the mucus coat up to the mouth.

The skin and mucous membranes also create a chemical environment that is hostile to many microorganisms. The surface of the skin is acidic, which prevents bacterial growth. Saliva, mucus, and the tears of the eye contain an enzyme that breaks down bacterial cell walls. The stomach secretions create a highly acidic environment, which kills many pathogens entering the digestive system.

Finally, the surface of the body and the lower digestive system have a community of microorganisms such as bacteria, archaea, and fungi that coexist without harming the body. There is evidence that these organisms are highly beneficial to their host, combating disease-causing organisms and outcompeting them for nutritional resources provided by the host body. Despite these defenses, pathogens may enter the body through skin abrasions or punctures, or by collecting on mucosal surfaces in large numbers that overcome the protections of mucus or cilia.

Internal Defenses

When pathogens enter the body, the innate immune system responds with a variety of internal defenses. These include the inflammatory response, phagocytosis, natural killer cells, and the complement system. White blood cells in the blood and lymph recognize pathogens as foreign to the body. A **white blood cell** is larger than a red blood cell, is nucleated, and is typically able to move using amoeboid locomotion. Because they can move on their own, white blood cells can leave the blood to go to infected tissues. For example, a **monocyte** is a type of white blood cell that circulates in the blood and lymph and develops into a macrophage after it moves into infected tissue. A **macrophage** is a large cell that engulfs foreign particles and pathogens. **Mast cells** are produced in the same way as white blood cells, but unlike circulating white blood cells, mast cells take up residence in connective tissues and especially mucosal tissues. They are responsible for releasing chemicals in response to physical injury. They also play a role in the allergic response, which will be discussed later in the chapter.

When a pathogen is recognized as foreign, chemicals called cytokines are released. A **cytokine** is a chemical messenger that regulates cell differentiation (form and function), proliferation (production), and gene expression to produce a variety of immune responses. Approximately 40 types of cytokines exist in humans. In addition to being released from white blood cells after pathogen recognition, cytokines are also released by the infected

cells and bind to nearby uninfected cells, inducing those cells to release cytokines. This positive feedback loop results in a burst of cytokine production.

One class of early-acting cytokines is the interferons, which are released by infected cells as a warning to nearby uninfected cells. An **interferon** is a small protein that signals a viral infection to other cells. The interferons stimulate uninfected cells to produce compounds that interfere with viral replication. Interferons also activate macrophages and other cells.

The Inflammatory Response and Phagocytosis

The first cytokines to be produced encourage **inflammation**, a localized redness, swelling, heat, and pain. Inflammation is a response to physical trauma, such as a cut or a blow, chemical irritation, and infection by pathogens (viruses, bacteria, or fungi). The chemical signals that trigger an inflammatory response enter the extracellular fluid and cause capillaries to dilate (expand) and capillary walls to become more permeable, or leaky. The serum and other compounds leaking from capillaries cause swelling of the area, which in turn causes pain. Various kinds of white blood cells are attracted to the area of inflammation. The types of white blood cells that arrive at an inflamed site depend on the nature of the injury or infecting pathogen. For example, a **neutrophil** is an early arriving white blood cell that engulfs and digests pathogens. Neutrophils are the most abundant white blood cells of the immune system (**Figure 15.9**). Macrophages follow neutrophils and take over the phagocytosis function and are involved in the resolution of an inflamed site, cleaning up cell debris and pathogens.

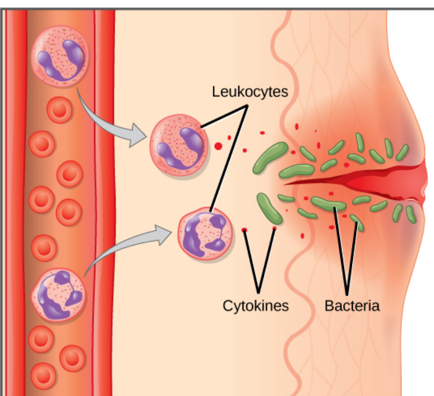


Figure 15.9 White blood cells (leukocytes) release chemicals to stimulate the inflammatory response following a cut in the skin.

Cytokines also send feedback to cells of the nervous system to bring about the overall symptoms of feeling sick, which include lethargy, muscle pain, and nausea. Cytokines also increase the core body temperature, causing a fever. The elevated temperatures of a fever inhibit the growth of pathogens and speed up cellular repair processes. For these reasons, suppression of fevers should be limited to those that are dangerously high.

Check out this **23-second, stop-motion video (https://commons.wikimedia.org/wiki/File:S1-Polymorphonuclear_Cells_with_Conidia_in_Liquid_Media.ogv)** showing a neutrophil that searches and engulfs fungus spores during an elapsed time of 79 minutes.

Natural Killer Cells

A **lymphocyte** is a white blood cell that contains a large nucleus (**Figure 15.10**). Most lymphocytes are associated with the adaptive immune response, but infected cells are identified and destroyed by natural killer cells, the only lymphocytes of the innate immune system. A **natural killer (NK) cell** is a lymphocyte that can kill cells infected with viruses (or cancerous cells). NK cells identify intracellular infections, especially from viruses, by the altered expression of **major histocompatibility class (MHC) I molecules** on the surface of infected cells. MHC class I molecules are proteins on the surfaces of all nucleated cells that provide a sample of the cell's internal environment at any given time. Unhealthy cells, whether infected or cancerous, display an altered MHC

class I complement on their cell surfaces.

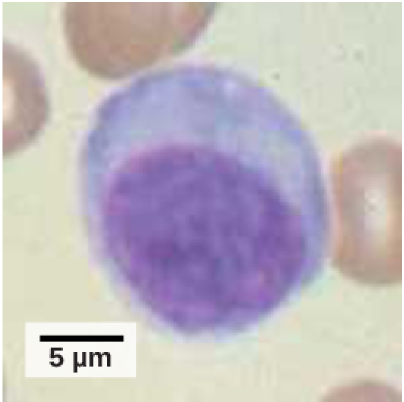


Figure 15.10 Lymphocytes, such as NK cells, are characterized by their large nuclei that actively absorb Wright stain and therefore appear dark colored under a microscope. (credit: scale-bar data from Matt Russell)

After the NK cell detects an infected or tumor cell, it induces programmed cell death, or apoptosis. Phagocytic cells then come along and digest the cell debris left behind. NK cells are constantly patrolling the body and are an effective mechanism for controlling potential infections and preventing cancer progression. The various types of immune cells are shown in Figure 15.11.

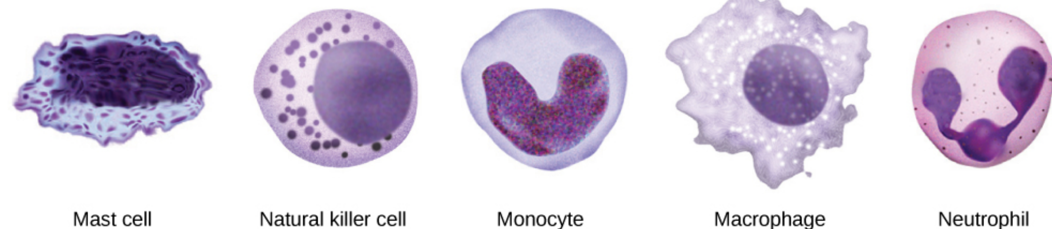


Figure 15.11 Cells involved in the innate immune response include mast cells, natural killer cells, and white blood cells, such as monocytes, macrophages and neutrophils.

Complement

An array of approximately 20 types of proteins, called a **complement system**, is also activated by infection or the activity of the cells of the adaptive immune system and functions to destroy extracellular pathogens. Liver cells and macrophages synthesize inactive forms of complement proteins continuously; these proteins are abundant in the blood serum and are capable of responding immediately to infecting microorganisms. The complement system is so named because it is complementary to the innate and adaptive immune system. Complement proteins bind to the surfaces of microorganisms and are particularly attracted to pathogens that are already tagged by the adaptive immune system. This “tagging” involves the attachment of specific proteins called antibodies (discussed in detail later) to the pathogen. When they attach, the antibodies change shape providing a binding site for one of the complement proteins. After the first few complement proteins bind, a cascade of binding in a specific sequence of proteins follows in which the pathogen rapidly becomes coated in complement proteins.

Complement proteins perform several functions, one of which is to serve as a marker to indicate the presence of a pathogen to phagocytic cells and enhance engulfment. Certain complement proteins can combine to open pores in microbial cell membranes and cause lysis of the cells.

15.3 | Adaptive Immunity

By the end of this section, you will be able to:

- Explain adaptive immunity
- Describe cell-mediated immune response and humoral immune response
- Describe immune tolerance

The adaptive, or acquired, immune response takes days or even weeks to become established—much longer than the innate response; however, adaptive immunity is more specific to an invading pathogen. **Adaptive immunity** is an immunity that occurs after exposure to an antigen either from a pathogen or a vaccination. An **antigen** is a molecule that stimulates a response in the immune system. This part of the immune system is activated when the innate immune response is insufficient to control an infection. In fact, without information from the innate immune system, the adaptive response could not be mobilized. There are two types of adaptive responses: the **cell-mediated immune response**, which is controlled by activated **T cells**, and the **humoral immune response**, which is controlled by activated **B cells** and antibodies. Activated T and B cells whose surface binding sites are specific to the molecules on the pathogen greatly increase in numbers and attack the invading pathogen. Their attack can kill pathogens directly or they can secrete antibodies that enhance the phagocytosis of pathogens and disrupt the infection. Adaptive immunity also involves a memory to give the host long-term protection from reinfection with the same type of pathogen; on reexposure, this host memory will facilitate a rapid and powerful response.

B and T Cells

Lymphocytes, which are white blood cells, are formed with other blood cells in the red bone marrow found in many flat bones, such as the shoulder or pelvic bones. The two types of lymphocytes of the adaptive immune response are B and T cells (**Figure 15.12**). Whether an immature lymphocyte becomes a B cell or T cell depends on where in the body it matures. The B cells remain in the bone marrow to mature (hence the name “B” for “bone marrow”), while T cells migrate to the thymus, where they mature (hence the name “T” for “thymus”).

Maturation of a B or T cell involves becoming immunocompetent, meaning that it can recognize, by binding, a specific molecule or antigen (discussed below). During the maturation process, B and T cells that bind too strongly to the body’s own cells are eliminated in order to minimize an immune response against the body’s own tissues. Those cells that react weakly to the body’s own cells, but have highly specific receptors on their cell surfaces that allow them to recognize a foreign molecule, or antigen, remain. This process occurs during fetal development and continues throughout life. The specificity of this receptor is determined by the genetics of the individual and is present before a foreign molecule is introduced to the body or encountered. Thus, it is genetics and not experience that initially provides a vast array of cells, each capable of binding to a different specific foreign molecule. Once they are immunocompetent, the T and B cells will migrate to the spleen and lymph nodes where they will remain until they are called on during an infection. B cells are involved in the humoral immune response, which targets pathogens loose in blood and lymph, and T cells are involved in the cell-mediated immune response, which targets infected cells.

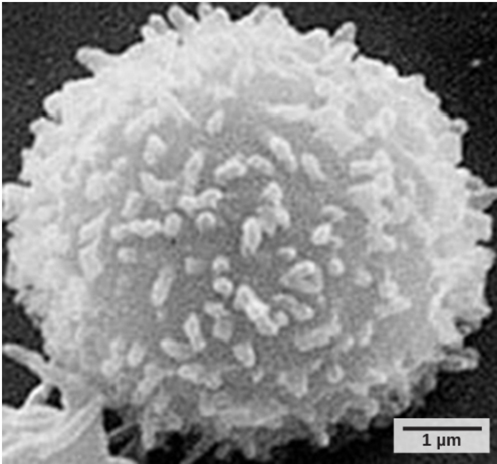


Figure 15.12 This scanning electron micrograph shows a T lymphocyte. T and B cells are indistinguishable by light microscopy but can be differentiated experimentally by probing their surface receptors. (credit: modification of work by NCI; scale-bar data from Matt Russell)

Humoral Immune Response

As mentioned, an antigen is a molecule that stimulates a response in the immune system. Not every molecule is antigenic. B cells participate in a chemical response to antigens present in the body by producing specific antibodies that circulate throughout the body and bind with the antigen whenever it is encountered. This is known as the humoral immune response. As discussed, during maturation of B cells, a set of highly specific B cells are produced that have many antigen receptor molecules in their membrane (Figure 15.13).

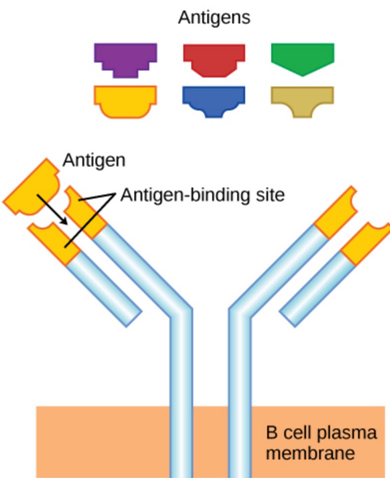


Figure 15.13 B cell receptors are embedded in the membranes of B cells and bind a variety of antigens through their variable regions.

Each B cell has only one kind of antigen receptor, which makes every B cell different. Once the B cells mature in the bone marrow, they migrate to lymph nodes or other lymphatic organs. When a B cell encounters the antigen that binds to its receptor, the antigen molecule is brought into the cell by endocytosis and reappears on the surface of the cell bound to an **MHC class II molecule**. When this process is complete, the B cell is sensitized. In most cases, the sensitized B cell must then encounter a specific kind of T cell, called a helper T cell, before it is activated. The helper T cell must already have been activated through an encounter with the antigen (discussed below).

The helper T cell binds to the antigen-MHC class II complex and is induced to release cytokines that induce the B cell to divide rapidly, which makes thousands of identical (clonal) cells. These daughter cells become either plasma cells or memory B cells. The memory B cells remain inactive at this point, until another later encounter with the antigen, caused by a reinfection by the same bacteria or virus, results in them dividing into a new population of plasma cells. The plasma cells, on the other hand, produce and secrete large quantities, up to 100 million molecules per hour, of antibody molecules. An **antibody**, also known as an immunoglobulin (Ig), is

a protein that is produced by plasma cells after stimulation by an antigen. Antibodies are the agents of humoral immunity. Antibodies occur in the blood, in gastric and mucus secretions, and in breast milk. Antibodies in these bodily fluids can bind pathogens and mark them for destruction by phagocytes before they can infect cells.

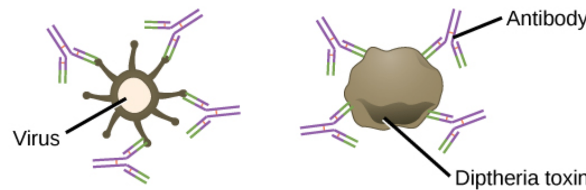
These antibodies circulate in the blood stream and lymphatic system and bind with the antigen whenever it is encountered. The binding can fight infection in several ways. Antibodies can bind to viruses or bacteria and interfere with the chemical interactions required for them to infect or bind to other cells. The antibodies may create bridges between different particles containing antigenic sites clumping them all together and preventing their proper functioning. The antigen-antibody complex stimulates the complement system described previously, destroying the cell bearing the antigen. Phagocytic cells, such as those already described, are attracted by the antigen-antibody complexes, and phagocytosis is enhanced when the complexes are present. Finally, antibodies stimulate inflammation, and their presence in mucus and on the skin prevents pathogen attack.

Antibodies coat extracellular pathogens and neutralize them by blocking key sites on the pathogen that enhance their infectivity (such as receptors that “dock” pathogens on host cells) (**Figure 15.14**). Antibody neutralization can prevent pathogens from entering and infecting host cells. The neutralized antibody-coated pathogens can then be filtered by the spleen and eliminated in urine or feces.

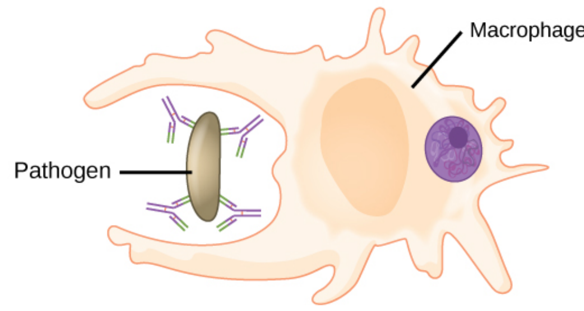
Antibodies also mark pathogens for destruction by phagocytic cells, such as macrophages or neutrophils, in a process called opsonization. In a process called complement fixation, some antibodies provide a place for complement proteins to bind. The combination of antibodies and complement promotes rapid clearing of pathogens.

The production of antibodies by plasma cells in response to an antigen is called **active immunity** and describes the host’s active response of the immune system to an infection or to a vaccination. There is also a **passive immune** response where antibodies come from an outside source, instead of the individual’s own plasma cells, and are introduced into the host. For example, antibodies circulating in a pregnant woman’s body move across the placenta into the developing fetus. The child benefits from the presence of these antibodies for up to several months after birth. In addition, a passive immune response is possible by injecting antibodies into an individual in the form of an antivenom to a snake-bite toxin or antibodies in blood serum to help fight a hepatitis infection. This gives immediate protection since the body does not need the time required to mount its own response.

(a) Neutralization Antibodies prevent a virus or toxic protein from binding their target.



(b) Opsonization A pathogen tagged by antibodies is consumed by a macrophage or neutrophil.



(c) Complement activation Antibodies attached to the surface of a pathogen cell activate the complement system.

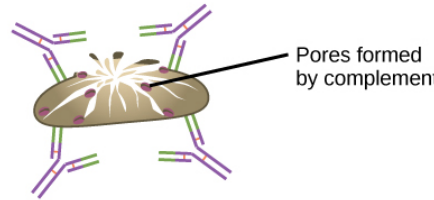


Figure 15.14 Antibodies may inhibit infection by (a) preventing the antigen from binding its target, (b) tagging a pathogen for destruction by macrophages or neutrophils, or (c) activating the complement cascade.

Cell-Mediated Immunity

Unlike B cells, T lymphocytes are unable to recognize pathogens without assistance. Instead, dendritic cells and macrophages first engulf and digest pathogens into hundreds or thousands of antigens. Then, an **antigen-presenting cell (APC)** detects, engulfs, and informs the adaptive immune response about an infection. When a pathogen is detected, these APCs will engulf and break it down through phagocytosis. Antigen fragments will then be transported to the surface of the APC, where they will serve as an indicator to other immune cells. A **dendritic cell** is an immune cell that mops up antigenic materials in its surroundings and presents them on its surface. Dendritic cells are located in the skin, the linings of the nose, lungs, stomach, and intestines. These positions are ideal locations to encounter invading pathogens. Once they are activated by pathogens and mature to become APCs they migrate to the spleen or a lymph node. Macrophages also function as APCs. After phagocytosis by a macrophage, the phagocytic vesicle fuses with an intracellular lysosome. Within the resulting phagolysosome, the components are broken down into fragments; the fragments are then loaded onto MHC class II molecules and are transported to the cell surface for antigen presentation (Figure 15.15). Helper T cells cannot properly respond to an antigen unless it is processed and embedded in an MHC class II molecule. The APCs express MHC class II on their surfaces, and when combined with a foreign antigen, these complexes signal an invader.

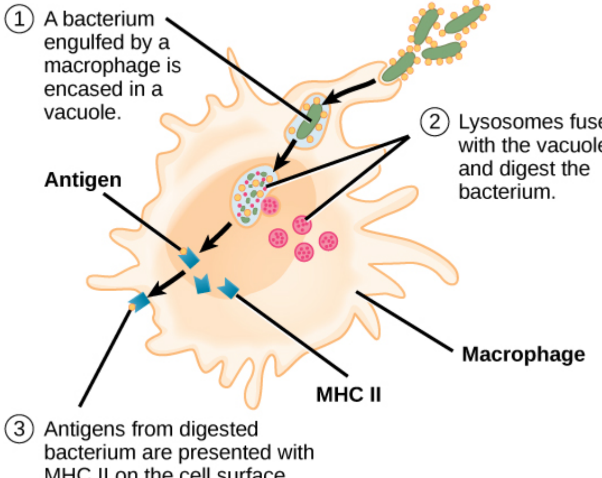


Figure 15.15 An antigen-presenting cell (APC), such as a macrophage, engulfs a foreign antigen, partially digests it in a lysosome, and then embeds it in an MHC class II molecule for presentation at the cell surface. Lymphocytes of the adaptive immune response must interact with antigen-embedded MHC class II molecules to mature into functional immune cells.

View this **animation from Rockefeller University (http://openstax.org/l/immune_system2)** to see how dendritic cells act as sentinels in the body's immune system.

T cells have many functions. Some respond to APCs of the innate immune system and indirectly induce immune responses by releasing cytokines. Others stimulate B cells to start the humoral response as described previously. Another type of T cell detects APC signals and directly kills the infected cells, while some are involved in suppressing inappropriate immune reactions to harmless or "self" antigens.

There are two main types of T cells: helper T lymphocytes (T_H) and the cytotoxic T lymphocytes (T_C). The T_H lymphocytes function indirectly to tell other immune cells about potential pathogens. T_H lymphocytes recognize specific antigens presented by the MHC class II complexes of APCs. There are two populations of T_H cells: T_H1 and T_H2. T_H1 cells secrete cytokines to enhance the activities of macrophages and other T cells. T_H2 cells stimulate naïve B cells to secrete antibodies. Whether a T_H1 or a T_H2 immune response develops depends on the specific types of cytokines secreted by cells of the innate immune system, which in turn depends on the nature of the invading pathogen.

Cytotoxic T cells (T_C) are the key component of the cell-mediated part of the adaptive immune system and attack and destroy infected cells. T_C cells are particularly important in protecting against viral infections; this is because viruses replicate within cells where they are shielded from extracellular contact with circulating antibodies. Once activated, the T_C creates a large clone of cells with one specific set of cell-surface receptors, as in the case with proliferation of activated B cells. As with B cells, the clone includes active T_C cells and inactive memory T_C cells. The resulting active T_C cells then identify infected host cells. Because of the time required to generate a population of clonal T and B cells, there is a delay in the adaptive immune response compared to the innate immune response.

T_C cells attempt to identify and destroy infected cells before the pathogen can replicate and escape, thereby halting the progression of intracellular infections. T_C cells also support NK lymphocytes to destroy early cancers. Cytokines secreted by the T_H1 response that stimulates macrophages also stimulate T_C cells and enhance their ability to identify and destroy infected cells and tumors. A summary of how the humoral and cell-mediated immune responses are activated appears in **Figure 15.16**.

B plasma cells and T_C cells are collectively called **effector cells** because they are involved in "effecting"

(bringing about) the immune response of killing pathogens and infected host cells.

Figure 15.16 A helper T cell becomes activated by binding to an antigen presented by an APC via the MHCII receptor, causing it to release cytokines. Depending on the cytokines released, this activates either the humoral or the cell-mediated immune response.

Immunological Memory

The adaptive immune system has a memory component that allows for a rapid and large response upon reinvasion of the same pathogen. During the adaptive immune response to a pathogen that has not been encountered before, known as the **primary immune response**, plasma cells secreting antibodies and differentiated T cells increase, then plateau over time. As B and T cells mature into effector cells, a subset of the naïve populations differentiates into B and T memory cells with the same antigen specificities (**Figure 15.17**). A **memory cell** is an antigen-specific B or T lymphocyte that does not differentiate into an effector cell during the primary immune response, but that can immediately become an effector cell on reexposure to the same pathogen. As the infection is cleared and pathogenic stimuli subside, the effectors are no longer needed and they undergo apoptosis. In contrast, the memory cells persist in the circulation.

Visual Connection

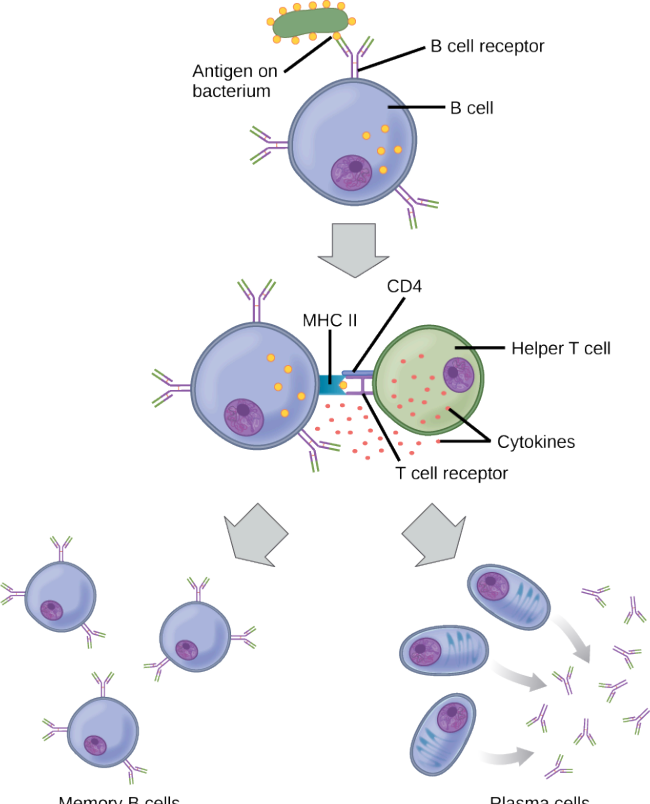


Figure 15.17 After initially binding an antigen to the B cell receptor, a B cell internalizes the antigen and presents it on MHC class II. A helper T cell recognizes the MHC class II- antigen complex and activates the B cell. As a result, memory B cells and plasma cells are made.

The Rh antigen is found on Rh-positive red blood cells. An Rh-negative female can usually carry an Rh-positive fetus to term without difficulty. However, if she has a second Rh-positive fetus, her body may launch an immune attack that causes hemolytic disease of the newborn. Why do you think hemolytic disease is only a problem during the second or subsequent pregnancies?

If the pathogen is never encountered again during the individual's lifetime, B and T memory cells will circulate for a few years or even several decades and will gradually die off, having never functioned as effector cells. However, if the host is re-exposed to the same pathogen type, circulating memory cells will immediately differentiate into plasma cells and T_C cells without input from APCs or T_H cells. This is known as the **secondary immune response**. One reason why the adaptive immune response is delayed is because it takes time for naïve B and T cells with the appropriate antigen specificities to be identified, activated, and proliferate. On reinfection, this step is skipped, and the result is a more rapid production of immune defenses. Memory B cells that differentiate into plasma cells output tens to hundreds-fold greater antibody amounts than were secreted during the primary response (Figure 15.18). This rapid and dramatic antibody response may stop the infection before it can even become established, and the individual may not realize they had been exposed.

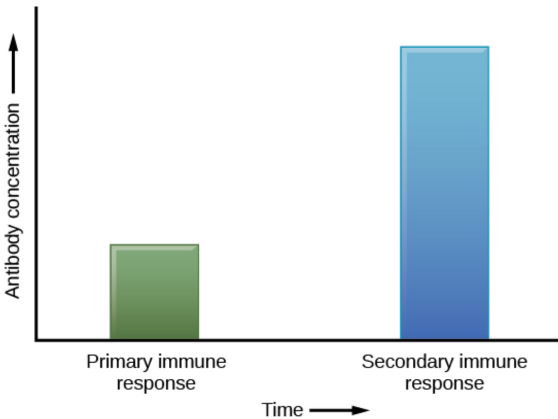


Figure 15.18 In the primary response to infection, antibodies are secreted first from plasma cells. Upon re-exposure to the same pathogen, memory cells differentiate into antibody-secreting plasma cells that output a greater amount of antibody for a longer period of time.

Vaccination is based on the knowledge that exposure to noninfectious antigens, derived from known pathogens, generates a mild primary immune response. The immune response to vaccination may not be perceived by the host as illness but still confers immune memory. When exposed to the corresponding pathogen to which an individual was vaccinated, the reaction is similar to a secondary exposure. Because each reinfection generates more memory cells and increased resistance to the pathogen, some vaccine courses involve one or more booster vaccinations to mimic repeat exposures.

The Lymphatic System

Lymph is the watery fluid that bathes tissues and organs and contains protective white blood cells but does not contain erythrocytes. Lymph moves about the body through the lymphatic system, which is made up of vessels, lymph ducts, lymph glands, and organs, such as tonsils, adenoids, thymus, and spleen.

Although the immune system is characterized by circulating cells throughout the body, the regulation, maturation, and intercommunication of immune factors occur at specific sites. The blood circulates immune cells, proteins, and other factors through the body. Approximately 0.1 percent of all cells in the blood are leukocytes, which include monocytes (the precursor of macrophages) and lymphocytes. Most cells in the blood are red blood cells. Cells of the immune system can travel between the distinct lymphatic and blood circulatory systems, which are separated by interstitial space, by a process called extravasation (passing through to surrounding tissue).

Recall that cells of the immune system originate from stem cells in the bone marrow. B cell maturation occurs in the bone marrow, whereas progenitor cells migrate from the bone marrow and develop and mature into naïve T cells in the organ called the thymus.

On maturation, T and B lymphocytes circulate to various destinations. Lymph nodes scattered throughout the body house large populations of T and B cells, dendritic cells, and macrophages (Figure 15.19). Lymph gathers antigens as it drains from tissues. These antigens then are filtered through lymph nodes before the lymph is returned to circulation. APCs in the lymph nodes capture and process antigens and inform nearby lymphocytes about potential pathogens.

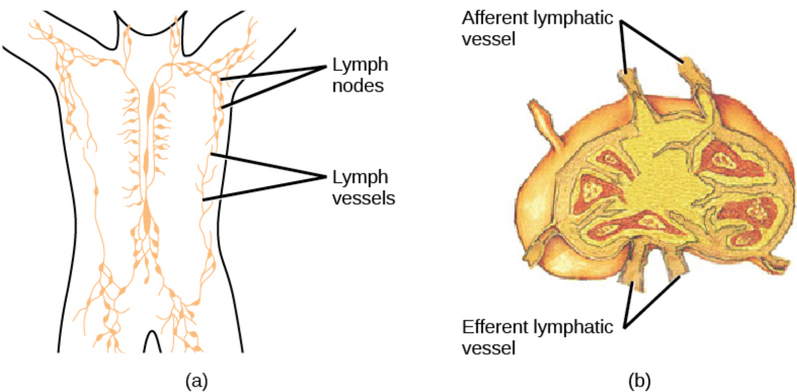


Figure 15.19 (a) Lymphatic vessels carry a clear fluid called lymph throughout the body. The liquid passes through (b) lymph nodes that filter the lymph that enters the node through afferent vessels and leaves through efferent vessels; lymph nodes are filled with lymphocytes that purge infecting cells. (credit a: modification of work by NIH; credit b: modification of work by NCI, NIH)

The spleen houses B and T cells, macrophages, dendritic cells, and NK cells (**Figure 15.20**). The spleen is the site where APCs that have trapped foreign particles in the blood can communicate with lymphocytes. Antibodies are synthesized and secreted by activated plasma cells in the spleen, and the spleen filters foreign substances and antibody-complexed pathogens from the blood. Functionally, the spleen is to the blood as lymph nodes are to the lymph.

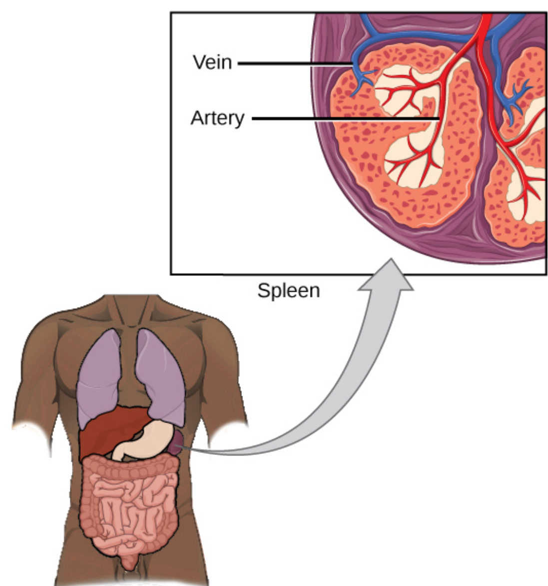


Figure 15.20 The spleen functions to immunologically filter the blood and allow for communication between cells corresponding to the innate and adaptive immune responses. (credit: modification of work by NCI, NIH)

Mucosal Immune System

The innate and adaptive immune responses compose the systemic immune system (affecting the whole body), which is distinct from the mucosal immune system. Mucosa associated lymphoid tissue (MALT) is a crucial component of a functional immune system because mucosal surfaces, such as the nasal passages, are the first tissues onto which inhaled or ingested pathogens are deposited. The mucosal tissue includes the mouth, pharynx, and esophagus, and the gastrointestinal, respiratory, and urogenital tracts.

Mucosal immunity is formed by MALT, which functions independently of the systemic immune system, and which has its own innate and adaptive components. MALT is a collection of lymphatic tissue that combines with epithelial tissue lining the mucosa throughout the body. This tissue functions as the immune barrier and response in areas of the body with direct contact to the external environment. The systemic and mucosal immune systems use many of the same cell types. Foreign particles that make their way to MALT are taken up by absorptive

epithelial cells and delivered to APCs located directly below the mucosal tissue. APCs of the mucosal immune system are primarily dendritic cells, with B cells and macrophages having minor roles. Processed antigens displayed on APCs are detected by T cells in the MALT and at the tonsils, adenoids, appendix, or the mesenteric lymph nodes of the intestine. Activated T cells then migrate through the lymphatic system and into the circulatory system to mucosal sites of infection.

Immune Tolerance

The immune system has to be regulated to prevent wasteful, unnecessary responses to harmless substances, and more importantly, so that it does not attack “self.” The acquired ability to prevent an unnecessary or harmful immune response to a detected foreign substance known not to cause disease, or self-antigens, is described as **immune tolerance**. The primary mechanism for developing immune tolerance to self-antigens occurs during the selection for weakly self-binding cells during T and B lymphocyte maturation. There are populations of T cells that suppress the immune response to self-antigens and that suppress the immune response after the infection has cleared to minimize host cell damage induced by inflammation and cell lysis. Immune tolerance is especially well developed in the mucosa of the upper digestive system because of the tremendous number of foreign substances (such as food proteins) that APCs of the oral cavity, pharynx, and gastrointestinal mucosa encounter. Immune tolerance is brought about by specialized APCs in the liver, lymph nodes, small intestine, and lung that present harmless antigens to a diverse population of regulatory T (T_{reg}) cells, specialized lymphocytes that suppress local inflammation and inhibit the secretion of stimulatory immune factors. The combined result of T_{reg} cells is to prevent immunologic activation and inflammation in undesired tissue compartments and to allow the immune system to focus on pathogens instead.

15.4 | Isolation, Culture, and Identification of Viruses

Learning Objectives

By the end of this section, you will be able to:

- Discuss why viruses were originally described as filterable agents
- Describe the cultivation of viruses and specimen collection and handling
- Compare in vivo and in vitro techniques used to cultivate viruses

At the beginning of this chapter, we described how porcelain Chamberland filters with pores small enough to allow viruses to pass through were used to discover TMV. Today, porcelain filters have been replaced with membrane filters and other devices used to isolate and identify viruses.

Isolation of Viruses

Unlike bacteria, many of which can be grown on an artificial nutrient medium, viruses require a living host cell for replication. Infected host cells (eukaryotic or prokaryotic) can be cultured and grown, and then the growth medium can be harvested as a source of virus. Virions in the liquid medium can be separated from the host cells by either centrifugation or filtration. Filters can physically remove anything present in the solution that is larger than the virions; the viruses can then be collected in the filtrate (see Figure 15.21).

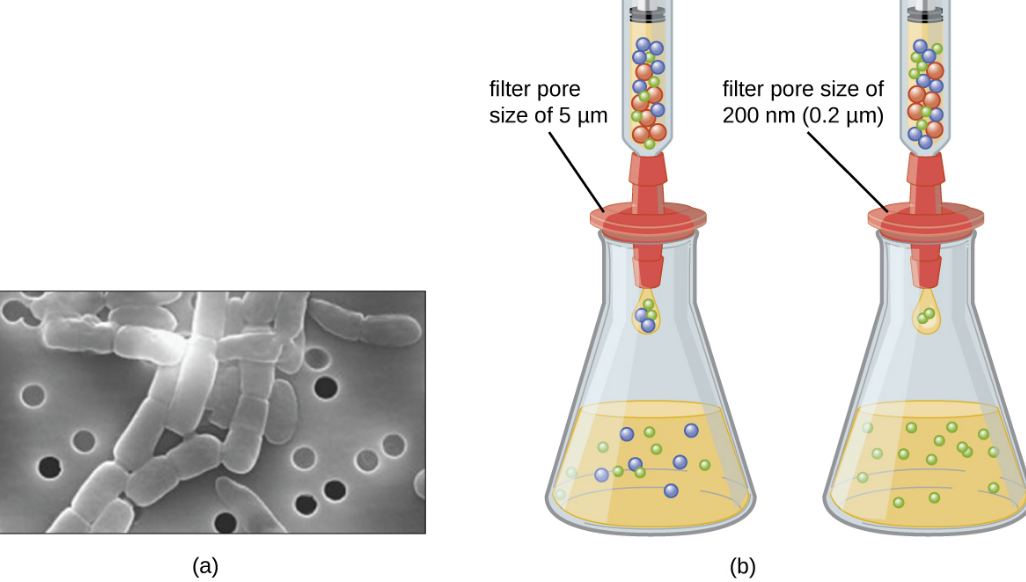
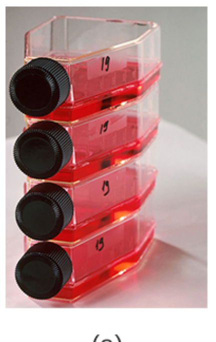


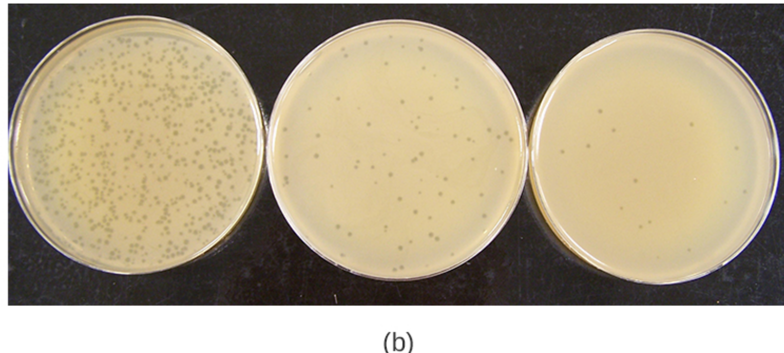
Figure 15.21 Membrane filters can be used to remove cells or viruses from a solution. (a) This scanning electron micrograph shows rod-shaped bacterial cells captured on the surface of a membrane filter. Note differences in the comparative size of the membrane pores and bacteria. Viruses will pass through this filter. (b) The size of the pores in the filter determines what is captured on the surface of the filter (animal [red] and bacteria [blue]) and removed from liquid passing through. Note the viruses (green) pass through the finer filter. (credit a: modification of work by U.S. Department of Energy)

- What size filter pore is needed to collect a virus?

Cultivation of Viruses

Viruses can be grown **in vivo** (within a whole living organism, plant, or animal) or **in vitro** (outside a living organism in cells in an artificial environment. Flat horizontal cell culture flasks (see Figure 15.22**(a)**) are a common vessel used for in vitro work. Bacteriophages can be grown in the presence of a dense layer of bacteria (also called a **bacterial lawn**) grown in a 0.7 % soft agar in a Petri dish or flat (horizontal) flask (see Figure 15.22**(b)**). As the phage kills the bacteria, many plaques are observed among the cloudy bacterial lawn.





(a) (b)

Figure 15.22 (a) Flasks like this may be used to culture human or animal cells for viral culturing. (b) These plates contain bacteriophage T4 grown on an *Escherichia coli* lawn. Clear plaques are visible where host bacterial cells have been lysed. Viral titers increase on the plates to the left. (credit a: modification of work by National Institutes of Health; credit b: modification of work by American Society for Microbiology)

Animal viruses require cells within a host animal or tissue-culture cells derived from an animal. Animal virus cultivation is important for 1) identification and diagnosis of pathogenic viruses in clinical specimens, 2) production of vaccines, and 3) basic research studies. In vivo host sources can be a developing embryo in an embryonated bird's egg (e.g., chicken, turkey) or a whole animal. For example, most of the influenza vaccine manufactured for annual flu vaccination programs is cultured in hens' eggs.

The embryo or host animal serves as an incubator for viral replication (see **Figure 15.23**). Location within the embryo or host animal is important. Many viruses have a tissue tropism, and must therefore be introduced into a specific site for growth. Within an embryo, target sites include the amniotic cavity, the chorioallantoic membrane, or the yolk sac. Viral infection may damage tissue membranes, producing lesions called pox; disrupt embryonic development; or cause the death of the embryo.

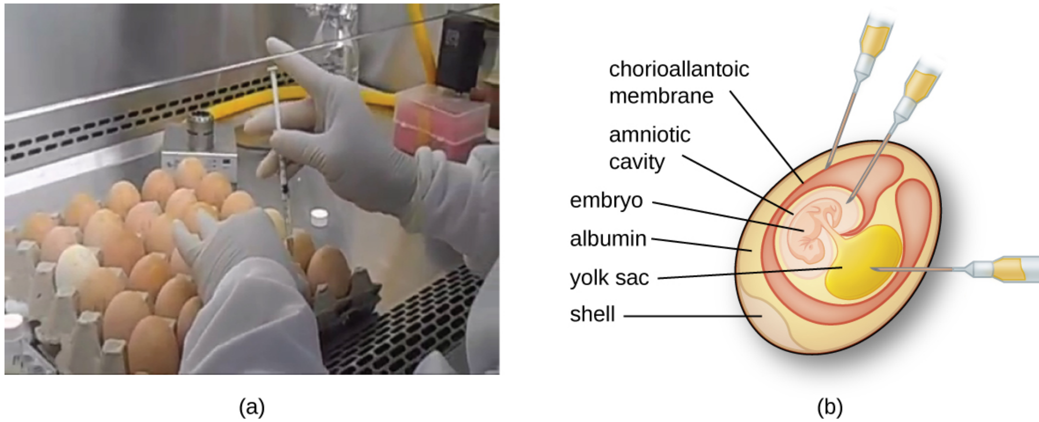


Figure 15.23 (a) The cells within chicken eggs are used to culture different types of viruses. (b) Viruses can be replicated in various locations within the egg, including the chorioallantoic membrane, the amniotic cavity, and the yolk sac. (credit a: modification of work by “Chung Hoang”/YouTube)

For in vitro studies, various types of cells can be used to support the growth of viruses. A primary cell culture is freshly prepared from animal organs or tissues. Cells are extracted from tissues by mechanical scraping or mincing to release cells or by an enzymatic method using trypsin or collagenase to break up tissue and release single cells into suspension. Because of anchorage-dependence requirements, primary cell cultures require a liquid culture medium in a Petri dish or tissue-culture flask so cells have a solid surface such as glass or plastic for attachment and growth. Primary cultures usually have a limited life span. When cells in a primary culture undergo mitosis and a sufficient density of cells is produced, cells come in contact with other cells. When this cell-to-cell-contact occurs, mitosis is triggered to stop. This is called contact inhibition and it prevents the density of the cells from becoming too high. To prevent contact inhibition, cells from the primary cell culture must be transferred to another vessel with fresh growth medium. This is called a secondary cell culture. Periodically, cell density must be reduced by pouring off some cells and adding fresh medium to provide space and nutrients to maintain cell growth. In contrast to primary cell cultures, continuous cell lines, usually derived from transformed cells or tumors, are often able to be subcultured many times or even grown indefinitely (in which case they are called immortal). Continuous cell lines may not exhibit anchorage dependency (they will grow in suspension) and may have lost their contact inhibition. As a result, continuous cell lines can grow in piles or lumps resembling small tumor growths (see **Figure 15.24**).

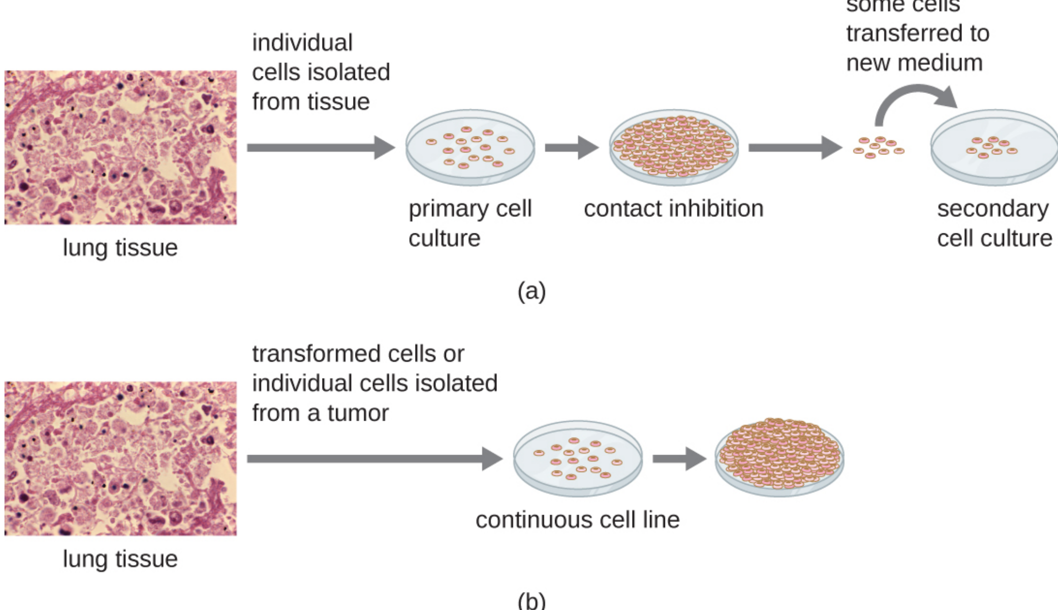


Figure 15.24 Cells for culture are prepared by separating them from their tissue matrix. (a) Primary cell cultures grow attached to the surface of the culture container. Contact inhibition slows the growth of the cells once they become too dense and begin touching each other. At this point, growth can only be sustained by making a secondary culture. (b) Continuous cell cultures are not affected by contact inhibition. They continue to grow regardless of cell density. (credit "micrographs": modification of work by Centers for Disease Control and Prevention)

An example of an immortal cell line is the HeLa cell line, which was originally cultivated from tumor cells obtained from Henrietta Lacks, a patient who died of cervical cancer in 1951. HeLa cells were the first continuous tissue-culture cell line and were used to establish tissue culture as an important technology for research in cell biology, virology, and medicine. Prior to the discovery of HeLa cells, scientists were not able to establish tissue cultures with any reliability or stability. More than six decades later, this cell line is still alive and being used for medical research. See **Eye on Ethics: The Immortal Cell Line of Henrietta Lacks** to read more about this important cell line and the controversial means by which it was obtained.

- What property of cells makes periodic dilutions of primary cell cultures necessary?

The Immortal Cell Line of Henrietta Lacks

In January 1951, Henrietta Lacks, a 30-year-old African American woman from Baltimore, was diagnosed with cervical cancer at Johns Hopkins Hospital. We now know her cancer was caused by the human papillomavirus (HPV). Cytopathic effects of the virus altered the characteristics of her cells in a process called transformation, which gives the cells the ability to divide continuously. This ability, of course, resulted in a cancerous tumor that eventually killed Mrs. Lacks in October at age 31. Before her death, samples of her cancerous cells were taken without her knowledge or permission. The samples eventually ended up in the possession of Dr. George Gey, a biomedical researcher at Johns Hopkins University. Gey was able to grow some of the cells from Lacks's sample, creating what is known today as the immortal HeLa cell line. These cells have the ability to live and grow indefinitely and, even today, are still widely used in many areas of research.

According to Lacks's husband, neither Henrietta nor the family gave the hospital permission to collect her tissue specimen. Indeed, the family was not aware until 20 years after Lacks's death that her cells were still alive and actively being used for commercial and research purposes. Yet HeLa cells have been pivotal in numerous research discoveries related to polio, cancer, and AIDS, among other diseases. The cells have also been commercialized, although they have never themselves been patented. Despite this, Henrietta Lacks's estate has never benefited from the use of the cells, although, in 2013, the Lacks family was given control over the publication of the genetic sequence of her cells.

This case raises several bioethical issues surrounding patients' informed consent and the right to know. At the time Lacks's tissues were taken, there were no laws or guidelines about informed consent. Does that

mean she was treated fairly at the time? Certainly by today's standards, the answer would be no. Harvesting tissue or organs from a dying patient without consent is not only considered unethical but illegal, regardless of whether such an act could save other patients' lives. Is it ethical, then, for scientists to continue to use Lacks's tissues for research, even though they were obtained illegally by today's standards?

Ethical or not, Lacks's cells are widely used today for so many applications that it is impossible to list them all. Is this a case in which the ends justify the means? Would Lacks be pleased to know about her contribution to science and the millions of people who have benefited? Would she want her family to be compensated for the commercial products that have been developed using her cells? Or would she feel violated and exploited by the researchers who took part of her body without her consent? Because she was never asked, we will never know.

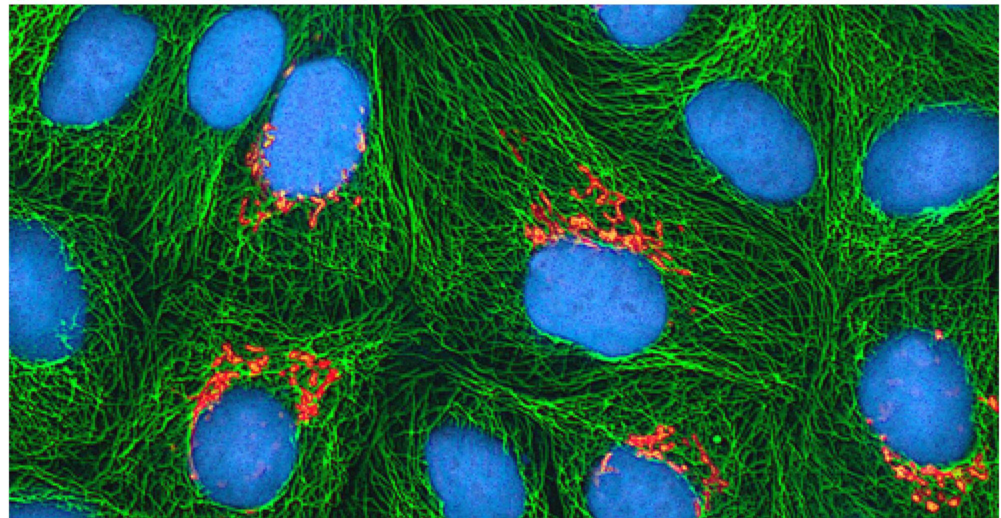


Figure 15.25 A multiphoton fluorescence image of HeLa cells in culture. Various fluorescent stains have been used to show the DNA (cyan), microtubules (green), and Golgi apparatus (orange). (credit: modification of work by National Institutes of Health)

Detection of a Virus

Regardless of the method of cultivation, once a virus has been introduced into a whole host organism, embryo, or tissue-culture cell, a sample can be prepared from the infected host, embryo, or cell line for further analysis under a brightfield, electron, or fluorescent microscope. **Cytopathic effects (CPEs)** are distinct observable cell abnormalities due to viral infection. CPEs can include loss of adherence to the surface of the container, changes in cell shape from flat to round, shrinkage of the nucleus, vacuoles in the cytoplasm, fusion of cytoplasmic membranes and the formation of multinucleated syncytia, inclusion bodies in the nucleus or cytoplasm, and complete cell lysis (see **Figure 15.26**).

Further pathological changes include viral disruption of the host genome and altering normal cells into transformed cells, which are the types of cells associated with carcinomas and sarcomas. The type or severity of the CPE depends on the type of virus involved. **Figure 15.26** lists CPEs for specific viruses.

Cytopathic Effects of Specific Viruses		
Virus	**Cytopathic Effect**	**Example**
Paramyxovirus	Syncytium and faint basophilic cytoplasmic inclusion bodies	
Poxvirus	Pink eosinophilic cytoplasmic inclusion bodies (arrows) and cell swelling	
Herpesvirus	Cytoplasmic stranding (arrow) and nuclear inclusion bodies (dashed arrow)	
Adenovirus	Cell enlargement, rounding, and distinctive “grape-like” clusters	

Figure 15.26 (credit “micrographs”: modification of work by American Society for Microbiology)

Watch this **video (https://www.openstax.org/l/22virusesoncell)** to learn about the effects of viruses on cells.

Hemagglutination Assay

A serological assay is used to detect the presence of certain types of viruses in patient serum. Serum is the straw-colored liquid fraction of blood plasma from which clotting factors have been removed. Serum can be used in a direct assay called a hemagglutination assay to detect specific types of viruses in the patient’s sample. Hemagglutination is the agglutination (clumping) together of erythrocytes (red blood cells). Many viruses produce surface proteins or spikes called hemagglutinins that can bind to receptors on the membranes of erythrocytes and cause the cells to agglutinate. Hemagglutination is observable without using the microscope, but this method does not always differentiate between infectious and noninfectious viral particles, since both can agglutinate erythrocytes.

To identify a specific pathogenic virus using hemagglutination, we must use an indirect approach. Proteins called antibodies, generated by the patient’s immune system to fight a specific virus, can be used to bind to

components such as hemagglutinins that are uniquely associated with specific types of viruses. The binding of the antibodies with the hemagglutinins found on the virus subsequently prevent erythrocytes from directly interacting with the virus. So when erythrocytes are added to the antibody-coated viruses, there is no appearance of agglutination; agglutination has been inhibited. We call these types of indirect assays for virus-specific antibodies hemagglutination inhibition (HAI) assays. HAI can be used to detect the presence of antibodies specific to many types of viruses that may be causing or have caused an infection in a patient even months or years after infection (see **Figure 15.27**). This assay is described in greater detail in **Agglutination Assays**.

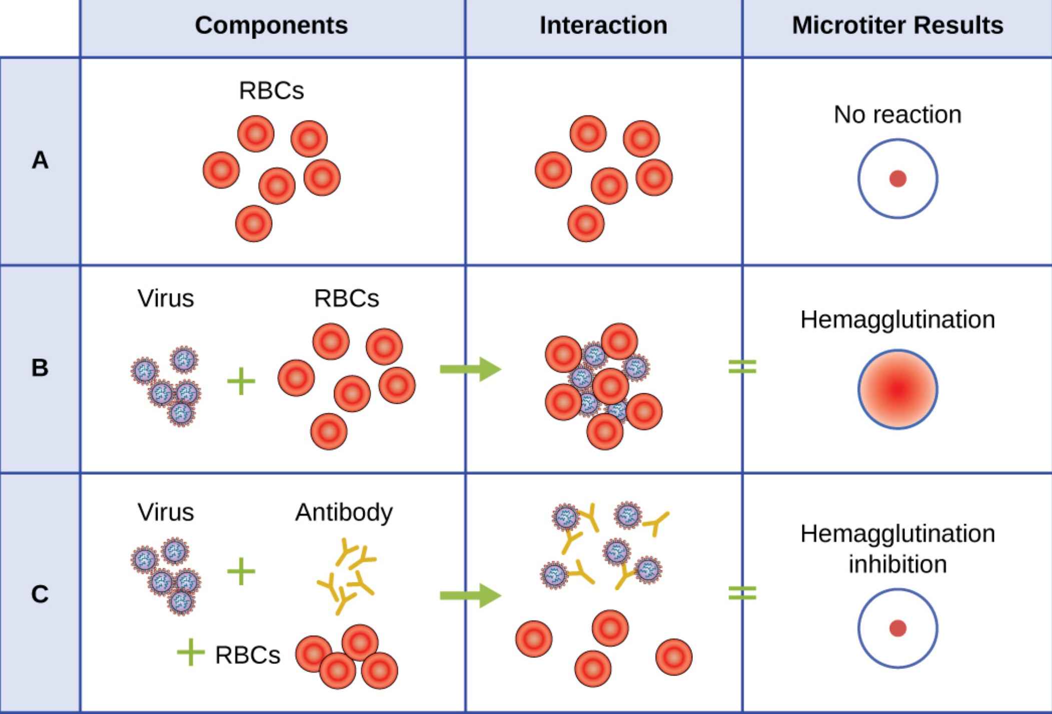


Figure 15.27 This chart shows the possible outcomes of a hemagglutination test. Row A: Erythrocytes do not bind together and will sink to the bottom of the well plate; this becomes visible as a red dot in the center of the well. Row B: Many viruses have hemagglutinins that causes agglutination of erythrocytes; the resulting hemagglutination forms a lattice structure that results in red color throughout the well. Row C: Virus-specific antibody, the viruses, and the erythrocytes are added to the well plate. The virus-specific antibodies inhibit agglutination, as can be seen as a red dot in the bottom of the well. (credit: modification of work by Centers for Disease Control and Prevention)

- What is the outcome of a positive HIA test?

Nucleic Acid Amplification Test

Nucleic acid amplification tests (NAAT) are used in molecular biology to detect unique nucleic acid sequences of viruses in patient samples. Polymerase chain reaction (PCR) is an NAAT used to detect the presence of viral DNA in a patient's tissue or body fluid sample. PCR is a technique that amplifies (i.e., synthesizes many copies) of a viral DNA segment of interest. Using PCR, short nucleotide sequences called primers bind to specific sequences of viral DNA, enabling identification of the virus.

Reverse transcriptase-PCR (RT-PCR) is an NAAT used to detect the presence of RNA viruses. RT-PCR differs from PCR in that the enzyme reverse transcriptase (RT) is used to make a cDNA from the small amount of viral RNA in the specimen. The cDNA can then be amplified by PCR. Both PCR and RT-PCR are used to detect and confirm the presence of the viral nucleic acid in patient specimens.

HPV Scare

Michelle, a 21-year-old nursing student, came to the university clinic worried that she might have been exposed to a sexually transmitted disease (STD). Her sexual partner had recently developed several bumps on the base of his penis. He had put off going to the doctor, but Michelle suspects they are genital warts caused by HPV. She is especially concerned because she knows that HPV not only causes warts but is a

prominent cause of cervical cancer. She and her partner always use condoms for contraception, but she is not confident that this precaution will protect her from HPV.

Michelle's physician finds no physical signs of genital warts or any other STDs, but recommends that Michelle get a Pap smear along with an HPV test. The Pap smear will screen for abnormal cervical cells and the CPEs associated with HPV; the HPV test will test for the presence of the virus. If both tests are negative, Michelle can be more assured that she most likely has not become infected with HPV. However, her doctor suggests it might be wise for Michelle to get vaccinated against HPV to protect herself from possible future exposure.

- Why does Michelle's physician order two different tests instead of relying on one or the other?

Enzyme Immunoassay

Enzyme immunoassays (EIAs) rely on the ability of antibodies to detect and attach to specific biomolecules called antigens. The detecting antibody attaches to the target antigen with a high degree of specificity in what might be a complex mixture of biomolecules. Also included in this type of assay is a colorless enzyme attached to the detecting antibody. The enzyme acts as a tag on the detecting antibody and can interact with a colorless substrate, leading to the production of a colored end product. EIAs often rely on layers of antibodies to capture and react with antigens, all of which are attached to a membrane filter (see **Figure 15.28**). EIAs for viral antigens are often used as preliminary screening tests. If the results are positive, further confirmation will require tests with even greater sensitivity, such as a western blot or an NAAT. EIAs are discussed in more detail in **EIAs and ELISAs**.

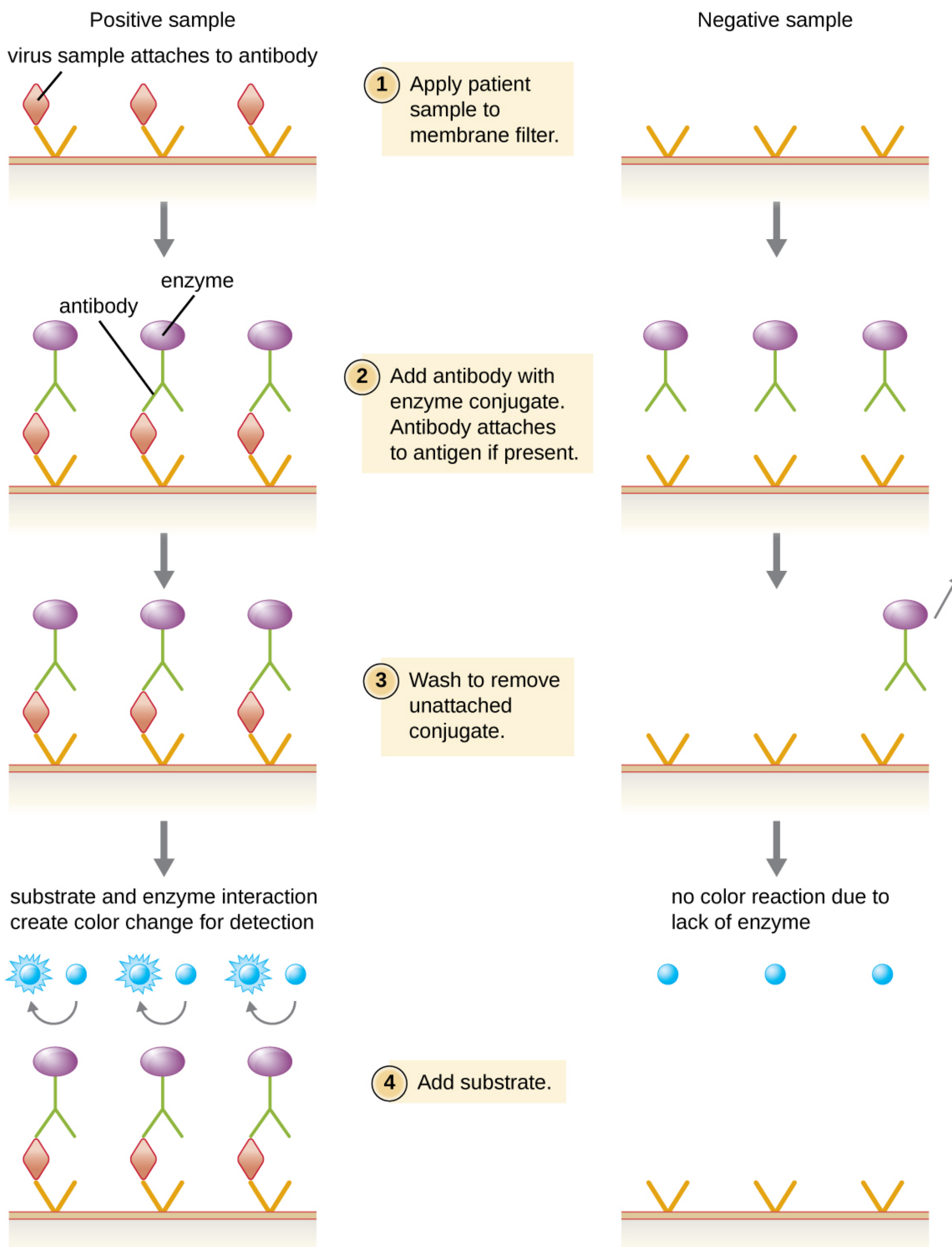


Figure 15.28 Similar to rapid, over-the-counter pregnancy tests, EIAs for viral antigens require a few drops of diluted patient serum or plasma applied to a membrane filter. The membrane filter has been previously modified and embedded with antibody to viral antigen and internal controls. Antibody conjugate is added to the filter, with the targeted antibody attached to the antigen (in the case of a positive test). Excess conjugate is washed off the filter. Substrate is added to activate the enzyme-mediated reaction to reveal the color change of a positive test. (credit: modification of work by “Cavitri”/Wikimedia Commons)

- What typically indicates a positive EIA test?

Part 3

Along with the RT/PCR analysis, David’s saliva was also collected for viral cultivation. In general, no single

diagnostic test is sufficient for antemortem diagnosis, since the results will depend on the sensitivity of the assay, the quantity of virions present at the time of testing, and the timing of the assay, since release of virions in the saliva can vary. As it turns out, the result was negative for viral cultivation from the saliva. This is not surprising to David's doctor, because one negative result is not an absolute indication of the absence of infection. It may be that the number of virions in the saliva is low at the time of sampling. It is not unusual to repeat the test at intervals to enhance the chance of detecting higher virus loads.

- Should David's doctor modify his course of treatment based on these test results?

Jump to the ***next (https://legacy.cnx.org/content/m58811/latest/#fs-id1168328231014)*** *Clinical Focus box. Go back to the* ***previous (https://legacy.cnx.org/content/m58808/latest/#fs-id1168328286182)*** *Clinical Focus box.*

Fill in the Blank

Exercise 15.1

Viruses can be diagnosed and observed using a(n) ____________ microscope.

Solution
Electron

Exercise 15.2

Cell abnormalities resulting from a viral infection are called ____________ ____________.

Solution
cytopathic effects

Short Answer

Exercise 15.3

Briefly explain the various methods of culturing viruses.

15.5 | Vaccines

Learning Objectives

By the end of this section, you will be able to:

- Compare the various kinds of artificial immunity
- Differentiate between variolation and vaccination
- Describe different types of vaccines and explain their respective advantages and disadvantages

For many diseases, prevention is the best form of treatment, and few strategies for disease prevention are as effective as vaccination. Vaccination is a form of artificial immunity. By artificially stimulating the adaptive immune defenses, a vaccine triggers memory cell production similar to that which would occur during a primary response. In so doing, the patient is able to mount a strong secondary response upon exposure to the pathogen—but without having to first suffer through an initial infection. In this section, we will explore several different kinds of artificial immunity along with various types of vaccines and the mechanisms by which they induce artificial immunity.

Classifications of Adaptive Immunity

All forms of adaptive immunity can be described as either active or passive. **Active immunity** refers to the

activation of an individual's own adaptive immune defenses, whereas **passive immunity** refers to the transfer of adaptive immune defenses from another individual or animal. Active and passive immunity can be further subdivided based on whether the protection is acquired naturally or artificially.

Natural active immunity is adaptive immunity that develops after natural exposure to a pathogen (**Figure 15.29**). Examples would include the lifelong immunity that develops after recovery from a chickenpox or measles infection (although an acute infection is not always necessary to activate adaptive immunity). The length of time that an individual is protected can vary substantially depending upon the pathogen and antigens involved. For example, activation of adaptive immunity by protein spike structures during an intracellular viral infection can activate lifelong immunity, whereas activation by carbohydrate capsule antigens during an extracellular bacterial infection may activate shorter-term immunity.

Natural passive immunity involves the natural passage of antibodies from a mother to her child before and after birth. IgG is the only antibody class that can cross the placenta from mother's blood to the fetal blood supply. Placental transfer of IgG is an important passive immune defense for the infant, lasting up to six months after birth. Secretory IgA can also be transferred from mother to infant through breast milk.

Artificial passive immunity refers to the transfer of antibodies produced by a donor (human or animal) to another individual. This transfer of antibodies may be done as a prophylactic measure (i.e., to prevent disease after exposure to a pathogen) or as a strategy for treating an active infection. For example, artificial passive immunity is commonly used for post-exposure prophylaxis against rabies, hepatitis A, hepatitis B, and chickenpox (in high risk individuals). Active infections treated by artificial passive immunity include cytomegalovirus infections in immunocompromised patients and Ebola virus infections. In 1995, eight patients in the Democratic Republic of the Congo with active Ebola infections were treated with blood transfusions from patients who were recovering from Ebola. Only one of the eight patients died (a 12.5% mortality rate), which was much lower than the expected 80% mortality rate for Ebola in untreated patients.[1] Artificial passive immunity is also used for the treatment of diseases caused by bacterial toxins, including tetanus, botulism, and diphtheria.

Artificial active immunity is the foundation for vaccination. It involves the activation of adaptive immunity through the deliberate exposure of an individual to weakened or inactivated pathogens, or preparations consisting of key pathogen antigens.

1. K. Mupapa, M. Massamba, K. Kibadi, K. Kivula, A. Bwaka, M. Kipasa, R. Colebunders, J. J. Muyembe-Tamfum. "Treatment of Ebola Hemorrhagic Fever with Blood Transfusions from Convalescent Patients." *Journal of Infectious Diseases* 179 Suppl. (1999): S18–S23.

Mechanisms of Acquisition of Immunity		
	Natural acquired	**Artificial acquired**
Passive	Immunity acquired from antibodies passed in breast milk or through placenta	Immunity gained through antibodies harvested from another person or an animal
Active	Immunity gained through illness and recovery	Immunity acquired through a vaccine

Figure 15.29 The four classifications of immunity. (credit top left photo: modification of work by USDA; credit top right photo: modification of work by "Michaelberry"/Wikimedia; credit bottom left photo: modification of work by Centers for Disease Control and Prevention; credit bottom right photo: Airman 1st Class Destinee Doughert / U.S. Air Force; Public Domain)

- What is the difference between active and passive immunity?
- What kind of immunity is conferred by a vaccine?

Herd Immunity

The four kinds of immunity just described result from an individual's adaptive immune system. For any given disease, an individual may be considered immune or susceptible depending on his or her ability to mount an effective immune response upon exposure. Thus, any given population is likely to have some individuals who are immune and other individuals who are susceptible. If a population has very few susceptible individuals, even those susceptible individuals will be protected by a phenomenon called **herd immunity**. Herd immunity has nothing to do with an individual's ability to mount an effective immune response; rather, it occurs because there are too few susceptible individuals in a population for the disease to spread effectively.

Vaccination programs create herd immunity by greatly reducing the number of susceptible individuals in a population. Even if some individuals in the population are not vaccinated, as long as a certain percentage is immune (either naturally or artificially), the few susceptible individuals are unlikely to be exposed to the pathogen. However, because new individuals are constantly entering populations (for example, through birth or relocation), vaccination programs are necessary to maintain herd immunity.

Vaccination: Obligation or Choice

A growing number of parents are choosing not to vaccinate their children. They are dubbed " antivaxxers,"

and the majority of them believe that vaccines are a cause of autism (or other disease conditions), a link that has now been thoroughly disproven. Others object to vaccines on religious or moral grounds (e.g., the argument that Gardasil vaccination against HPV may promote sexual promiscuity), on personal ethical grounds (e.g., a conscientious objection to any medical intervention), or on political grounds (e.g., the notion that mandatory vaccinations are a violation of individual liberties).[2]

It is believed that this growing number of unvaccinated individuals has led to new outbreaks of whooping cough and measles. We would expect that herd immunity would protect those unvaccinated in our population, but herd immunity can only be maintained if enough individuals are being vaccinated.

Vaccination is clearly beneficial for public health. But from the individual parent's perspective the view can be murkier. Vaccines, like all medical interventions, have associated risks, and while the risks of vaccination may be extremely low compared to the risks of infection, parents may not always understand or accept the consensus of the medical community. Do such parents have a right to withhold vaccination from their children? Should they be allowed to put their children—and society at large—at risk?

Many governments insist on childhood vaccinations as a condition for entering public school, but it has become easy in most states to opt out of the requirement or to keep children out of the public system. Since the 1970s, West Virginia and Mississippi have had in place a stringent requirement for childhood vaccination, without exceptions, and neither state has had a case of measles since the early 1990s. California lawmakers recently passed a similar law in response to a measles outbreak in 2015, making it much more difficult for parents to opt out of vaccines if their children are attending public schools. Given this track record and renewed legislative efforts, should other states adopt similarly strict requirements?

What role should health-care providers play in promoting or enforcing universal vaccination? Studies have shown that many parents' minds can be changed in response to information delivered by health-care workers, but is it the place of health-care workers to try to persuade parents to have their children vaccinated? Some health-care providers are understandably reluctant to treat unvaccinated patients. Do they have the right to refuse service to patients who decline vaccines? Do insurance companies have the right to deny coverage to antivaxxers? These are all ethical questions that policymakers may be forced to address as more parents skirt vaccination norms.

Variolation and Vaccination

Thousands of years ago, it was first recognized that individuals who survived a smallpox infection were immune to subsequent infections. The practice of inoculating individuals to actively protect them from smallpox appears to have originated in the 10th century in China, when the practice of **variolation** was described (**Figure 15.30**). Variolation refers to the deliberate inoculation of individuals with infectious material from scabs or pustules of smallpox victims. Infectious materials were either injected into the skin or introduced through the nasal route. The infection that developed was usually milder than naturally acquired smallpox, and recovery from the milder infection provided protection against the more serious disease.

Although the majority of individuals treated by variolation developed only mild infections, the practice was not without risks. More serious and sometimes fatal infections did occur, and because smallpox was contagious, infections resulting from variolation could lead to epidemics. Even so, the practice of variolation for smallpox prevention spread to other regions, including India, Africa, and Europe.

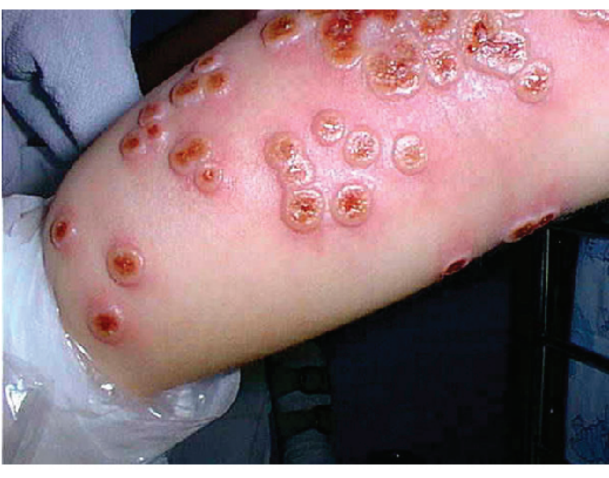
2. Elizabeth Yale. "Why Anti-Vaccination Movements Can Never Be Tamed." *Religion & Politics*, July 22, 2014. http://religionandpolitics.org/2014/07/22/why-anti-vaccination-movements-can-never-be-tamed.

Figure 15.30 Variolation for smallpox originated in the Far East and the practice later spread to Europe and Africa. This Japanese relief depicts a patient receiving a smallpox variolation from the physician Ogata Shunsaku (1748–1810).

Although variolation had been practiced for centuries, the English physician Edward Jenner (1749–1823) is generally credited with developing the modern process of vaccination. Jenner observed that milkmaids who developed cowpox, a disease similar to smallpox but milder, were immune to the more serious smallpox. This led Jenner to hypothesize that exposure to a less virulent pathogen could provide immune protection against a more virulent pathogen, providing a safer alternative to variolation. In 1796, Jenner tested his hypothesis by obtaining infectious samples from a milkmaid's active cowpox lesion and injecting the materials into a young boy (**Figure 15.31**). The boy developed a mild infection that included a low-grade fever, discomfort in his axillae (armpit) and loss of appetite. When the boy was later infected with infectious samples from smallpox lesions, he did not contract smallpox.[3] This new approach was termed **vaccination**, a name deriving from the use of cowpox (Latin *vacca* meaning "cow") to protect against smallpox. Today, we know that Jenner's vaccine worked because the cowpox virus is genetically and antigenically related to the *Variola* viruses that caused smallpox. Exposure to cowpox antigens resulted in a primary response and the production of memory cells that identical or related epitopes of Variola virus upon a later exposure to smallpox.

The success of Jenner's smallpox vaccination led other scientists to develop vaccines for other diseases. Perhaps the most notable was Louis Pasteur, who developed vaccines for rabies, cholera, and anthrax. During the 20th and 21st centuries, effective vaccines were developed to prevent a wide range of diseases caused by viruses (e.g., chickenpox and shingles, hepatitis, measles, mumps, polio, and yellow fever) and bacteria (e.g., diphtheria, pneumococcal pneumonia, tetanus, and whooping cough,).

3. N. J. Willis. "Edward Jenner and the Eradication of Smallpox." *Scottish Medical Journal* 42 (1997): 118–121.



(a) (b)

Figure 15.31 (a) A painting of Edward Jenner depicts a cow and a milkmaid in the background. (b) Lesions on a patient infected with cowpox, a zoonotic disease caused by a virus closely related to the one that causes smallpox. (credit b: modification of work by the Centers for Disease Control and Prevention)

- What is the difference between variolation and vaccination for smallpox?
- Explain why vaccination is less risky than variolation.

Classes of Vaccines

For a vaccine to provide protection against a disease, it must expose an individual to pathogen-specific antigens that will stimulate a protective adaptive immune response. By its very nature, this entails some risk. As with any pharmaceutical drug, vaccines have the potential to cause adverse effects. However, the ideal vaccine causes no severe adverse effects and poses no risk of contracting the disease that it is intended to prevent. Various types of vaccines have been developed with these goals in mind. These different classes of vaccines are described in the next section and summarized in **Table 15.1**.

Live Attenuated Vaccines

Live attenuated vaccines expose an individual to a weakened strain of a pathogen with the goal of establishing a subclinical infection that will activate the adaptive immune defenses. Pathogens are attenuated to decrease their virulence using methods such as genetic manipulation (to eliminate key virulence factors) or long-term culturing in an unnatural host or environment (to promote mutations and decrease virulence).

By establishing an active infection, live attenuated vaccines stimulate a more comprehensive immune response than some other types of vaccines. Live attenuated vaccines activate both cellular and humoral immunity and stimulate the development of memory for long-lasting immunity. In some cases, vaccination of one individual with a live attenuated pathogen can even lead to natural transmission of the attenuated pathogen to other individuals. This can cause the other individuals to also develop an active, subclinical infection that activates their adaptive immune defenses.

Disadvantages associated with live attenuated vaccines include the challenges associated with long-term storage and transport as well as the potential for a patient to develop signs and symptoms of disease during the active infection (particularly in immunocompromised patients). There is also a risk of the attenuated pathogen reverting back to full virulence. **Table 15.1** lists examples live attenuated vaccines.

Inactivated Vaccines

Inactivated vaccines contain whole pathogens that have been killed or inactivated with heat, chemicals, or radiation. For inactivated vaccines to be effective, the inactivation process must not affect the structure of key antigens on the pathogen.

Because the pathogen is killed or inactive, inactivated vaccines do not produce an active infection, and the resulting immune response is weaker and less comprehensive than that provoked by a live attenuated vaccine. Typically the response involves only humoral immunity, and the pathogen cannot be transmitted to other individuals. In addition, inactivated vaccines usually require higher doses and multiple boosters, possibly causing

inflammatory reactions at the site of injection.

Despite these disadvantages, inactivated vaccines do have the advantages of long-term storage stability and ease of transport. Also, there is no risk of causing severe active infections. However, inactivated vaccines are not without their side effects. **Table 15.1** lists examples of inactivated vaccines.

Subunit Vaccines

Whereas live attenuated and inactive vaccines expose an individual to a weakened or dead pathogen, **subunit vaccines** only expose the patient to the key antigens of a pathogen—not whole cells or viruses. Subunit vaccines can be produced either by chemically degrading a pathogen and isolating its key antigens or by producing the antigens through genetic engineering. Because these vaccines contain only the essential antigens of a pathogen, the risk of side effects is relatively low. **Table 15.1** lists examples of subunit vaccines.

Toxoid Vaccines

Like subunit vaccines, **toxoid vaccines** do not introduce a whole pathogen to the patient; they contain inactivated bacterial toxins, called toxoids. Toxoid vaccines are used to prevent diseases in which bacterial toxins play an important role in pathogenesis. These vaccines activate humoral immunity that neutralizes the toxins. **Table 15.1** lists examples of toxoid vaccines.

Conjugate Vaccines

A **conjugate vaccine** is a type of subunit vaccine that consists of a protein conjugated to a capsule polysaccharide. Conjugate vaccines have been developed to enhance the efficacy of subunit vaccines against pathogens that have protective polysaccharide capsules that help them evade phagocytosis, causing invasive infections that can lead to meningitis and other serious conditions. The subunit vaccines against these pathogens introduce T-independent capsular polysaccharide antigens that result in the production of antibodies that can opsonize the capsule and thus combat the infection; however, children under the age of two years do not respond effectively to these vaccines. Children do respond effectively when vaccinated with the conjugate vaccine, in which a protein with T-dependent antigens is conjugated to the capsule polysaccharide. The conjugated protein-polysaccharide antigen stimulates production of antibodies against both the protein and the capsule polysaccharide. **Table 15.1** lists examples of conjugate vaccines.

Classes of Vaccines

<table>
<tr><th>Class</th><th>Description</th><th>Advantages</th><th>Disadvantages</th><th>Examples</th></tr>
<tr><td rowspan="3">Live attenuated</td><td rowspan="3">Weakened strain of whole pathogen</td><td>Cellular and humoral immunity</td><td>Difficult to store and transport</td><td rowspan="3">Chickenpox, German measles, measles, mumps, tuberculosis, typhoid fever, yellow fever</td></tr>
<tr><td>Long-lasting immunity</td><td>Risk of infection in immunocompromised patients</td></tr>
<tr><td>Transmission to contacts</td><td>Risk of reversion</td></tr>
<tr><td rowspan="2">Inactivated</td><td rowspan="2">Whole pathogen killed or inactivated with heat, chemicals, or radiation</td><td>Ease of storage and transport</td><td>Weaker immunity (humoral only)</td><td rowspan="2">Cholera, hepatitis A, influenza, plague, rabies</td></tr>
<tr><td>No risk of severe active infection</td><td>Higher doses and more boosters required</td></tr>
<tr><td rowspan="3">Subunit</td><td rowspan="3">Immunogenic antigens</td><td rowspan="3">Lower risk of side effects</td><td>Limited longevity</td><td rowspan="3">Anthrax, hepatitis B, influenza, meningitis, papillomavirus, pneumococcal pneumonia, whooping cough</td></tr>
<tr><td>Multiple doses required</td></tr>
<tr><td>No protection against antigenic variation</td></tr>
</table>

Table 15.1

Classes of Vaccines

Class	Description	Advantages	Disadvantages	Examples
Toxoid	Inactivated bacterial toxin	Humoral immunity to neutralize toxin	Does not prevent infection	Botulism, diphtheria, pertussis, tetanus
Conjugate	Capsule polysaccharide conjugated to protein	T-dependent response to capsule	Costly to produce	Meningitis (*Haemophilus influenzae*, *Streptococcus pneumoniae*, *Neisseria meningitides*)
			No protection against antigenic variation	
		Better response in young children	May interfere with other vaccines	

Table 15.1

- What is the risk associated with a live attenuated vaccine?
- Why is a conjugated vaccine necessary in some cases?

DNA Vaccines

DNA vaccines represent a relatively new and promising approach to vaccination. A DNA vaccine is produced by incorporating genes for antigens into a recombinant plasmid vaccine. Introduction of the DNA vaccine into a patient leads to uptake of the recombinant plasmid by some of the patient's cells, followed by transcription and translation of antigens and presentation of these antigens with MHC I to activate adaptive immunity. This results in the stimulation of both humoral and cellular immunity without the risk of active disease associated with live attenuated vaccines.

Although most DNA vaccines for humans are still in development, it is likely that they will become more prevalent in the near future as researchers are working on engineering DNA vaccines that will activate adaptive immunity against several different pathogens at once. First-generation DNA vaccines tested in the 1990s looked promising in animal models but were disappointing when tested in human subjects. Poor cellular uptake of the DNA plasmids was one of the major problems impacting their efficacy. Trials of second-generation DNA vaccines have been more promising thanks to new techniques for enhancing cellular uptake and optimizing antigens. DNA vaccines for various cancers and viral pathogens such as HIV, HPV, and hepatitis B and C are currently in development.

Some DNA vaccines are already in use. In 2005, a DNA vaccine against West Nile virus was approved for use in horses in the United States. Canada has also approved a DNA vaccine to protect fish from infectious hematopoietic necrosis virus.[4] A DNA vaccine against Japanese encephalitis virus was approved for use in humans in 2010 in Australia.[5]

Resolution

Based on Olivia's symptoms, her physician made a preliminary diagnosis of bacterial meningitis without waiting for positive identification from the blood and CSF samples sent to the lab. Olivia was admitted to the hospital and treated with intravenous broad-spectrum antibiotics and rehydration therapy. Over the next several days, her condition began to improve, and new blood samples and lumbar puncture samples showed an absence of microbes in the blood and CSF with levels of white blood cells returning to normal. During this time, the lab produced a positive identification of *Neisseria meningitidis*, the causative agent of meningococcal meningitis, in her original CSF sample.

N. meningitidis produces a polysaccharide capsule that serves as a virulence factor. *N. meningitidis* tends

4. M. Alonso and J. C. Leong. "Licensed DNA Vaccines Against Infectious Hematopoietic Necrosis Virus (IHNV)." *Recent Patents on DNA & Gene Sequences (Discontinued)* 7 no. 1 (2013): 62–65, issn 1872-2156/2212-3431. doi 10.2174/1872215611307010009.
5. S.B. Halstead and S. J. Thomas. "New Japanese Encephalitis Vaccines: Alternatives to Production in Mouse Brain." *Expert Review of Vaccines* 10 no. 3 (2011): 355–64.

to affect infants after they begin to lose the natural passive immunity provided by maternal antibodies. At one year of age, Olivia's maternal IgG antibodies would have disappeared, and she would not have developed memory cells capable of recognizing antigens associated with the polysaccharide capsule of the *N. meningitidis.* As a result, her adaptive immune system was unable to produce protective antibodies to combat the infection, and without antibiotics she may not have survived. Olivia's infection likely would have been avoided altogether had she been vaccinated. A conjugate vaccine to prevent meningococcal meningitis is available and approved for infants as young as two months of age. However, current vaccination schedules in the United States recommend that the vaccine be administered at age 11–12 with a booster at age 16.

*Go back to the **previous (https://legacy.cnx.org/content/m58886/latest/#fs-id1167662393469)** Clinical Focus box.*

In countries with developed public health systems, many vaccines are routinely administered to children and adults. Vaccine schedules are changed periodically, based on new information and research results gathered by public health agencies. In the United States, the CDC publishes **schedules and other updated information (https://www.openstax.org/l/22CDCVacSched)** about vaccines.

Matching

Exercise 15.4

Match each type of vaccine with the corresponding example.

___inactivated vaccine	A. Weakened influenza virions that can only replicate in the slightly lower temperatures of the nasal passages are sprayed into the nose. They do not cause serious flu symptoms, but still produce an active infection that induces a protective adaptive immune response.
___live attenuated vaccine	B. Tetanus toxin molecules are harvested and chemically treated to render them harmless. They are then injected into a patient's arm.
___toxoid vaccine	C. Influenza virus particles grown in chicken eggs are harvested and chemically treated to render them noninfectious. These immunogenic particles are then purified and packaged and administered as an injection.
___subunit vaccine	D. The gene for hepatitis B virus surface antigen is inserted into a yeast genome. The modified yeast is grown and the virus protein is produced, harvested, purified, and used in a vaccine.

Solution
C, A, B, D

Fill in the Blank

Exercise 15.5

A(n) ________ pathogen is in a weakened state; it is still capable of stimulating an immune response but does not cause a disease.

Solution
attenuated

Exercise 15.6

________ immunity occurs when antibodies from one individual are harvested and given to another to protect

against disease or treat active disease.

Solution
Artificial passive

Exercise 15.7

In the practice of ________, scabs from smallpox victims were used to immunize susceptible individuals against smallpox.

Solution
variolation

Short answer

Exercise 15.8

Briefly compare the pros and cons of inactivated versus live attenuated vaccines.

KEY TERMS

acellular lacking cells

active immunity an immunity that occurs as a result of the activity of the body's own cells rather than from antibodies acquired from an external source

adaptive immunity a specific immune response that occurs after exposure to an antigen either from a pathogen or a vaccination

antibody a protein that is produced by plasma cells after stimulation by an antigen; also known as an immunoglobulin

antigen a macromolecule that reacts with cells of the immune system and which may or may not have a stimulatory effect

antigen-presenting cell (APC) an immune cell that detects, engulfs, and informs the adaptive immune response about an infection by presenting the processed antigen on its cell surface

apoptosis the cell death caused by induction of a cell's own internal mechanisms either as a natural step in the development of a multicellular organism or by other environmental factors such as signals from cells of the immune system

attenuation the weakening of a virus during vaccine development

B cell a lymphocyte that matures in the bone marrow

capsid the protein coating of the viral core

cell-mediated immune response an adaptive immune response that is controlled by T cells

complement system an array of approximately 20 soluble proteins of the innate immune system that enhance phagocytosis, bore holes in pathogens, and recruit lymphocytes

cytokine a chemical messenger that regulates cell differentiation, proliferation, and gene expression to effect immune responses

cytopathic causing cell damage

cytotoxic T lymphocyte (T_C) an adaptive immune cell that directly kills infected cells via enzymes, and that releases cytokines to enhance the immune response

dendritic cell an immune cell that processes antigen material and presents it on the surface of its cell in MHC class II molecules and induces an immune response in other cells

effector cell a lymphocyte that has differentiated, such as a B cell, plasma cell, or cytotoxic T cell

glycoprotein a protein molecule with attached carbohydrate molecules

helper T lymphocyte (T_H) a cell of the adaptive immune system that binds APCs via MHC class II molecules and stimulates B cells or secretes cytokines to initiate the immune response

humoral immune response the adaptive immune response that is controlled by activated B cells and antibodies

immune tolerance an acquired ability to prevent an unnecessary or harmful immune response to a detected foreign body known not to cause disease

inflammation the localized redness, swelling, heat, and pain that results from the movement of leukocytes through opened capillaries to a site of infection

innate immunity an immunity that occurs naturally because of genetic factors or physiology, and is not caused

by infection or vaccination

interferon a cytokine that inhibits viral replication

lymph the watery fluid present in the lymphatic circulatory system that bathes tissues and organs with protective white blood cells and does not contain erythrocytes

lymphocyte a type of white blood cell that includes natural killer cells of the innate immune system and B and T cells of the adaptive immune system

macrophage a large phagocytic cell that engulfs foreign particles and pathogens

major histocompatibility class (MHC) I a group of proteins found on the surface of all nucleated cells that signals to immune cells whether the cell is normal or is infected or cancerous; it also provides the appropriate sites into which antigens can be loaded for recognition by lymphocytes

major histocompatibility class (MHC) II molecule a protein found on the surface of antigen-presenting cells that signals to immune cells whether the cell is normal or is infected or cancerous; it provides the appropriate template into which antigens can be loaded for recognition by lymphocytes

mast cell a leukocyte that produces inflammatory molecules, such as histamine, in response to large pathogens

memory cell an antigen-specific B or T lymphocyte that does not differentiate into an effector cell during the primary immune response but that can immediately become an effector cell on reexposure to the same pathogen

monocyte a type of white blood cell that circulates in the blood and lymph and differentiates into a macrophage after it moves into infected tissue

natural killer (NK) cell a lymphocyte that can kill cells infected with viruses or tumor cells

neutrophil a phagocytic leukocyte that engulfs and digests pathogens

passive immunity an immunity that does not result from the activity of the body's own immune cells but by transfer of antibodies from one individual to another

primary immune response the response of the adaptive immune system to the first exposure to an antigen

secondary immune response the response of the adaptive immune system to a second or later exposure to an antigen mediated by memory cells

T cell a lymphocyte that matures in the thymus gland

vaccine a weakened solution of virus components, viruses, or other agents that produce an immune response

viral envelope a lipid bilayer that envelops some viruses

virion an individual virus particle outside a host cell

white blood cell a nucleated cell found in the blood that is a part of the immune system; also called leukocytes

CHAPTER SUMMARY

15.1 Viruses

Viruses are acellular entities that can usually only be seen with an electron microscope. Their genomes contain either DNA or RNA, and they replicate using the replication proteins of a host cell. Viruses are diverse, infecting archaea, bacteria, fungi, plants, and animals. Viruses consist of a nucleic-acid core surrounded by a protein capsid with or without an outer lipid envelope.

Viral replication within a living cell always produces changes in the cell, sometimes resulting in cell death and sometimes slowly killing the infected cells. There are six basic stages in the virus replication cycle: attachment, penetration, uncoating, replication, assembly, and release. A viral infection may be productive, resulting in new

virions, or nonproductive, meaning the virus remains inside the cell without producing new virions.

Viruses cause a variety of diseases in humans. Many of these diseases can be prevented by the use of viral vaccines, which stimulate protective immunity against the virus without causing major disease. Viral vaccines may also be used in active viral infections, boosting the ability of the immune system to control or destroy the virus. Antiviral drugs that target enzymes and other protein products of viral genes have been developed and used with mixed success. Combinations of anti-HIV drugs have been used to effectively control the virus, extending the lifespan of infected individuals.

15.2 Innate Immunity

The innate immune system consists first of physical and chemical barriers to infection including the skin and mucous membranes and their secretions, ciliated surfaces, and body hairs. The second line of defense is an internal defense system designed to counter pathogenic threats that bypass the physical and chemical barriers of the body. Using a combination of cellular and molecular responses, the innate immune system identifies the nature of a pathogen and responds with inflammation, phagocytosis, cytokine release, destruction by NK cells, or the complement system.

15.3 Adaptive Immunity

The adaptive immune response is a slower-acting, longer-lasting, and more specific response than the innate response. However, the adaptive response requires information from the innate immune system to function. APCs display antigens on MHC molecules to naïve T cells. T cells with cell-surface receptors that bind a specific antigen will bind to that APC. In response, the T cells differentiate and proliferate, becoming T_H cells or T_C cells. T_H cells stimulate B cells that have engulfed and presented pathogen-derived antigens. B cells differentiate into plasma cells that secrete antibodies, whereas T_C cells destroy infected or cancerous cells. Memory cells are produced by activated and proliferating B and T cells and persist after a primary exposure to a pathogen. If re-exposure occurs, memory cells differentiate into effector cells without input from the innate immune system. The mucosal immune system is largely independent of the systemic immune system but functions in parallel to protect the extensive mucosal surfaces of the body. Immune tolerance is brought about by T_{reg} cells to limit reactions to harmless antigens and the body’s own molecules.

15.4 Isolation, Culture, and Identification of Viruses

- Viral cultivation requires the presence of some form of host cell (whole organism, embryo, or cell culture).
- Viruses can be isolated from samples by filtration.
- Viral filtrate is a rich source of released virions.
- Bacteriophages are detected by presence of clear **plaques** on bacterial lawn.
- Animal and plant viruses are detected by **cytopathic effects**, molecular techniques (PCR, RT-PCR), enzyme immunoassays, and serological assays (hemagglutination assay, hemagglutination inhibition assay).

15.5 Vaccines

- Adaptive immunity can be divided into four distinct classifications: **natural active immunity, natural passive immunity, artificial passive immunity,** and **artificial active immunity.**
- Artificial active immunity is the foundation for **vaccination** and vaccine development. Vaccination programs not only confer artificial immunity on individuals, but also foster **herd immunity** in populations.
- **Variolation** against smallpox originated in the 10th century in China, but the procedure was risky because it could cause the disease it was intended to prevent. Modern vaccination was developed by Edward Jenner, who developed the practice of inoculating patients with infectious materials from cowpox lesions to prevent smallpox.
- **Live attenuated vaccines** and **inactivated vaccines** contain whole pathogens that are weak, killed, or inactivated. **Subunit vaccines, toxoid vaccines,** and **conjugate vaccines** contain acellular components

with antigens that stimulate an immune response.

VISUAL CONNECTION QUESTIONS

15.1 Viruses

9. Figure 15.5 Which of the following statements about virus structure is true?

a. All viruses are encased in a viral membrane.
b. The capsomere is made up of small protein subunits called capsids.
c. DNA is the genetic material in all viruses.
d. Glycoproteins help the virus attach to the host cell.

10. Figure 15.6 Influenza virus is packaged in a viral envelope, which fuses with the plasma membrane. This way, the virus can exit the host cell without killing it. What advantage does the virus gain by keeping the host cell alive?

15.3 Adaptive Immunity

11. Figure 15.17 The Rh antigen is found on Rh-positive red blood cells. An Rh-negative female can usually carry an Rh-positive fetus to term without difficulty. However, if she has a second Rh-positive fetus, her body may launch an immune attack that causes hemolytic disease of the newborn. Why do you think hemolytic disease is only a problem during the second or subsequent pregnancies?

REVIEW QUESTIONS

12. Which statement is true?

a. A virion contains DNA and RNA.
b. Viruses are acellular.
c. Viruses replicate outside of the cell.
d. Most viruses are easily visualized with a light microscope.

13. The viral ________ plays a role in attaching a virion to the host cell.

a. core
b. capsid
c. envelope
d. both b and c

14. Which statement is true of viral replication?

a. In the process of apoptosis, the cell survives.
b. During attachment, the virus attaches at specific sites on the cell surface.
c. The viral capsid helps the host cell produce more copies of the viral genome.
d. mRNA works outside of the host cell to produce enzymes and proteins.

15. Which of the following is a barrier against pathogens provided by the skin?

a. low pH
b. mucus
c. tears
d. cilia

16. Although interferons have several effects, they are particularly useful against infections with which type of pathogen?

a. bacteria
b. viruses
c. fungi
d. helminths

17. Which innate immune system component uses MHC class I molecules directly in its defense strategy?

a. macrophages
b. neutrophils
c. NK cells
d. interferon

18. The humoral immune response depends on which cells?

a. T_C cells
b. B cells
c. B and T_H cells
d. T_C and T_H cells

19. The fact that the body does not normally mount an immune response to the molecules in food is an example of _______.

a. secondary immune response
b. immunological memory
c. immune tolerance
d. passive immunity

20. Foreign particles circulating in the blood are filtered by the ____________.

a. spleen
b. lymph nodes
c. MALT
d. lymph

21. Which of the followings cannot be used to culture viruses?

A. tissue culture
B. liquid medium only
C. embryo
D. animal host

22. Which of the following tests can be used to detect the presence of a specific virus?

A. EIA
B. RT-PCR
C. PCR
D. all of the above

23. Which of the following is NOT a cytopathic effect?

A. transformation
B. cell fusion
C. mononucleated cell
D. inclusion bodies

24. A patient is bitten by a dog with confirmed rabies infection. After treating the bite wound, the physician injects the patient with antibodies that are specific for the rabies virus to prevent the development of an active infection. This is an example of:

a. Natural active immunity
b. Artificial active immunity
c. Natural passive immunity
d. Artificial passive immunity

25. A patient gets a cold, and recovers a few days later. The patient's classmates come down with the same cold roughly a week later, but the original patient does not get the same cold again. This is an example of:

a. Natural active immunity
b. Artificial active immunity
c. Natural passive immunity
d. Artificial passive immunity

CRITICAL THINKING QUESTIONS

26. Why can't dogs catch the measles?

27. Why is immunization after being bitten by a rabid animal so effective?

28. Different MHC class I molecules between donor and recipient cells can lead to rejection of a transplanted organ or tissue. Suggest a reason for this.

29. If a series of genetic mutations prevented some, but not all, of the complement proteins from binding antibodies or pathogens, would the entire complement system be compromised?

30. How do B and T cells differ with respect to antigens that they bind?

31. Why is the immune response after reinfection much faster than the adaptive immune response after the initial infection?

32. Label the components indicated by arrows.

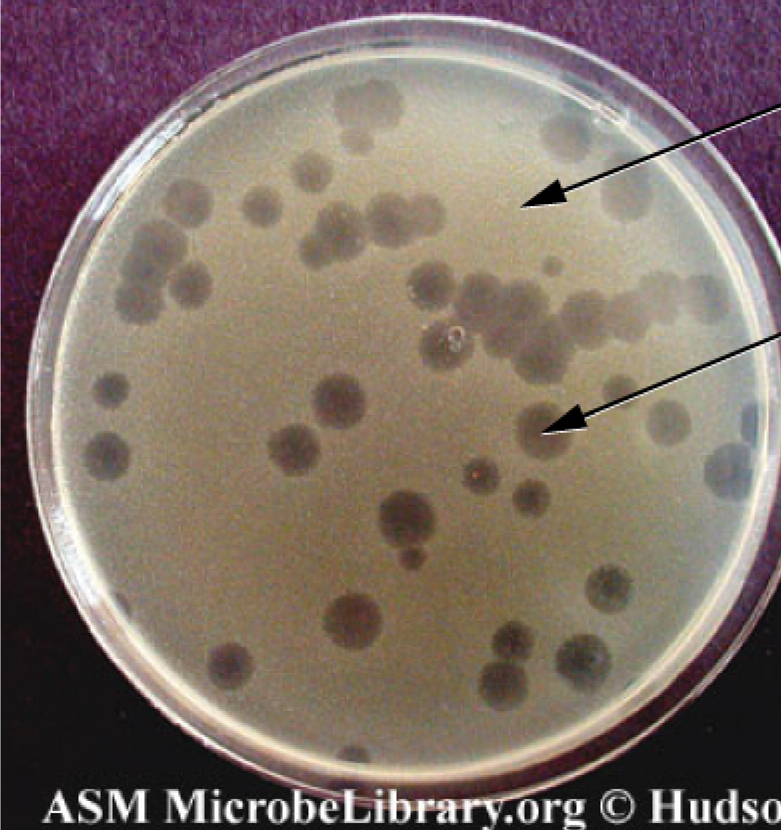


Figure 15.32 (credit: modification of work by American Society for Microbiology)

33. What are some characteristics of the viruses that are similar to a computer virus?

16 | IMMUNOCHEMISTRY

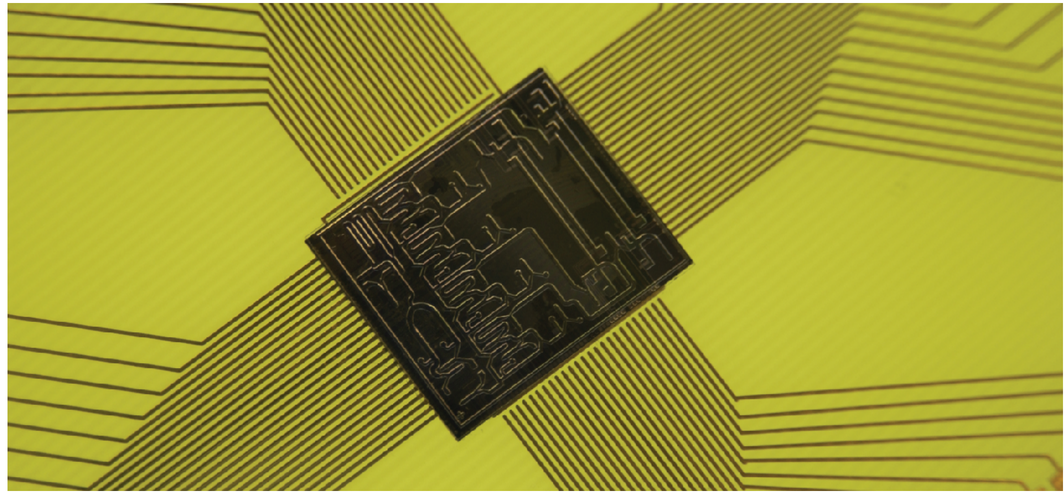


Figure 16.1 Lab-on-a-chip technology allows immunological assays to be miniaturized so tests can be done rapidly with minimum quantities of expensive reagents. The chips contain tiny flow tubes to allow movement of fluids by capillary action, reactions sites with embedded reagents, and data output through electronic sensors. (credit: modification of work by Maggie Bartlett, NHGRI)

Chapter Outline

16.1: Polyclonal and Monoclonal Antibody Production
16.2: Detecting Antigen-Antibody Complexes
16.3: Agglutination Assays
16.4: EIAs and ELISAs
16.5: Fluorescent Antibody Techniques

Introduction

Many laboratory tests are designed to confirm a presumptive diagnosis by detecting antibodies specific to a suspected pathogen. Unfortunately, many such tests are time-consuming and expensive. That is now changing, however, with the development of new, miniaturized technologies that are fast and inexpensive. For example, researchers at Columbia University are developing a “lab-on-a-chip” technology that will test a single drop of blood for 15 different infectious diseases, including HIV and syphilis, in a matter of minutes.[1] The blood is pulled through tiny capillaries into reaction chambers where the patient’s antibodies mix with reagents. A chip reader that attaches to a cell phone analyzes the results and sends them to the patient’s healthcare provider. Currently the device is being field tested in Rwanda to check pregnant women for chronic diseases. Researchers estimate that the chip readers will sell for about $100 and individual chips for $1.[2]

16.1 | Polyclonal and Monoclonal Antibody Production

1. Chin, Curtis D. et al., “Mobile Device for Disease Diagnosis and Data Tracking in Resource-Limited Settings,” *Clinical Chemistry* 59, no. 4 (2013): 629-40.
2. Evarts, H., “Fast, Low-Cost Device Uses the Cloud to Speed Up Testing for HIV and More,” January 24, 2013. Accessed July 14, 2016. http://engineering.columbia.edu/fast-low-cost-device-uses-cloud-speed-diagnostic-testing-hiv-and-more.

Learning Objectives

By the end of this section, you will be able to:

- Compare the method of development, use, and characteristics of monoclonal and polyclonal antibodies
- Explain the nature of antibody cross-reactivity and why this is less of a problem with monoclonal antibodies

Part 1

In an unfortunate incident, a healthcare worker struggling with addiction was caught stealing syringes of painkillers and replacing them with syringes filled with unknown substances. The hospital immediately fired the employee and had him arrested; however, two patients that he had worked with later tested positive for HIV.

While there was no proof that the infections originated from the tainted syringes, the hospital's public health physician took immediate steps to determine whether any other patients had been put at risk. Although the worker had only been employed for a short time, it was determined that he had come into contact with more than 1300 patients. The hospital decided to contact all of these patients and have them tested for HIV.

- Why does the hospital feel it is necessary to test every patient for HIV?
- What types of tests can be used to determine if a patient has HIV?

*Jump to the **next** Clinical Focus box.*

In addition to being crucial for our normal immune response, antibodies provide powerful tools for research and diagnostic purposes. The high specificity of antibodies makes them an excellent tool for detecting and quantifying a broad array of targets, from drugs to serum proteins to microorganisms. With *in vitro* assays, antibodies can be used to precipitate soluble antigens, agglutinate (clump) cells, opsonize and kill bacteria with the assistance of complement, and neutralize drugs, toxins, and viruses.

An antibody's **specificity** results from the antigen-binding site formed within the variable regions—regions of the antibody that have unique patterns of amino acids that can only bind to target antigens with a molecular sequence that provides complementary charges and noncovalent bonds. There are limitations to antibody specificity, however. Some antigens are so chemically similar that cross-reactivity occurs; in other words, antibodies raised against one antigen bind to a chemically similar but different antigen. Consider an antigen that consists of a single protein with multiple epitopes (**Figure 16.2**). This single protein may stimulate the production of many different antibodies, some of which may bind to chemically identical epitopes on other proteins.

Cross-reactivity is more likely to occur between antibodies and antigens that have low **affinity** or **avidity**. Affinity, which can be determined experimentally, is a measure of the binding strength between an antibody's binding site and an epitope, whereas avidity is the total strength of all the interactions in an antibody-antigen complex (which may have more than one bonding site). Avidity is influenced by affinity as well as the structural arrangements of the epitope and the variable regions of the antibody. If an antibody has a high affinity/avidity for a specific antigen, it is less likely to cross-react with an antigen for which it has a lower affinity/avidity.

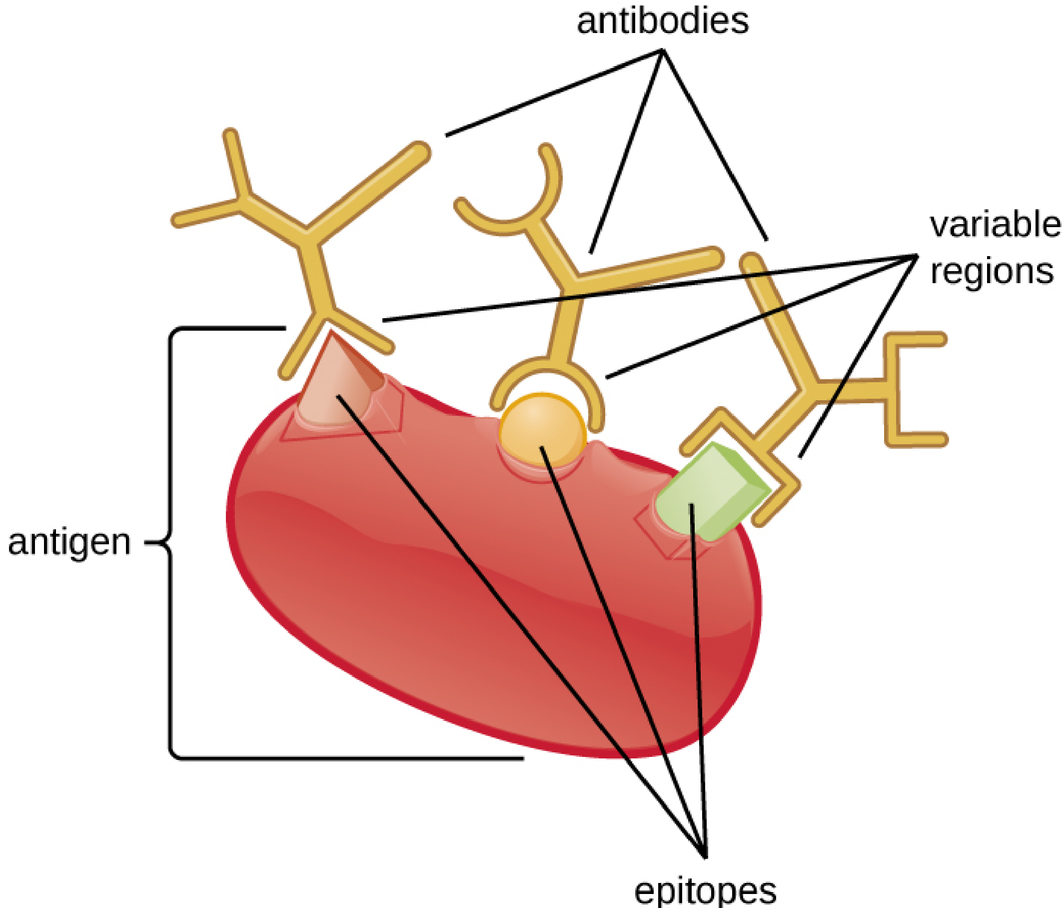


Figure 16.2 An antibody binds to a specific region on an antigen called an epitope. A single antigen can have multiple epitopes for different, specific antibodies.

- What property makes antibodies useful for research and clinical diagnosis?
- What is cross-reactivity and why does it occur?

Producing Polyclonal Antibodies

Antibodies used for research and diagnostic purposes are often obtained by injecting a lab animal such as a rabbit or a goat with a specific antigen. Within a few weeks, the animal's immune system will produce high levels of antibodies specific for the antigen. These antibodies can be harvested in an **antiserum**, which is whole serum collected from an animal following exposure to an antigen. Because most antigens are complex structures with multiple epitopes, they result in the production of multiple antibodies in the lab animal. This so-called **polyclonal antibody** response is also typical of the response to infection by the human immune system. Antiserum drawn from an animal will thus contain antibodies from multiple clones of B cells, with each B cell responding to a specific epitope on the antigen (Figure 16.3).

Lab animals are usually injected at least twice with antigen when being used to produce antiserum. The second injection will activate memory cells that make class IgG antibodies against the antigen. The memory cells also undergo **affinity maturation**, resulting in a pool of antibodies with higher average affinity. Affinity maturation occurs because of mutations in the immunoglobulin gene variable regions, resulting in B cells with slightly altered antigen-binding sites. On re-exposure to the antigen, those B cells capable of producing antibody with higher affinity antigen-binding sites will be stimulated to proliferate and produce more antibody than their lower-affinity peers. An adjuvant, which is a chemical that provokes a generalized activation of the immune system that stimulates greater antibody production, is often mixed with the antigen prior to injection.

Antiserum obtained from animals will not only contain antibodies against the antigen artificially introduced in the laboratory, but it will also contain antibodies to any other antigens to which the animal has been exposed during its lifetime. For this reason, antisera must first be "purified" to remove other antibodies before using the

antibodies for research or diagnostic assays.

1 Inject antigen into rabbit.
2 Antigen activates B cells.
3 Plasma B cells produce polyclonal antibodies.
4 Obtain antiserum from rabbit containing polyclonal antibodies.
B cells
antigen
memory B cells
plasma B cells
secreted antibodies

Figure 16.3 This diagram illustrates the process for harvesting polyclonal antibodies produced in response to an antigen.

Clinical Uses of Polyclonal Antisera

Polyclonal antisera are used in many clinical tests that are designed to determine whether a patient is producing antibodies in response to a particular pathogen. While these tests are certainly powerful diagnostic tools, they have their limitations, because they are an indirect means of determining whether a particular pathogen is present. Tests based on a polyclonal response can sometimes lead to a **false-positive** result—in other words, a test that confirms the presence of an antigen that is, in fact, not present. Antibody-based tests can also result in a **false-negative** result, which occurs when the test fails to detect an antibody that is, in fact, present.

The accuracy of antibody tests can be described in terms of **test sensitivity** and **test specificity**. Test sensitivity is the probability of getting a positive test result when the patient is indeed infected. If a test has high sensitivity, the probability of a false negative is low. Test specificity, on the other hand, is the probability of getting a negative test result when the patient is not infected. If a test has high specificity, the probability of a false positive is low.

False positives often occur due to cross-reactivity, which can occur when epitopes from a different pathogen are similar to those found on the pathogen being tested for. For this reason, antibody-based tests are often used only as screening tests; if the results are positive, other confirmatory tests are used to make sure that the results were not a false positive.

For example, a blood sample from a patient suspected of having hepatitis C can be screened for the virus using antibodies that bind to antigens on hepatitis C virus. If the patient is indeed infected with hepatitis C virus, the antibodies will bind to the antigens, yielding a positive test result. If the patient is not infected with hepatitic C virus, the antibodies will generally not bind to anything and the test should be negative; however, a false positive may occur if the patient has been previously infected by any of a variety of pathogens that elicit antibodies that cross-react with the hepatitis C virus antigens. Antibody tests for hepatitis C have high sensitivity (a low probability of a false negative) but low specificity (a high probability of a false positive). Thus, patients who test positive must have a second, confirmatory test to rule out the possibility of a false positive. The confirmatory test is a more expensive and time-consuming test that directly tests for the presence of hepatitis C viral RNA in the blood. Only after the confirmatory test comes back positive can the patient be definitively diagnosed with a hepatitis C infection. Antibody-based tests can result in a false negative if, for any reason, the patient's immune system has not produced detectable levels of antibodies. For some diseases, it may take several weeks following infection before the immune system produces enough antibodies to cross the detection threshold of the assay. In immunocompromised patients, the immune system may not be capable of producing a detectable level of antibodies.

Another limitation of using antibody production as an indicator of disease is that antibodies in the blood will persist long after the infection has been cleared. Depending on the type of infection, antibodies will be present for many months; sometimes, they may be present for the remainder of the patient's life. Thus, a positive antibody-based test only means that the patient was infected at some point in time; it does not prove that the infection is

active.

In addition to their role in diagnosis, polyclonal antisera can activate complement, detect the presence of bacteria in clinical and food industry settings, and perform a wide array of precipitation reactions that can detect and quantify serum proteins, viruses, or other antigens. However, with the many specificities of antibody present in a polyclonal antiserum, there is a significant likelihood that the antiserum will cross-react with antigens to which the individual was never exposed. Therefore, we must always account for the possibility of false-positive results when working with a polyclonal antiserum.

- What is a false positive and what are some reasons that false positives occur?
- What is a false negative and what are some reasons that false positives occur?
- If a patient tests negative on a highly sensitive test, what is the likelihood that the person is infected with the pathogen?

Producing Monoclonal Antibodies

Some types of assays require better antibody specificity and affinity than can be obtained using a polyclonal antiserum. To attain this high specificity, all of the antibodies must bind with high affinity to a single epitope. This high specificity can be provided by **monoclonal antibodies (mAbs)**. **Table 16.1** compares some of the important characteristics of monoclonal and polyclonal antibodies.

Unlike polyclonal antibodies, which are produced in live animals, monoclonal antibodies are produced *in vitro* using tissue-culture techniques. mAbs are produced by immunizing an animal, often a mouse, multiple times with a specific antigen. B cells from the spleen of the immunized animal are then removed. Since normal B cells are unable to proliferate forever, they are fused with immortal, cancerous B cells called myeloma cells, to yield **hybridoma** cells. All of the cells are then placed in a selective medium that allows only the hybridomas to grow; unfused myeloma cells cannot grow, and any unfused B cells die off. The hybridomas, which are capable of growing continuously in culture while producing antibodies, are then screened for the desired mAb. Those producing the desired mAb are grown in tissue culture; the culture medium is harvested periodically and mAbs are purified from the medium. This is a very expensive and time-consuming process. It may take weeks of culturing and many liters of media to provide enough mAbs for an experiment or to treat a single patient. mAbs are expensive (**Figure 16.4**).

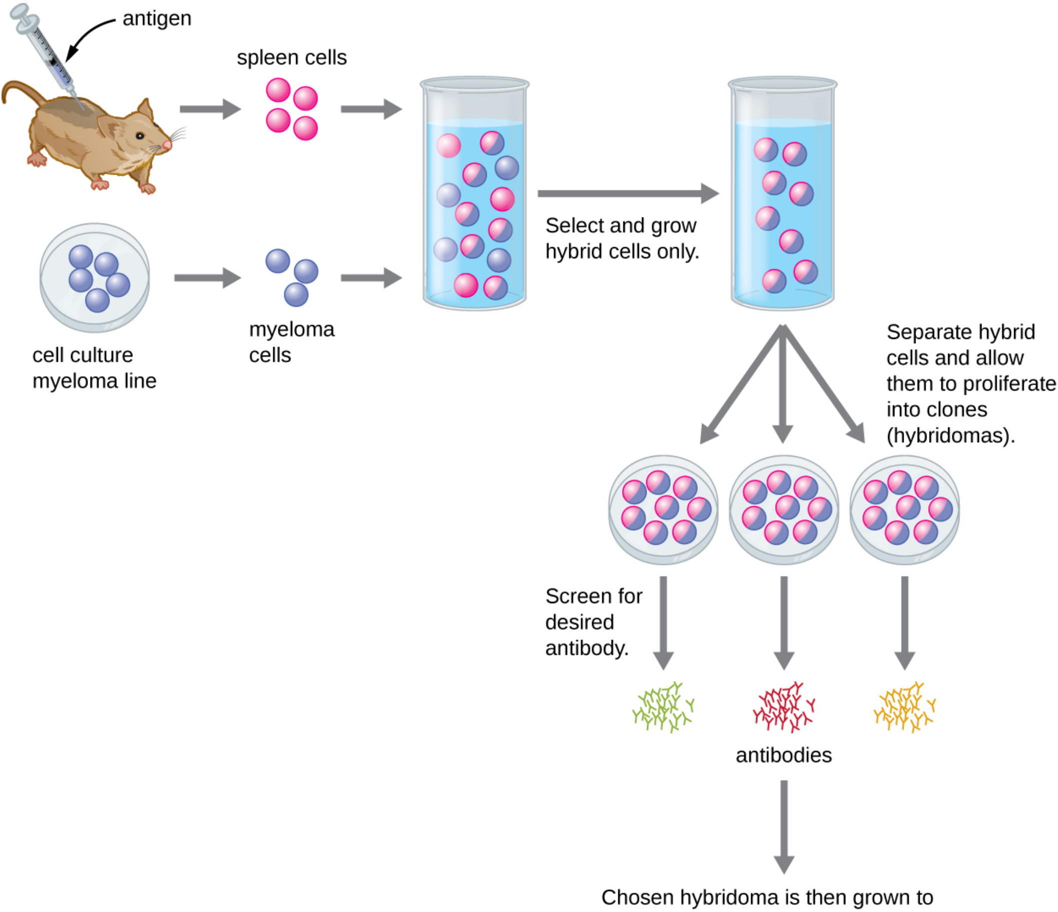


Figure 16.4 Monoclonal antibodies (mAbs) are produced by introducing an antigen to a mouse and then fusing polyclonal B cells from the mouse's spleen to myeloma cells. The resulting hybridoma cells are cultured and continue to produce antibodies to the antigen. Hybridomas producing the desired mAb are then grown in large numbers on a selective medium that is periodically harvested to obtain the desired mAbs.

Characteristics of Polyclonal and Monoclonal Antibodies

Monoclonal Antibodies	Polyclonal Antibodies
Expensive production	Inexpensive production
Long production time	Rapid production
Large quantities of specific antibodies	Large quantities of nonspecific antibodies
Recognize a single epitope on an antigen	Recognize multiple epitopes on an antigen
Production is continuous and uniform once the hybridoma is made	Different batches vary in composition

Table 16.1

Clinical Uses of Monoclonal Antibodies

Since the most common methods for producing monoclonal antibodies use mouse cells, it is necessary to create **humanized monoclonal antibodies** for human clinical use. Mouse antibodies cannot be injected repeatedly into humans, because the immune system will recognize them as being foreign and will respond to them with neutralizing antibodies. This problem can be minimized by genetically engineering the antibody in the mouse B cell. The variable regions of the mouse light and heavy chain genes are ligated to human constant regions, and the chimeric gene is then transferred into a host cell. This allows production of a mAb that is mostly "human"

with only the antigen-binding site being of mouse origin.

Humanized mAbs have been successfully used to treat cancer with minimal side effects. For example, the humanized monoclonal antibody drug Herceptin has been helpful for the treatment of some types of breast cancer. There have also been a few preliminary trials of humanized mAb for the treatment of infectious diseases, but none of these treatments are currently in use. In some cases, mAbs have proven too specific to treat infectious diseases, because they recognize some serovars of a pathogen but not others. Using a cocktail of multiple mAbs that target different strains of the pathogen can address this problem. However, the great cost associated with mAb production is another challenge that has prevented mAbs from becoming practical for use in treating microbial infections.[3]

One promising technology for inexpensive mAbs is the use of genetically engineered plants to produce antibodies (or **plantibodies**). This technology transforms plant cells into antibody factories rather than relying on tissue culture cells, which are expensive and technically demanding. In some cases, it may even be possible to deliver these antibodies by having patients eat the plants rather than by extracting and injecting the antibodies. For example, in 2013, a research group cloned antibody genes into plants that had the ability to neutralize an important toxin from bacteria that can cause severe gastrointestinal disease.[4] Eating the plants could potentially deliver the antibodies directly to the toxin.

- How are humanized monoclonal antibodies produced?
- What does the “monoclonal” of monoclonal antibodies mean?

Using Monoclonal Antibodies to Combat Ebola

During the 2014–2015 Ebola outbreak in West Africa, a few Ebola-infected patients were treated with ZMapp, a drug that had been shown to be effective in trials done in rhesus macaques only a few months before.[5] ZMapp is a combination of three mAbs produced by incorporating the antibody genes into tobacco plants using a viral vector. By using three mAbs, the drug is effective across multiple strains of the virus. Unfortunately, there was only enough ZMapp to treat a tiny number of patients.

While the current technology is not adequate for producing large quantities of ZMapp, it does show that plantibodies—plant-produced mAbs—are feasible for clinical use, potentially cost effective, and worth further development. The last several years have seen an explosion in the number of new mAb-based drugs for the treatment of cancer and infectious diseases; however, the widespread use of such drugs is currently inhibited by their exorbitant cost, especially in underdeveloped parts of the world, where a single dose might cost more than the patient’s lifetime income. Developing methods for cloning antibody genes into plants could reduce costs dramatically.

16.2 | Detecting Antigen-Antibody Complexes

Learning Objectives

By the end of this section, you will be able to:

- Describe various types of assays used to find antigen-antibody complexes
- Describe the circumstances under which antigen-antibody complexes precipitate out of solution
- Explain how antibodies in patient serum can be used to diagnose disease

Laboratory tests to detect antibodies and antigens outside of the body (e.g., in a test tube) are called *in vitro* assays. When both antibodies and their corresponding antigens are present in a solution, we can often observe a precipitation reaction in which large complexes (lattices) form and settle out of solution. In the next several

3. Saylor, Carolyn, Ekaterina Dadachova and Arturo Casadevall, “Monoclonal Antibody-Based Therapies for Microbial Diseases,” *Vaccine* 27 (2009): G38-G46.
4. Nakanishi, Katsuhiro et al., “Production of Hybrid-IgG/IgA Plantibodies with Neutralizing Activity against Shiga Toxin 1,” *PloS One* 8, no. 11 (2013): e80712.
5. Qiu, Xiangguo et al., “Reversion of Advanced Ebola Virus Disease in Nonhuman Primates with ZMapp,” *Nature* 514 (2014): 47–53.

sections, we will discuss several common *in vitro* assays.

Precipitin Reactions

A visible antigen-antibody complex is called a **precipitin**, and *in vitro* assays that produce a precipitin are called precipitin reactions. A precipitin reaction typically involves adding soluble antigens to a test tube containing a solution of antibodies. Each antibody has two arms, each of which can bind to an epitope. When an antibody binds to two antigens, the two antigens become bound together by the antibody. A lattice can form as antibodies bind more and more antigens together, resulting in a precipitin (Figure 16.5). Most precipitin tests use a polyclonal antiserum rather than monoclonal antibodies because polyclonal antibodies can bind to multiple epitopes, making lattice formation more likely. Although mAbs may bind some antigens, the binding will occur less often, making it much less likely that a visible precipitin will form.

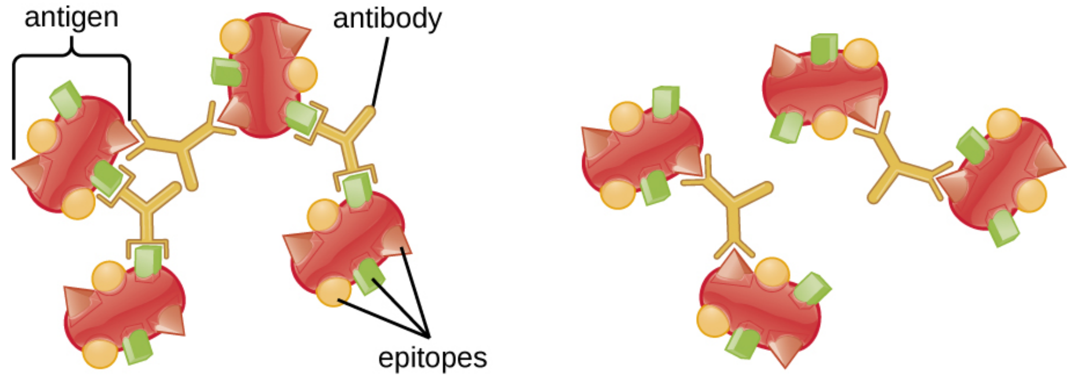


Figure 16.5 Polyclonal antiserum binds to multiple epitopes on an antigen, leading to lattice formation that results in a visible precipitin. Monoclonal antibodies can only bind to a single epitope; therefore, less binding occurs and lattice formation generally does not occur.

The amount of precipitation also depends on several other factors. For example, precipitation is enhanced when the antibodies have a high affinity for the antigen. While most antibodies bind antigen with high affinity, even high-affinity binding uses relatively weak noncovalent bonds, so that individual interactions will often break and new interactions will occur.

In addition, for precipitin formation to be visible, there must be an optimal ratio of antibody to antigen. The optimal ratio is not likely to be a 1:1 antigen-to-antibody ratio; it can vary dramatically, depending on the number of epitopes on the antigen and the class of antibody. Some antigens may have only one or two epitopes recognized by the antiserum, whereas other antigens may have many different epitopes and/or multiple instances of the same epitope on a single antigen molecule.

Figure 16.6 illustrates how the ratio of antigen and antibody affects the amount of precipitation. To achieve the optimal ratio, antigen is slowly added to a solution containing antibodies, and the amount of precipitin is determined qualitatively. Initially, there is not enough antigen to produce visible lattice formation; this is called the zone of antibody excess. As more antigen is added, the reaction enters the **equivalence zone** (or zone of equivalence), where both the optimal antigen-antibody interaction and maximal precipitation occur. If even more antigen were added, the amount of antigen would become excessive and actually cause the amount of precipitation to decline.

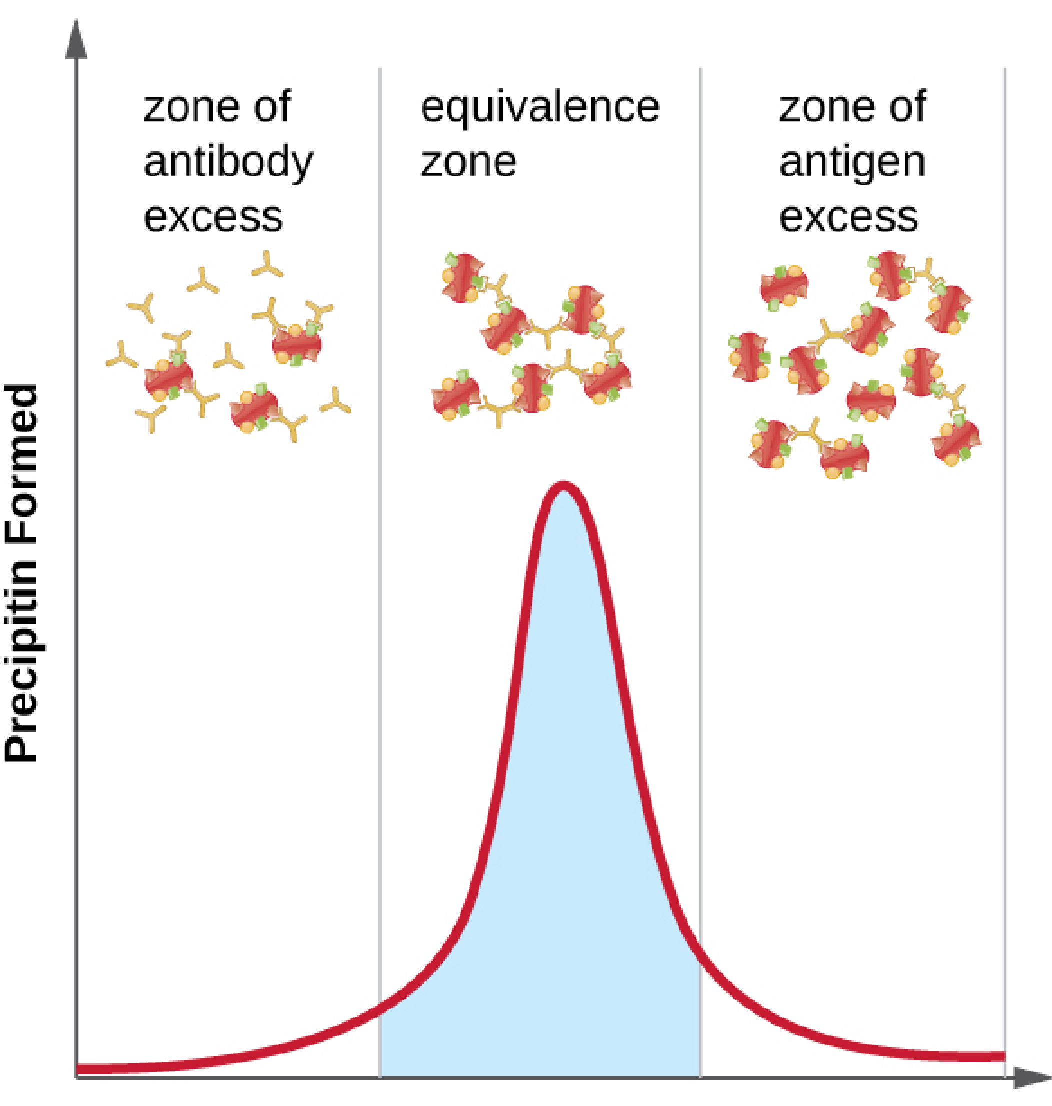


Figure 16.6 As antigen is slowly added to a solution containing a constant amount antibody, the amount of precipitin increases as the antibody-to-antigen ratio approaches the equivalence zone and decreases once the proportion of antigen exceeds the optimal ratio.

- What is a precipitin?
- Why do polyclonal antisera produce a better precipitin reaction?

Precipitin Ring Test

A variety of techniques allow us to use precipitin formation to quantify either antigen concentration or the amount of antibody present in an antiserum. One such technique is the **precipitin ring test** (Figure 16.7), which is used to determine the relative amount of antigen-specific antibody in a sample of serum. To perform this test, a set of test tubes is prepared by adding an antigen solution to the bottom of each tube. Each tube receives the same volume of solution, and the concentration of antigens is constant (e.g., 1 mg/mL). Next, glycerol is added to the antigen solution in each test tube, followed by a serial dilution of the antiserum. The glycerol prevents mixing of the antiserum with the antigen solution, allowing antigen-antibody binding to take place only at the interface of the two solutions. The result is a visible ring of precipitin in the tubes that have an antigen-antibody ratio within the equivalence zone. This highest dilution with a visible ring is used to determine the **titer** of the antibodies. The titer is the reciprocal of the highest dilution showing a positive result, expressed as a whole number. In Figure 16.7, the titer is 16.

While a measurement of titer does not tell us in absolute terms how much antibody is present, it does give a measure of biological activity, which is often more important than absolute amount. In this example, it would not be useful to know what mass of IgG were present in the antiserum, because there are many different specificities of antibody present; but it is important for us to know how much of the antibody activity in a patient's serum is directed against the antigen of interest (e.g., a particular pathogen or allergen).

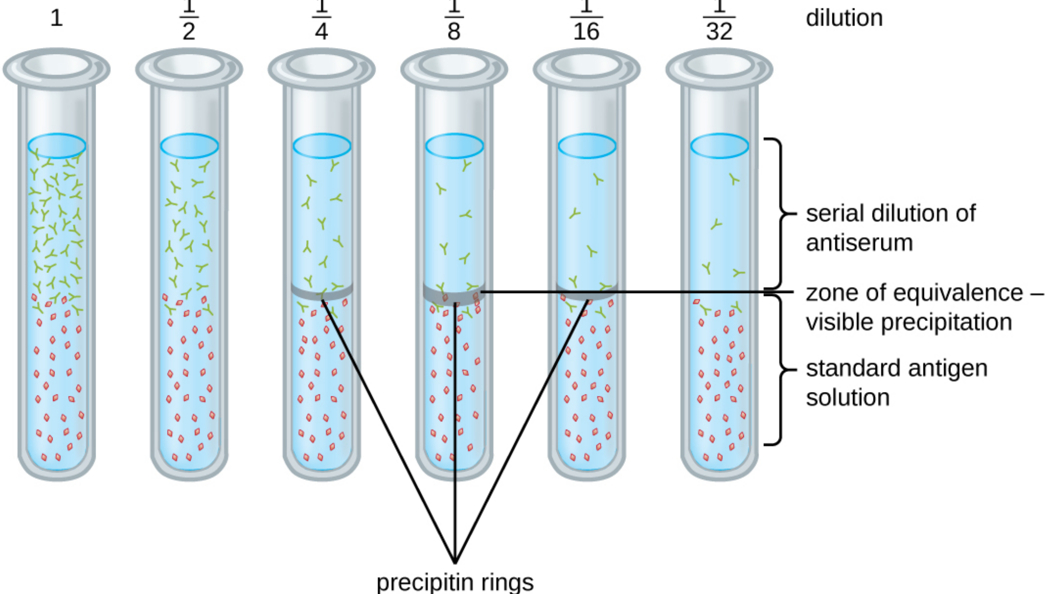


Figure 16.7 A precipitin ring test is performed using a standard antigen solution in the bottom of the tube and a serial dilution of antiserum in the top of the tube. Glycerol prevents the two solutions from mixing so that precipitation only occurs at the interface. A visible ring of precipitation is seen in the 1/4, 1/8, and 1/16 dilutions, indicating that these concentrations are within the equivalence zone. Since 1/16 is the highest dilution in which a precipitin is observed, the titer is the reciprocal, or 16.

Ouchterlony Assay

While the precipitin ring test provides insights into antibody-antigen interactions, it also has some drawbacks. It requires the use of large amounts of serum, and great care must be taken to avoid mixing the solutions and disrupting the ring. Performing a similar test in an agar gel matrix can minimize these problems. This type of assay is variously called **double immunodiffusion** or the **Ouchterlony assay** for Orjan Ouchterlony,[6] who first described the technique in 1948.

When agar is highly purified, it produces a clear, colorless gel. Holes are punched in the gel to form wells, and antigen and antisera are added to neighboring wells. Proteins are able to diffuse through the gel, and precipitin arcs form between the wells at the zone of equivalence. Because the precipitin lattice is too large to diffuse through the gel, the arcs are firmly locked in place and easy to see (**Figure 16.8**).

Although there are now more sensitive and quantitative methods of detecting antibody-antigen interactions, the Ouchterlony test provides a rapid and qualitative way of determining whether an antiserum has antibodies against a particular antigen. The Ouchterlony test is particularly useful when looking for cross-reactivity. We can check an antiserum against a group of closely related antigens and see which combinations form precipitin arcs.

6. Ouchterlony, Örjan, "In Vitro Method for Testing the Toxin-Producing Capacity of Diphtheria Bacteria," *Acta Pathologica Microbiologica Scandinavica* 26, no. 4 (1949): 516-24.

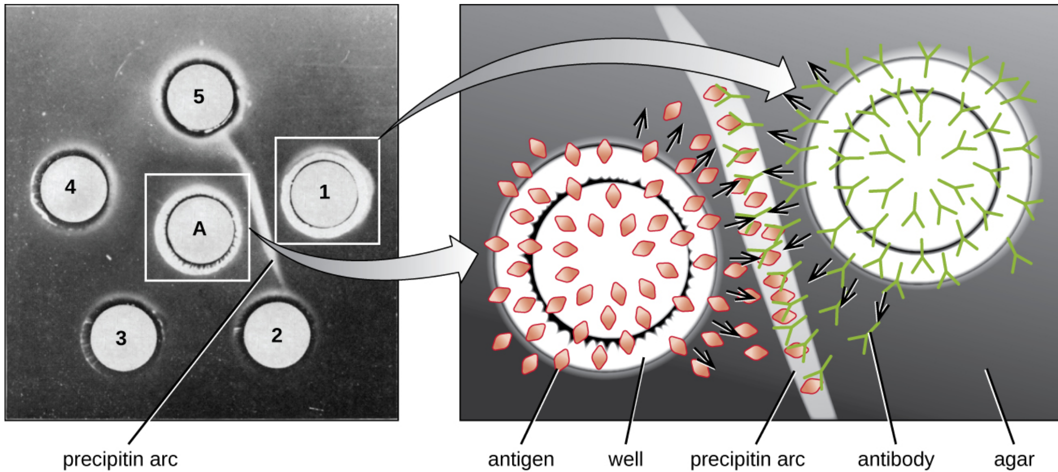


Figure 16.8 The Ouchterlony test places antigen (well A) and antisera (wells 1 through 5) in a gel. The antibodies and antigen diffuse through the gel, causing a precipitin arc to form at the zone of equivalence. In this example, only the antiserum in well 1 contains antibodies to the antigen. The resulting precipitin arc is stable because the lattice is too large to diffuse through the gel. (credit left: modification of work by Higgins PJ, Tong C, Borenfreund E, Okin RS, Bendich A)

Radial Immunodiffusion Assay

The **radial immunodiffusion** (RID) assay is similar to the Ouchterlony assay but is used to precisely quantify antigen concentration rather than to compare different antigens. In this assay, the antiserum is added to tempered agar (liquid agar at slightly above 45 °C), which is poured into a small petri dish or onto a glass slide and allowed to cool. Wells are cut in the cooled agar, and antigen is then added to the wells and allowed to diffuse. As the antigen and antibody interact, they form a zone of precipitation. The square of the diameter of the zone of precipitation is directly proportional to the concentration of antigen. By measuring the zones of precipitation produced by samples of known concentration (see the outer ring of samples in **Figure 16.9**), we can prepare a standard curve for determining the concentration of an unknown solution. The RID assay is a also useful test for determining the concentration of many serum proteins such as the C3 and C4 complement proteins, among others.

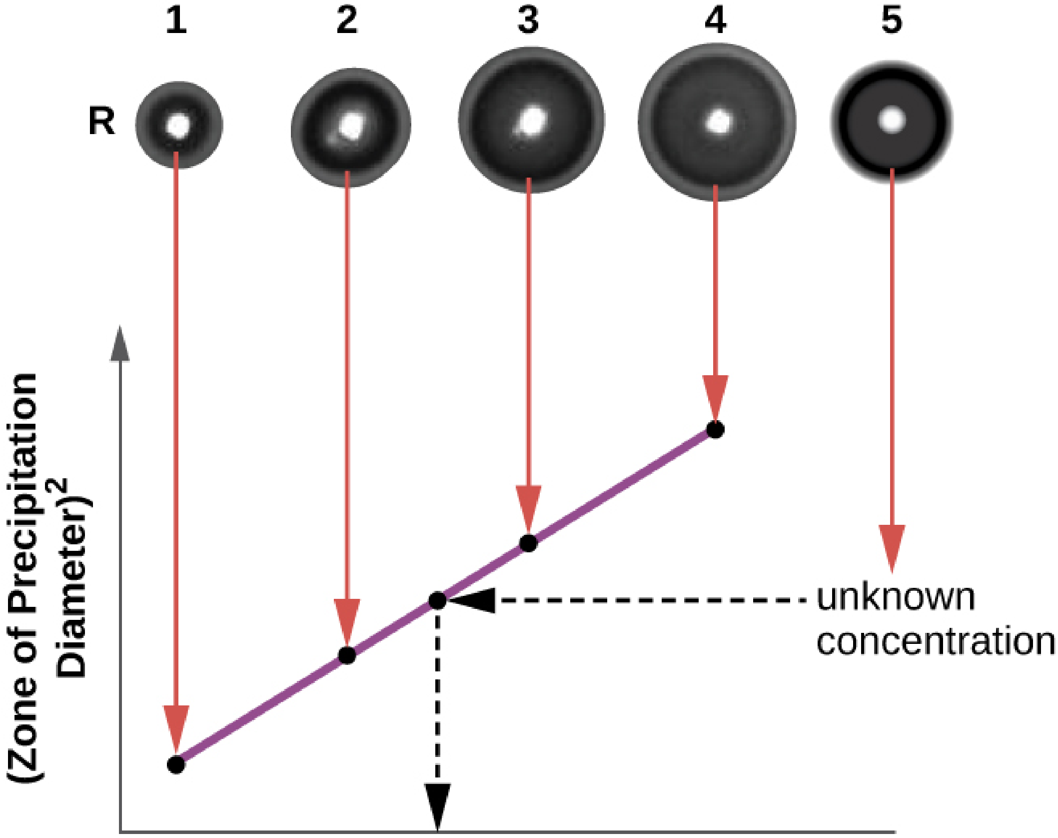


Figure 16.9 In this radial immunodiffusion (RID) assay, an antiserum is mixed with the agar before it is cooled, and solutions containing antigen are added to each well in increasing concentrations (wells 1–4). An antigen solution of an unknown concentration is added to well 5. The zones of precipitation are measured and plotted against a standard curve to determine the antigen concentration of the unknown sample. (credit circles: modification of work by Kangwa M, Yelemane V, Polat AN, Gorrepati KD, Grasselli M, Fernández-Lahore M)

- Why does a precipitin ring form in a precipitin ring test, and what are some reasons why a ring might not form?
- Compare and contrast the techniques used in an Ouchterlony assay and a radial immunodiffusion assay.

Flocculation Assays

A flocculation assay is similar to a precipitin reaction except that it involves insoluble antigens such as lipids. A **flocculant** is similar to a precipitin in that there is a visible lattice of antigen and antibody, but because lipids are insoluble in aqueous solution, they cannot precipitate. Instead of precipitation, flocculation (foaming) is observed in the test tube fluid.

Using Flocculation to Test for Syphilis

Syphilis is a sexually transmitted infection that can cause severe, chronic disease in adults. In addition, it is readily passed from infected mothers to their newborns during pregnancy and childbirth, often resulting in stillbirth or serious long-term health problems for the infant. Unfortunately, syphilis can also be difficult to diagnose in expectant mothers, because it is often asymptomatic, especially in women. In addition, the causative agent, the bacterium *Treponema pallidum*, is both difficult to grow on conventional lab media and too small to see using routine microcopy. For these reasons, presumptive diagnoses of syphilis are generally confirmed indirectly in the laboratory using tests that detect antibodies to treponemal antigens.

In 1906, German scientist August von Wassermann (1866–1925) introduced the first test for syphilis

that relied on detecting anti-treponemal antibodies in the patient's blood. The antibodies detected in the Wassermann test were antiphospholipid antibodies that are nonspecific to *T. pallidum*. Their presence can assist in the diagnosis of syphilis, but because they are nonspecific, they can also lead to false-positive results in patients with other diseases and autoimmune conditions. The original Wasserman test has been modified over the years to minimize false-positives and is now known as the Venereal Disease Research Lab test, better known by its acronym, the VDRL test.

To perform the VDRL test, patient serum or cerebral spinal fluid is placed on a slide with a mixture of cardiolipin (an antigenic phospholipid found in the mitochondrial membrane of various pathogens), lecithin, and cholesterol. The lecithin and cholesterol stabilize the reaction and diminish false positives. Anti-treponemal antibodies from an infected patient's serum will bind cardiolipin and form a flocculant. Although the VDRL test is more specific than the original Wassermann assay, false positives may still occur in patients with autoimmune diseases that cause extensive cell damage (e.g., systemic lupus erythematosus).

Neutralization Assay

To cause infection, viruses must bind to receptors on host cells. Antiviral antibodies can neutralize viral infections by coating the virions, blocking the binding (**m58884 (https://legacy.cnx.org/content/m58884/latest/#OSC_Microbio_18_04_neutral)**). This activity neutralizes virions and can result in the formation of large antibody-virus complexes (which are readily removed by phagocytosis) or by antibody binding to the virus and blocking its binding to host cell receptors. This neutralization activity is the basis of neutralization assays, sensitive assays used for diagnoses of viral infections.

When viruses infect cells, they often cause damage (cytopathic effects) that may include lysis of the host cells. Cytopathic effects can be visualized by growing host cells in a petri dish, covering the cells with a thin layer of agar, and then adding virus (see **Isolation, Culture, and Identification of Viruses**). The virus will diffuse very slowly through the agar. A virus will enter a host cell, proliferate (causing cell damage), be released from the dead host cell, and then move to neighboring cells. As more and more cells die, plaques of dead cells will form (**Figure 16.10**).

During the course of a viral infection, the patient will mount an antibody response to the virus, and we can quantify those antibodies using a plaque reduction assay. To perform the assay, a serial dilution is carried out on a serum sample. Each dilution is then mixed with a standardized amount of the suspect virus. Any virus-specific antibodies in the serum will neutralize some of the virus. The suspensions are then added to host cells in culture to allow any nonneutralized virus to infect the cells and form plaques after several days. The titer is defined as the reciprocal of the highest dilution showing a 50% reduction in plaques. Titer is always expressed as a whole number. For example, if a 1/64 dilution was the highest dilution to show 50% plaque reduction, then the titer is 64.

The presence of antibodies in the patient's serum does not tell us whether the patient is currently infected or was infected in the past. Current infections can be identified by waiting two weeks and testing another serum sample. A four-fold increase in neutralizing titer in this second sample indicates a new infection.

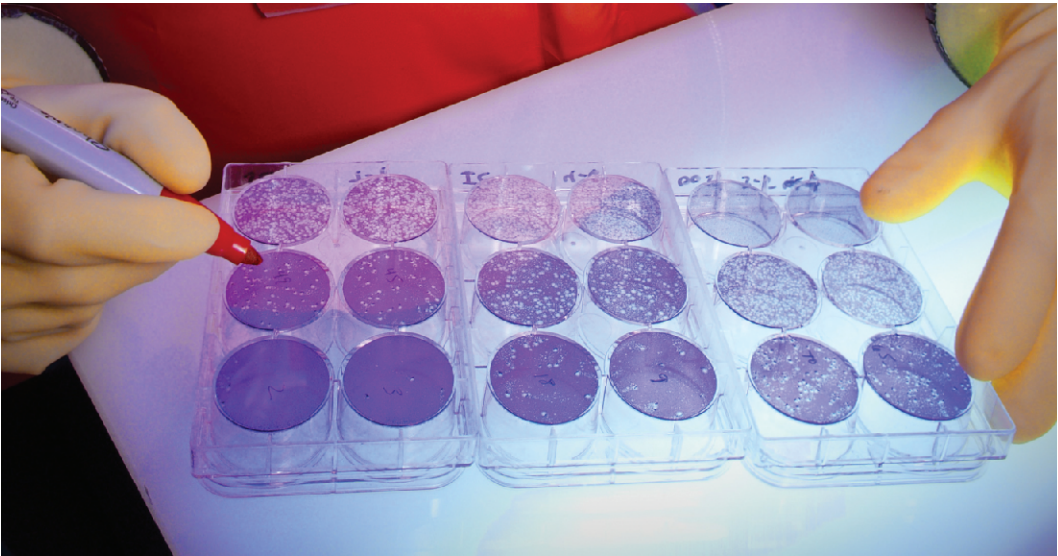


Figure 16.10 In a neutralization assay, antibodies in patient serum neutralize viruses added to the wells, preventing the formation of plaques. In the assay pictured, the wells with numerous plaques (white patches) contain a low concentration of antibodies. The wells with relatively few plaques have a high concentration of antibodies. (credit: modification of work by Centers for Disease Control and Prevention)

- In a neutralization assay, if a patient's serum has high numbers of antiviral antibodies, would you expect to see more or fewer plaques?

Immunoelectrophoresis

When a patient has elevated protein levels in the blood or is losing protein in the urine, a clinician will often order a polyacrylamide gel electrophoresis (PAGE) assay (see **Visualizing and Characterizing DNA, RNA, and Protein**). This assay compares the relative abundance of the various types of serum proteins. Abnormal protein electrophoresis patterns can be further studied using **immunoelectrophoresis (IEP)**. The IEP begins by running a PAGE. Antisera against selected serum proteins are added to troughs running parallel to the electrophoresis track, forming precipitin arcs similar to those seen in an Ouchterlony assay (**Figure 16.11**). This allows the identification of abnormal immunoglobulin proteins in the sample.

IEP is particularly useful in the diagnosis of multiple myeloma, a cancer of antibody-secreting cells. Patients with multiple myeloma cannot produce healthy antibodies; instead they produce abnormal antibodies that are monoclonal proteins (M proteins). Thus, patients with multiple myeloma will present with elevated serum protein levels that show a distinct band in the gamma globulin region of a protein electrophoresis gel and a sharp spike (in M protein) on the densitometer scan rather than the normal broad smear (**Figure 16.12**). When antibodies against the various types of antibody heavy and light chains are used to form precipitin arcs, the M protein will cause distinctly skewed arcs against one class of heavy chain and one class of light chain as seen in **Figure 16.11**.

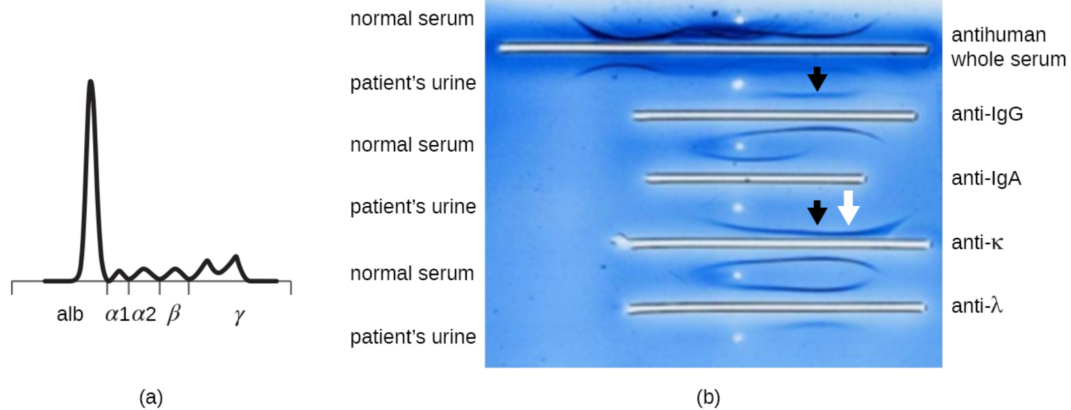


Figure 16.11 (a) This graph shows normal measurements of serum proteins. (b) This photograph shows an immunoelectrophoresis of urine. After electrophoresis, antisera were added to the troughs and the precipitin arcs formed, illustrating the distribution of specific proteins. The skewed arcs (arrows) help to diagnose multiple myeloma. (credit a, b: modification of work by Izawa S, Akimoto T, Ikeuchi H, Kusano E, Nagata D)

Protein Electrophoresis and the Characterization of Immunoglobulin Structure

The advent of electrophoresis ultimately led to researching and understanding the structure of antibodies. When Swedish biochemist Arne Tiselius (1902–1971) published the first protein electrophoresis results in 1937,[7] he could identify the protein albumin (the smallest and most abundant serum protein) by the sharp band it produced in the gel. The other serum proteins could not be resolved in a simple protein electrophoresis, so he named the three broad bands, with many proteins in each band, alpha, beta, and gamma globulins. Two years later, American immunologist Elvin Kabat (1914–2000) traveled to Sweden to work with Tiselius using this new technique and showed that antibodies migrated as gamma globulins.[8] With this new understanding in hand, researchers soon learned that multiple myeloma, because it is a cancer of antibody-secreting cells, could be tentatively diagnosed by the presence of a large M spike in the gamma-globulin region by protein electrophoresis. Prior to this discovery, studies on immunoglobulin structure had been minimal, because of the difficulty of obtaining pure samples to study. Sera from multiple myeloma patients proved to be an excellent source of highly enriched monoclonal immunoglobulin, providing the raw material for studies over the next 20-plus years that resulted in the elucidation of the structure of immunoglobulin.

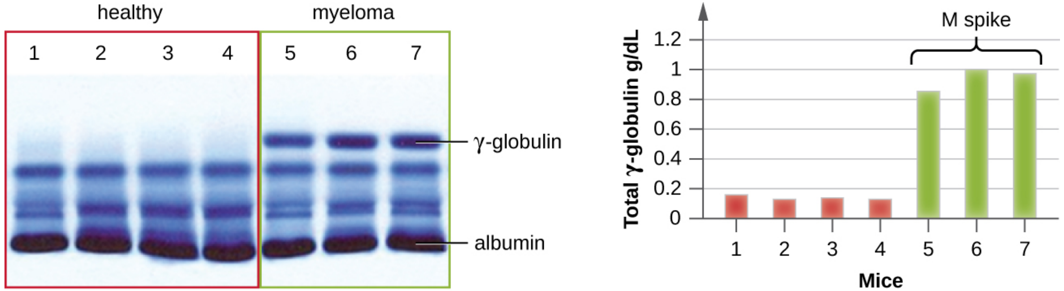


Figure 16.12 Electrophoresis patterns of myeloma (right) and normal sera (left). The proteins have been stained; when the density of each band is quantified by densitometry, the data produce the bar graph on the right. Both gels show the expected dense band of albumin at the bottom and an abnormal spike in the gamma-globulin region. (credit: modification of work by Soodgupta D, Hurchla MA, Jiang M, Zheleznyak A, Weilbaecher KN, Anderson CJ, Tomasson MH, Shokeen M)

- In general, what does an immunoelectrophoresis assay accomplish?

7. Tiselius, Arne, "Electrophoresis of Serum Globulin: Electrophoretic Analysis of Normal and Immune Sera," *Biochemical Journal* 31, no. 9 (1937): 1464.
8. Tiselius, Arne and Elvin A. Kabat. "An Electrophoretic Study of Immune Sera and Purified Antibody Preparations," *The Journal of Experimental Medicine* 69, no. 1 (1939): 119-31.

Immunoblot Assay: The Western Blot

After performing protein gel electrophoresis, specific proteins can be identified in the gel using antibodies. This technique is known as the **western blot**. Following separation of proteins by PAGE, the protein antigens in the gel are transferred to and immobilized on a nitrocellulose membrane. This membrane can then be exposed to a primary antibody produced to specifically bind to the protein of interest. A second antibody equipped with a molecular beacon will then bind to the first. These secondary antibodies are coupled to another molecule such as an enzyme or a **fluorophore** (a molecule that fluoresces when excited by light). When using antibodies coupled to enzymes, a **chromogenic substrate** for the enzyme is added. This substrate is usually colorless but will develop color in the presence of the antibody. The fluorescence or substrate coloring identifies the location of the specific protein in the membrane to which the antibodies are bound (**Figure 16.13**).

Typically, polyclonal antibodies are used for western blot assays. They are more sensitive than mAbs because of their ability to bind to various epitopes of the primary antigen, and the signal from polyclonal antibodies is typically stronger than that from mAbs. Monoclonal antibodies can also be used; however, they are much more expensive to produce and are less sensitive, since they are only able to recognize one specific epitope.

Several variations of the western blot are useful in research. In a southwestern blot, proteins are separated by SDS-PAGE, blotted onto a nitrocellulose membrane, allowed to renature, and then probed with a fluorescently or radioactively labeled DNA probe; the purpose of the southwestern is to identify specific DNA-protein interactions. Far-western blots are carried out to determine protein-protein interactions between immobilized proteins (separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and allowed to renature) and non-antibody protein probes. The bound non-antibody proteins that interact with the immobilized proteins in a far-western blot may be detected by radiolabeling, fluorescence, or the use of an antibody with an enzymatic molecular beacon.

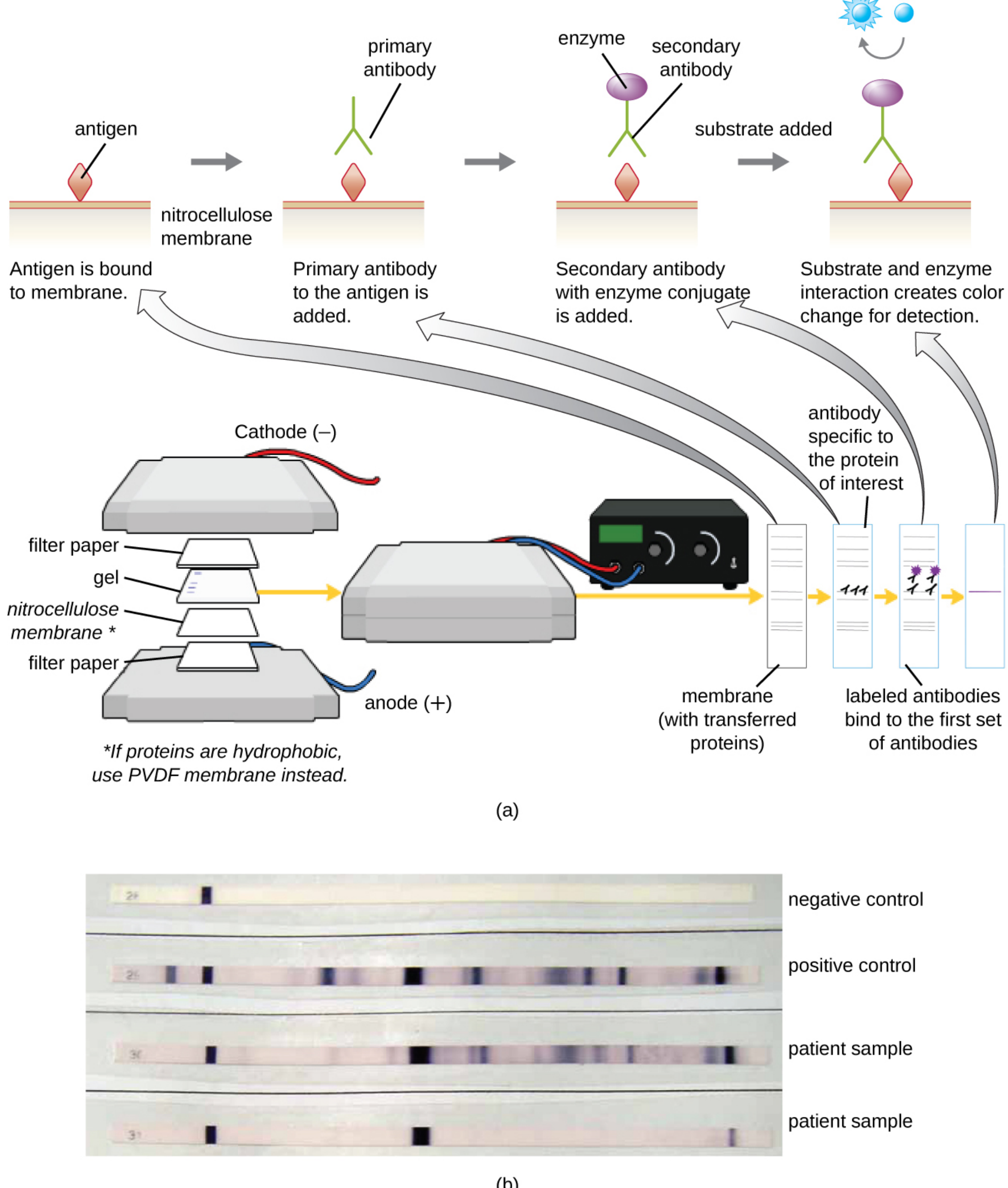


Figure 16.13 (a) This diagram summarizes the process of western blotting. Antibodies are used to identify specific bands on the protein gel. (b) A western blot test for antibodies against HIV. The top strip is the negative control; the next strip is the positive control. The bottom two strips are patient serum samples containing antibodies. (credit a: modification of work by “Bensaccount”/Wikimedia Commons)

- What is the function of the enzyme in the immunoblot assay?

Complement-Mediated Immunoassay

One of the key functions of antibodies is the activation (fixation) of complement. When antibody binds to bacteria, for example, certain complement proteins recognize the bound antibody and activate the complement cascade. In response, other complement proteins bind to the bacteria where some serve as opsonins to increase the efficiency of phagocytosis and others create holes in gram-negative bacterial cell membranes, causing lysis. This lytic activity can be used to detect the presence of antibodies against specific antigens in the serum.

Red blood cells are good indicator cells to use when evaluating complement-mediated cytolysis. Hemolysis of red blood cells releases hemoglobin, which is a brightly colored pigment, and hemolysis of even a small

number of red cells will cause the solution to become noticeably pink (**Figure 16.14**). This characteristic plays a role in the **complement fixation test**, which allows the detection of antibodies against specific pathogens. The complement fixation test can be used to check for antibodies against pathogens that are difficult to culture in the lab such as fungi, viruses, or the bacteria *Chlamydia*.

To perform the complement fixation test, antigen from a pathogen is added to patient serum. If antibodies to the antigen are present, the antibody will bind the antigen and fix all the available complement. When red blood cells and antibodies against red blood cells are subsequently added to the mix, there will be no complement left to lyse the red cells. Thus, if the solution remains clear, the test is positive. If there are no antipathogen antibodies in the patient's serum, the added antibodies will activate the complement to lyse the red cells, yielding a negative test (**Figure 16.14**).

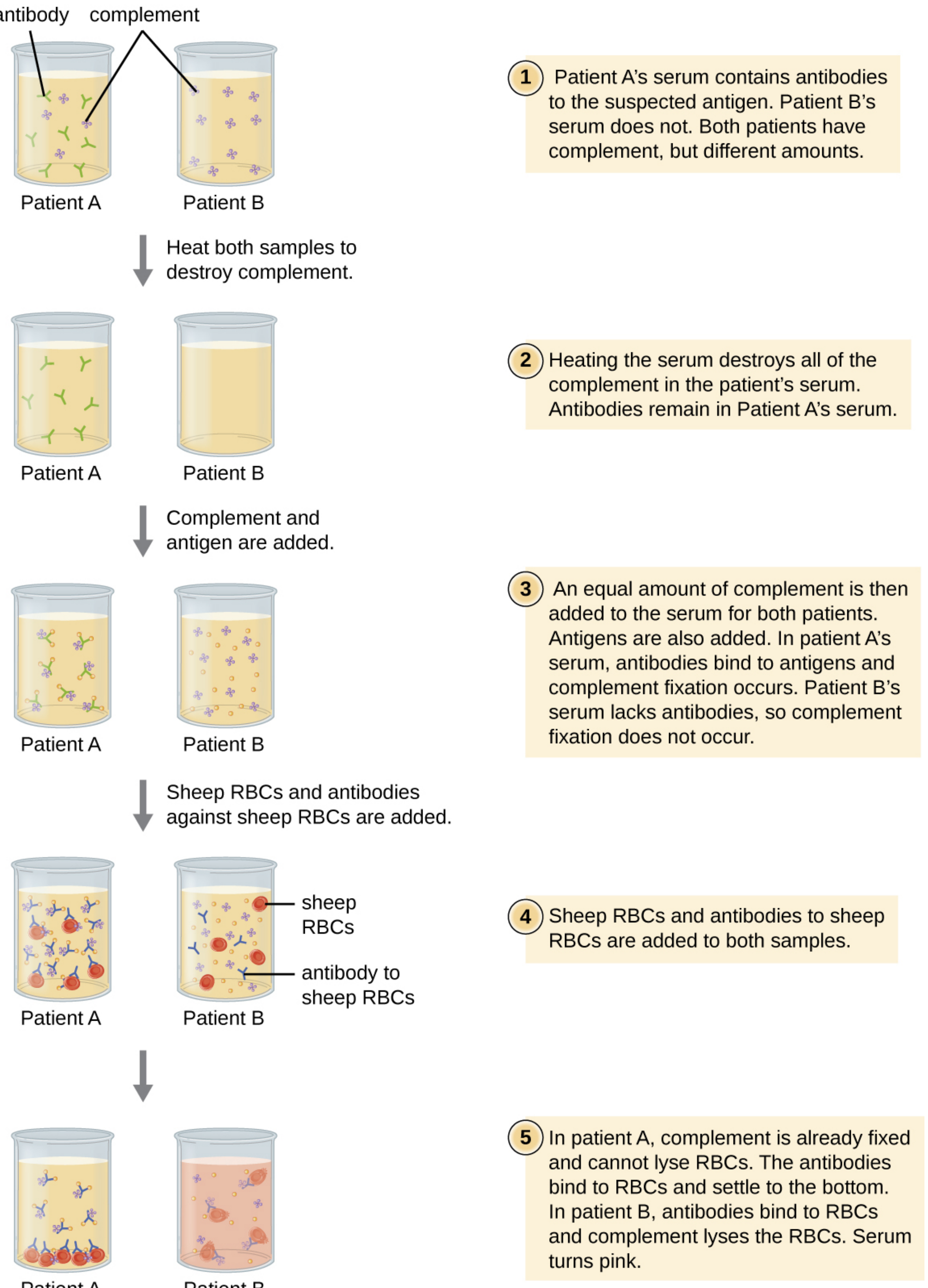


Figure 16.14 The complement fixation test is used to determine whether a patient's serum contains antibodies to a specific antigen. If it does, complement fixation will occur, and there will be no complement available to lyse the antibody-bound sheep red blood cells that are added to the solution in the next step. If the sample does not contain antibodies to the antigen, hemolysis of the sheep blood cells will be observed.

View this **video (https://openstax.org/l/22complfixatst)** to see an outline of the steps of the complement fixation test.

- In a complement fixation test, if the serum turns pink, does the patient have antibodies to the antigen

or not? Explain.

Table 16.2 summarizes the various types of antibody-antigen assays discussed in this section.

Mechanisms of Select Antibody-Antigen Assays

Type of Assay	Mechanism	Examples
Precipitation	Antibody binds to soluble antigen, forming a visible precipitin	Precipitin ring test to visualize lattice formation in solution
		Immunoelectrophoresis to examine distribution of antigens following electrophoresis
		Ouchterlony assay to compare diverse antigens
		Radial immunodiffusion assay to quantify antigens
Flocculation	Antibody binds to insoluble molecules in suspension, forming visible aggregates	VDRL test for syphilis
Neutralization	Antibody binds to virus, blocking viral entry into target cells and preventing formation of plaques	Plaque reduction assay for detecting presence of neutralizing antibodies in patient sera
Complement activation	Antibody binds to antigen, inducing complement activation and leaving no complement to lyse red blood cells	Complement fixation test for patient antibodies against hard-to-culture bacteria such as *Chlamydia*

Table 16.2

16.3 | Agglutination Assays

Learning Objectives

By the end of this section, you will be able to:

- Compare direct and indirect agglutination
- Identify various uses of hemagglutination in the diagnosis of disease
- Explain how blood types are determined
- Explain the steps used to cross-match blood to be used in a transfusion

In addition to causing precipitation of soluble molecules and flocculation of molecules in suspension, antibodies can also clump together cells or particles (e.g., antigen-coated latex beads) in a process called **agglutination** (m58884 (https://legacy.cnx.org/content/m58884/latest/#OSC_Microbio_18_04_agg)). Agglutination can be used as an indicator of the presence of antibodies against bacteria or red blood cells. Agglutination assays are usually quick and easy to perform on a glass slide or **microtiter plate** (Figure 16.15). Microtiter plates have an array of wells to hold small volumes of reagents and to observe reactions (e.g., agglutination) either visually or using a specially designed spectrophotometer. The wells come in many different sizes for assays involving different volumes of reagents.

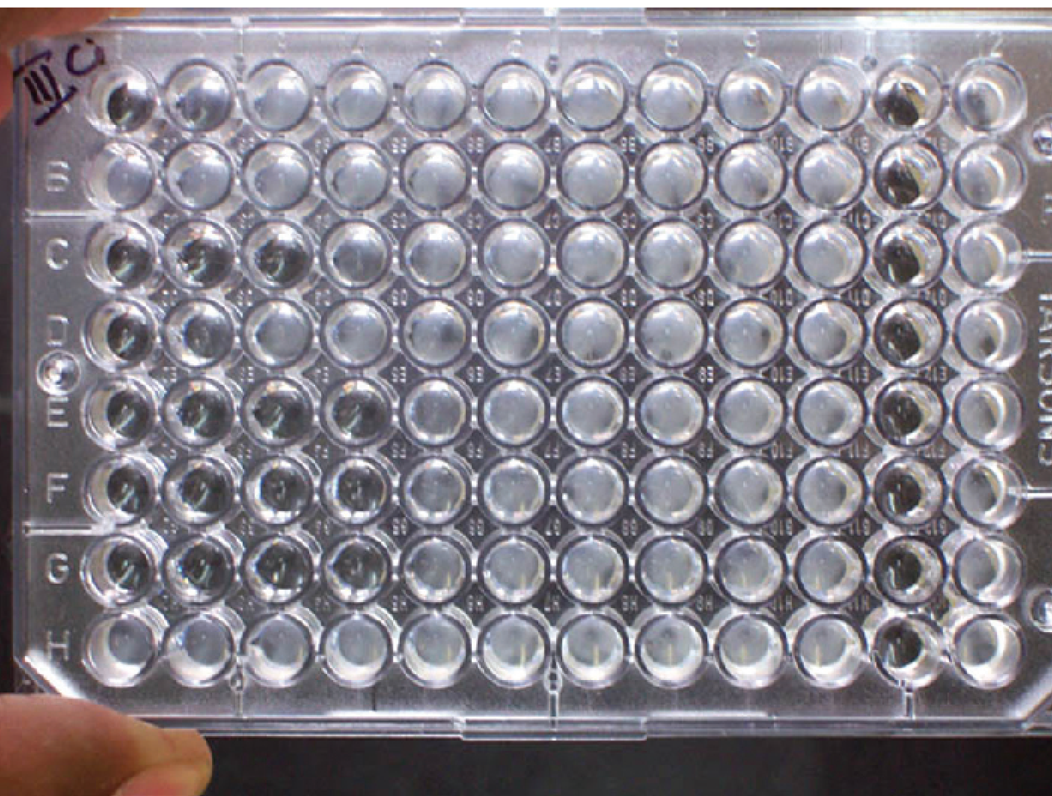


Figure 16.15 Microtiter plates are used for conducting numerous reactions simultaneously in an array of wells. (credit: modification of work by “Microrao”/Wikimedia)

Agglutination of Bacteria and Viruses

The use of agglutination tests to identify streptococcal bacteria was developed in the 1920s by Rebecca Lancefield working with her colleagues A.R. Dochez and Oswald Avery.[9] She used antibodies to identify M protein, a virulence factor on streptococci that is necessary for the bacteria’s ability to cause strep throat. Production of antibodies against M protein is crucial in mounting a protective response against the bacteria.

Lancefield used antisera to show that different strains of the same species of streptococci express different versions of M protein, which explains why children can come down with strep throat repeatedly. Lancefield classified beta-hemolytic streptococci into many groups based on antigenic differences in group-specific polysaccharides located in the bacterial cell wall. The strains are called **serovars** because they are differentiated using antisera. Identifying the serovars present in a disease outbreak is important because some serovars may cause more severe disease than others.

The method developed by Lancefield is a **direct agglutination assay**, since the bacterial cells themselves agglutinate. A similar strategy is more commonly used today when identifying serovars of bacteria and viruses; however, to improve visualization of the agglutination, the antibodies may be attached to inert latex beads. This technique is called an **indirect agglutination assay** (or latex fixation assay), because the agglutination of the beads is a marker for antibody binding to some other antigen (Figure 16.16). Indirect assays can be used to detect the presence of either antibodies or specific antigens.

9. Lancefield, Rebecca C., “The Antigenic Complex of *Streptococcus haemoliticus*. I. Demonstration of a Type-Specific Substance in Extracts of *Streptococcus haemolyticus*,” *The Journal of Experimental Medicine* 47, no. 1 (1928): 91-103.

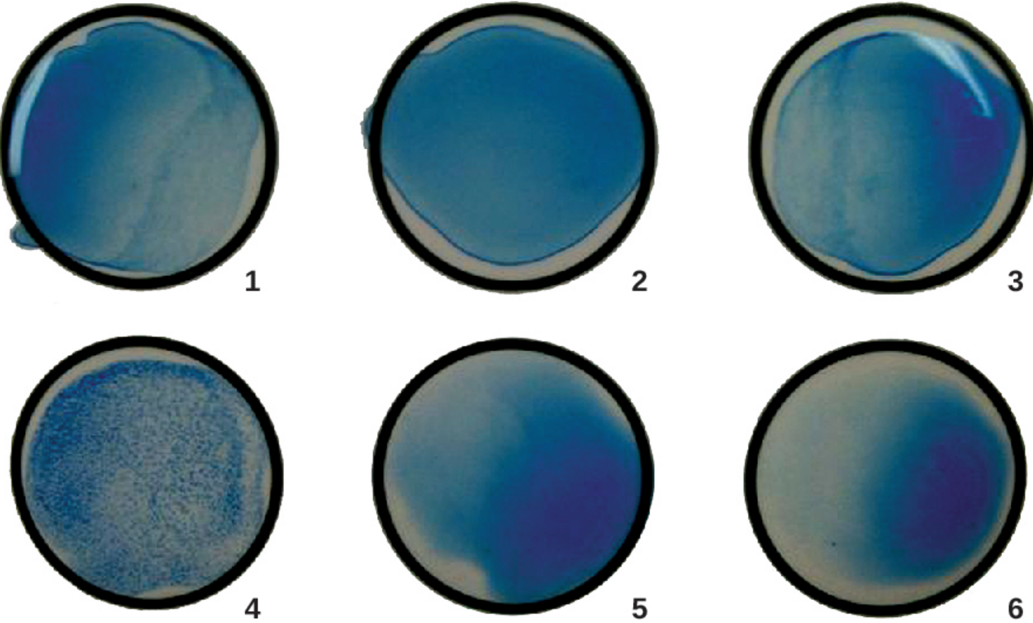


Figure 16.16 Antibodies against six different serovars of Group A strep were attached to latex beads. Each of the six antibody preparations was mixed with bacteria isolated from a patient. The tiny clumps seen in well 4 are indicative of agglutination, which is absent from all other wells. This indicates that the serovar associated with well 4 is present in the patient sample. (credit: modification of work by American Society for Microbiology)

To identify antibodies in a patient's serum, the antigen of interest is attached to latex beads. When mixed with patient serum, the antibodies will bind the antigen, cross-linking the latex beads and causing the beads to agglutinate indirectly; this indicates the presence of the antibody (**Figure 16.17**). This technique is most often used when looking for IgM antibodies, because their structure provides maximum cross-linking. One widely used example of this assay is a test for rheumatoid factor (RF) to confirm a diagnosis of rheumatoid arthritis. RF is, in fact, the presence of IgM antibodies that bind to the patient's own IgG. RF will agglutinate IgG-coated latex beads.

In the reverse test, soluble antigens can be detected in a patient's serum by attaching specific antibodies (commonly mAbs) to the latex beads and mixing this complex with the serum (**Figure 16.17**).

Agglutination tests are widely used in underdeveloped countries that may lack appropriate facilities for culturing bacteria. For example, the Widal test, used for the diagnosis of typhoid fever, looks for agglutination of *Salmonella enterica* subspecies *typhi* in patient sera. The Widal test is rapid, inexpensive, and useful for monitoring the extent of an outbreak; however, it is not as accurate as tests that involve culturing of the bacteria. The Widal test frequently produces false positives in patients with previous infections with other subspecies of *Salmonella*, as well as false negatives in patients with hyperproteinemia or immune deficiencies.

In addition, agglutination tests are limited by the fact that patients generally do not produce detectable levels of antibody during the first week (or longer) of an infection. A patient is said to have undergone **seroconversion** when antibody levels reach the threshold for detection. Typically, seroconversion coincides with the onset of signs and symptoms of disease. However, in an HIV infection, for example, it generally takes 3 weeks for seroconversion to take place, and in some instances, it may take much longer.

Similar to techniques for the precipitin ring test and plaque assays, it is routine to prepare serial two-fold dilutions of the patient's serum and determine the titer of agglutinating antibody present. Since antibody levels change over time in both primary and secondary immune responses, by checking samples over time, changes in antibody titer can be detected. For example, a comparison of the titer during the acute phase of an infection versus the titer from the convalescent phase will distinguish whether an infection is current or has occurred in the past. It is also possible to monitor how well the patient's immune system is responding to the pathogen.

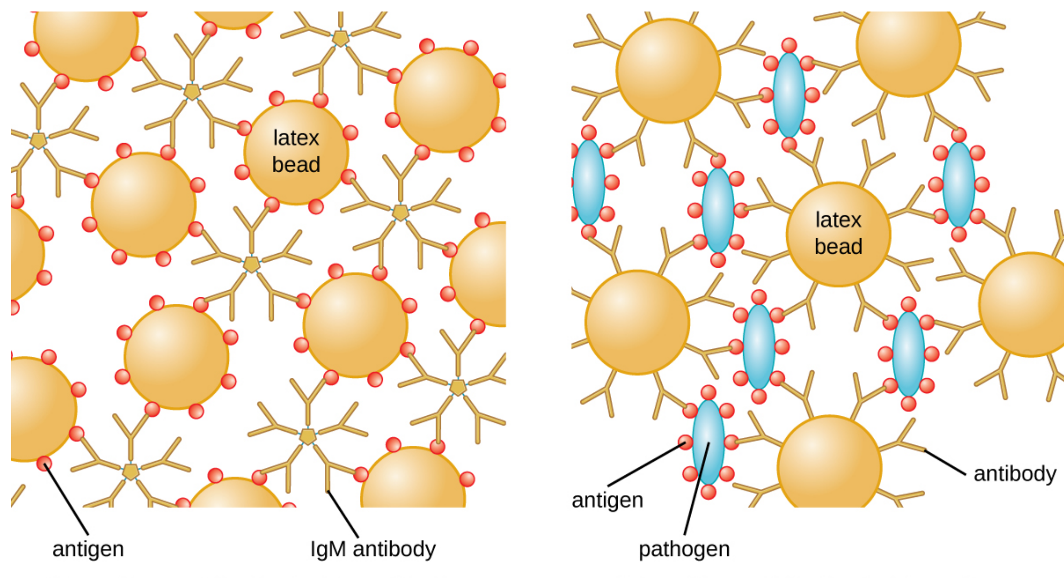


Figure 16.17 (a) Latex beads coated with an antigen will agglutinate when mixed with patient serum if the serum contains IgM antibodies against the antigen. (b) Latex beads coated with antibodies will agglutinate when mixed with patient serum if the serum contains antigens specific to the antibodies.

Watch this **video (https://openstax.org/l/22agglrealatbe)** that demonstrates agglutination reactions with latex beads.

- How is agglutination used to distinguish serovars from each other?
- In a latex bead assay to test for antibodies in a patient's serum, with what are the beads coated?
- What has happened when a patient has undergone seroconversion?

Hemagglutination

Agglutination of red blood cells is called **hemagglutination**. One common assay that uses hemagglutination is the **direct Coombs' test**, also called the **direct antihuman globulin test (DAT)**, which generally looks for nonagglutinating antibodies. The test can also detect complement attached to red blood cells.

The Coombs' test is often employed when a newborn has jaundice, yellowing of the skin caused by high blood concentrations of bilirubin, a product of the breakdown of hemoglobin in the blood. The Coombs' test is used to determine whether the child's red blood cells have been bound by the mother's antibodies. These antibodies would activate complement, leading to red blood cell lysis and the subsequent jaundice. Other conditions that can cause positive direct Coombs' tests include hemolytic transfusion reactions, autoimmune hemolytic anemia, infectious mononucleosis (caused by Epstein-Barr virus), syphilis, and *Mycoplasma* pneumonia. A positive direct Coombs' test may also be seen in some cancers and as an allergic reaction to some drugs (e.g., penicillin).

The antibodies bound to red blood cells in these conditions are most often IgG, and because of the orientation of the antigen-binding sites on IgG and the comparatively large size of a red blood cell, it is unlikely that any visible agglutination will occur. However, the presence of IgG bound to red blood cells can be detected by adding **Coombs' reagent**, an antiserum containing antihuman IgG antibodies (that may be combined with anti-complement) (**Figure 16.18**). The Coombs' reagent links the IgG attached to neighboring red blood cells and thus promotes agglutination.

There is also an **indirect Coombs' test** known as the **indirect antiglobulin test (IAT)**. This screens an individual for antibodies against red blood cell antigens (other than the A and B antigens) that are unbound in a patient's serum (**Figure 16.18**). IAT can be used to screen pregnant women for antibodies that may cause hemolytic disease of the newborn. It can also be used prior to giving blood transfusions. More detail on how the IAT is performed is discussed below.

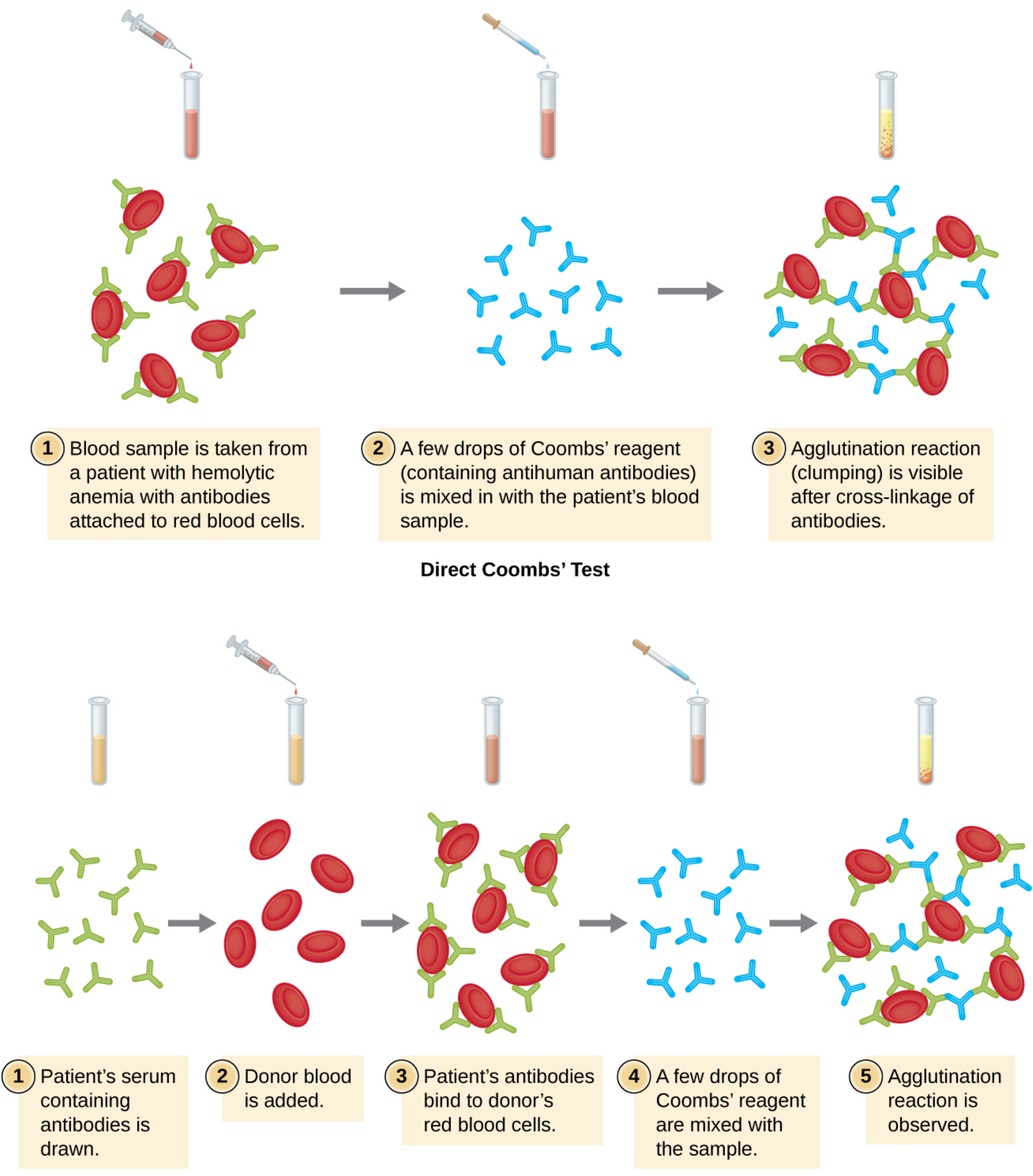


Figure 16.18 The steps in direct and indirect Coombs' tests are shown in the illustration.

Antibodies that bind to red blood cells are not the only cause of hemagglutination. Some viruses also bind to red blood cells, and this binding can cause agglutination when the viruses cross-link the red blood cells. For example, influenza viruses have two different types of viral spikes called neuraminidase (N) and hemagglutinin (H), the latter named for its ability to agglutinate red blood cells (see **Viruses (https://legacy.cnx.org/content/m58808/latest/)**). Thus, we can use red blood cells to detect the presence of influenza virus by **direct hemagglutination assays** (HA), in which the virus causes visible agglutination of red blood cells. The mumps and rubella viruses can also be detected using HA.

Most frequently, a serial dilution viral agglutination assay is used to measure the titer or estimate the amount of virus produced in cell culture or for vaccine production. A viral titer can be determined using a direct HA by making a serial dilution of the sample containing the virus, starting with a high concentration of sample that is then diluted in a series of wells. The highest dilution producing visible agglutination is the titer. The assay is carried out in a microtiter plate with V- or round-bottomed wells. In the presence of agglutinating viruses, the red blood cells and virus clump together and produce a diffuse mat over the bottom of the well. In the absence of virus, the red blood cells roll or sediment to the bottom of the well and form a dense pellet, which is why flat-bottomed wells cannot be used (**Figure 16.19**).

A modification of the HA assay can be used to determine the titer of antiviral antibodies. The presence of these antibodies in a patient's serum or in a lab-produced antiserum will neutralize the virus and block it from agglutinating the red cells, making this a **viral hemagglutination inhibition assay** (HIA). In this assay, patient serum is mixed with a standardized amount of virus. After a short incubation, a standardized amount of red blood cells is added and hemagglutination is observed. The titer of the patient's serum is the highest dilution that blocks agglutination (Figure 16.20).

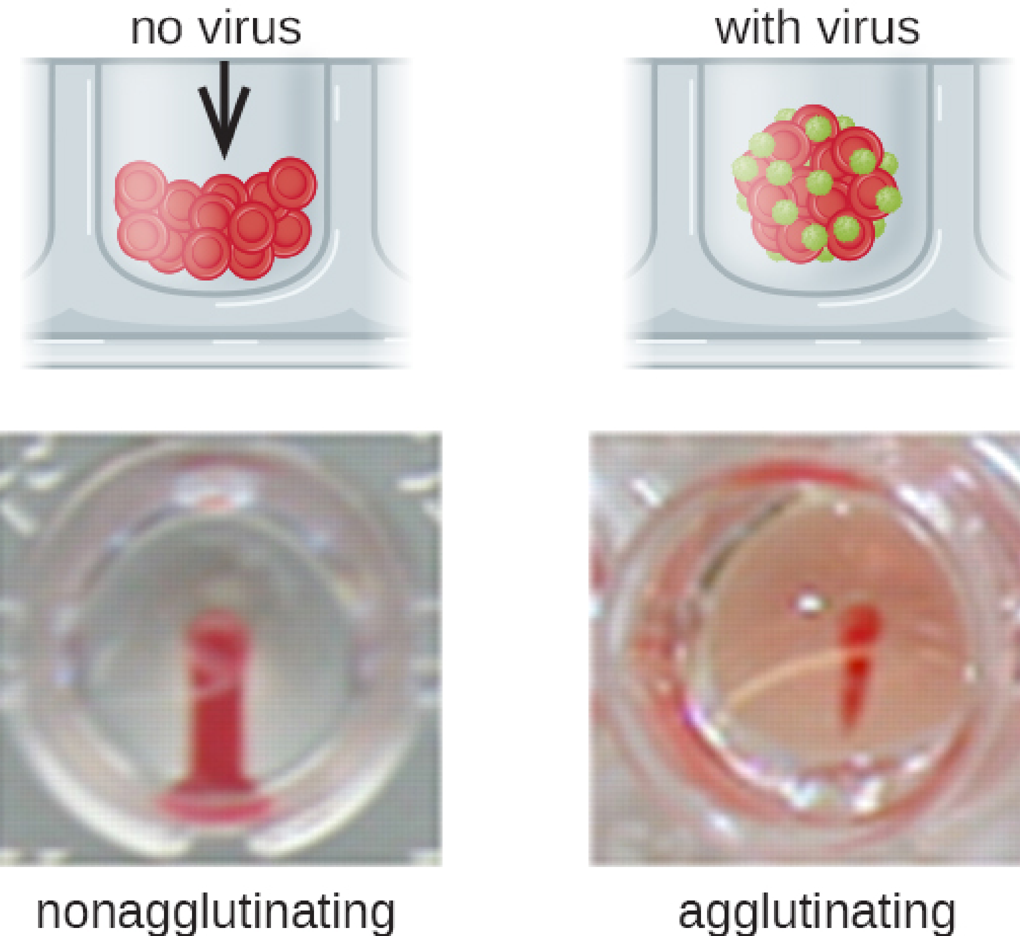


Figure 16.19 A viral suspension is mixed with a standardized amount of red blood cells. No agglutination of red blood cells is visible when the virus is absent, and the cells form a compact pellet at the bottom of the well. In the presence of virus, a diffuse pink precipitate forms in the well. (credit bottom: modification of work by American Society for Microbiology)

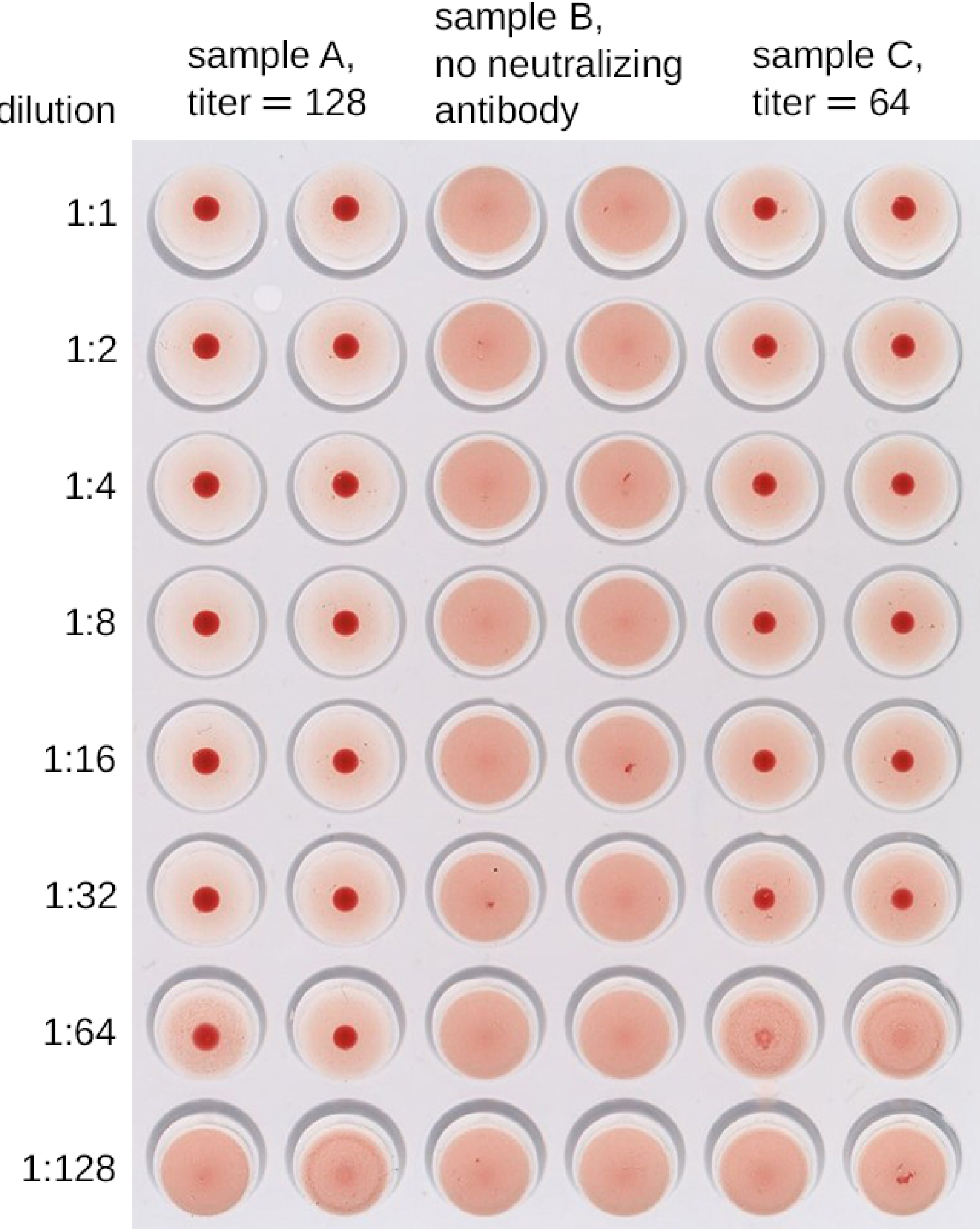


Figure 16.20 In this HIA, serum containing antibodies to influenzavirus underwent serial two-fold dilutions in a microtiter plate. Red blood cells were then added to the wells. Agglutination only occurred in those wells where the antibodies were too dilute to neutralize the virus. The highest dilution of patient serum that blocks agglutination is the titer of antibody in the patient's serum. In the case of this test, Sample A shows a titer of 64, and Sample C shows a titer of 32. (credit: modification of work by Evan Burkala)

- What is the mechanism by which viruses are detected in a hemagglutination assay?
- Which hemagglutination result tells us the titer of virus in a sample?

Animals in the Laboratory

Much of what we know today about the human immune system has been learned through research conducted using animals—primarily, mammals—as models. Besides research, mammals are also used for the production of most of the antibodies and other immune system components needed for

immunodiagnostics. Vaccines, diagnostics, therapies, and translational medicine in general have all been developed through research with animal models.

Consider some of the common uses of laboratory animals for producing immune system components. Guinea pigs are used as a source of complement, and mice are the primary source of cells for making mAbs. These mAbs can be used in research and for therapeutic purposes. Antisera are raised in a variety of species, including horses, sheep, goats, and rabbits. When producing an antiserum, the animal will usually be injected at least twice, and adjuvants may be used to boost the antibody response. The larger animals used for making antisera will have blood harvested repeatedly over long periods of time, with little harm to the animals, but that is not usually the case for rabbits. Although we can obtain a few milliliters of blood from the ear veins of rabbits, we usually need larger volumes, which results in the deaths of the animals.

We also use animals for the study of disease. The only way to grow *Treponema pallidum* for the study of syphilis is in living animals. Many viruses can be grown in cell culture, but growth in cell culture tells us very little about how the immune system will respond to the virus. When working on a newly discovered disease, we still employ Koch’s postulates, which require causing disease in lab animals using pathogens from pure culture as a crucial step in proving that a particular microorganism is the cause of a disease. Studying the proliferation of bacteria and viruses in animal hosts, and how the host immune system responds, has been central to microbiological research for well over 100 years.

While the practice of using laboratory animals is essential to scientific research and medical diagnostics, many people strongly object to the exploitation of animals for human benefit. This ethical argument is not a new one—indeed, one of Charles Darwin's daughters was an active antivivisectionist (vivisection is the practice of cutting or dissecting a live animal to study it). Most scientists acknowledge that there should be limits on the extent to which animals can be exploited for research purposes. Ethical considerations have led the National Institutes of Health (NIH) to develop strict regulations on the types of research that may be performed. These regulations also include guidelines for the humane treatment of lab animals, setting standards for their housing, care, and euthanization. The NIH document “Guide for the Care and Use of Laboratory Animals” makes it clear that the use of animals in research is a privilege granted by society to researchers.

The NIH guidelines are based on the principle of the three R’s: replace, refine, and reduce. Researchers should strive to *replace* animal models with nonliving models, *replace* vertebrates with invertebrates whenever possible, or use computer-models when applicable. They should *refine* husbandry and experimental procedures to reduce pain and suffering, and use experimental designs and procedures that *reduce* the number of animals needed to obtain the desired information. To obtain funding, researchers must satisfy NIH reviewers that the research justifies the use of animals and that their use is in accordance with the guidelines.

At the local level, any facility that uses animals and receives federal funding must have an Institutional Animal Care and Use Committee (IACUC) that ensures that the NIH guidelines are being followed. The IACUC must include researchers, administrators, a veterinarian, and at least one person with no ties to the institution, that is, a concerned citizen. This committee also performs inspections of laboratories and protocols. For research involving human subjects, an Institutional Review Board (IRB) ensures that proper guidelines are followed.

Visit this **site (https://openstax.org/l/22NIHcareuseani)** to view the NIH Guide for the Care and Use of Laboratory Animals.

Blood Typing and Cross-Matching

In addition to antibodies against bacteria and viruses to which they have previously been exposed, most individuals also carry antibodies against blood types other than their own. There are presently 33 immunologically important blood-type systems, many of which are restricted within various ethnic groups or rarely result in the production of antibodies. The most important and perhaps best known are the ABO and Rh blood groups (see **m58892 (https://legacy.cnx.org/content/m58892/latest/#OSC_Microbio_19_01_ABO)**).

When units of blood are being considered for transfusion, pretransfusion blood testing must be performed. For the blood unit, commercially prepared antibodies against the A, B, and Rh antigens are mixed with red blood

cells from the units to initially confirm that the blood type on the unit is accurate. Once a unit of blood has been requested for transfusion, it is vitally important to make sure the donor (unit of blood) and recipient (patient) are compatible for these crucial antigens. In addition to confirming the blood type of the unit, the patient's blood type is also confirmed using the same commercially prepared antibodies to A, B, and Rh. For example, as shown in Figure 16.21, if the donor blood is A-positive, it will agglutinate with the anti-A antiserum and with the anti-Rh antiserum. If no agglutination is observed with any of the sera, then the blood type would be O-negative.

Following determination of the blood type, immediately prior to releasing the blood for transfusion, a **cross-match** is performed in which a small aliquot of the donor red blood cells are mixed with serum from the patient awaiting transfusion. If the patient does have antibodies against the donor red blood cells, hemagglutination will occur. To confirm any negative test results and check for sensitized red blood cells, Coombs' reagent may be added to the mix to facilitate visualization of the antibody-red blood cell interaction.

Under some circumstances, a minor cross-match may be performed as well. In this assay, a small aliquot of donor serum is mixed with patient red blood cells. This allows the detection of agglutinizing antibodies in the donor serum. This test is rarely necessary because transfusions generally use packed red blood cells with most of the plasma removed by centrifugation.

Red blood cells have many other antigens in addition to ABO and Rh. While most people are unlikely to have antibodies against these antigens, women who have had multiple pregnancies or patients who have had multiple transfusions may have them because of repeated exposure. For this reason, an **antibody screen** test is used to determine if such antibodies are present. Patient serum is checked against commercially prepared, pooled, type O red blood cells that express these antigens. If agglutination occurs, the antigen to which the patient is responding must be identified and determined not to be present in the donor unit.

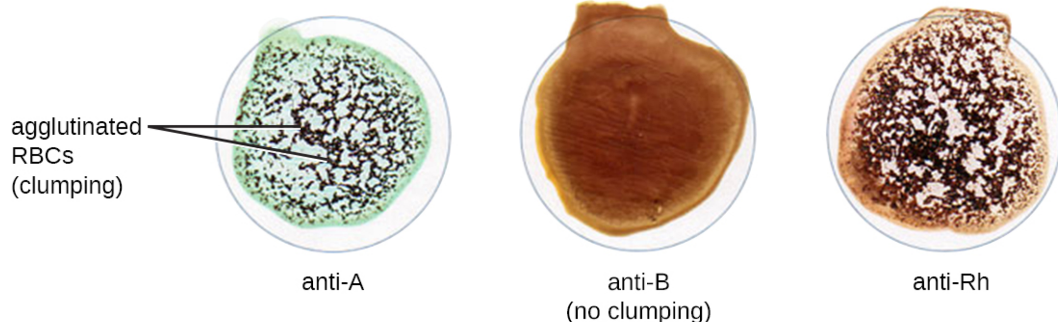


Figure 16.21 This sample of a commercially produced "bedside" card enables quick typing of both a recipient's and donor's blood before transfusion. The card contains three reaction sites or wells. One is coated with an anti-A antibody, one with an anti-B antibody, and one with an anti-Rh antibody. Agglutination of red blood cells in a given site indicates a positive identification of the blood antigens: in this case, A and Rh antigens for blood type A-positive.

- If a patient's blood agglutinates with anti-B serum, what is the patient's blood type?
- What is a cross-match assay, and why is it performed?

Table 16.3 summarizes the various kinds of agglutination assays discussed in this section.

Mechanisms of Select Antibody-Antigen Assays

Type of Assay	Mechanism	Example
Agglutination	Direct: Antibody is used to clump bacterial cells or other large structures	Serotyping bacteria
	Indirect: Latex beads are coupled with antigen or antibody to look for antibody or antigen, respectively, in patient serum	Confirming the presence of rheumatoid factor (IgM-binding Ig) in patient serum

Table 16.3

Mechanisms of Select Antibody-Antigen Assays

Type of Assay	Mechanism	Example
Hemagglutination	Direct: Some bacteria and viruses cross-link red blood cells and clump them together	Diagnosing influenza, mumps, and measles
	Direct Coombs' test (DAT): Detects nonagglutinating antibodies or complement proteins on red blood cells *in vivo*	Checking for maternal antibodies binding to neonatal red blood cells
	Indirect Coombs' test (IAT): Screens an individual for antibodies against red blood cell antigens (other than the A and B antigens) that are unbound in a patient's serum *in vitro*	Performing pretransfusion blood testing
	Viral hemagglutination inhibition: Uses antibodies from a patient to inhibit viral agglutination	Diagnosing various viral diseases by the presence of patient antibodies against the virus
	Blood typing and cross-matching: Detects ABO, Rh, and minor antigens in the blood	Matches donor blood to recipient immune requirements

Table 16.3

16.4 | EIAs and ELISAs

Learning Objectives

By the end of this section, you will be able to:

- Explain the differences and similarities between EIA, FEIA, and ELISA
- Describe the difference and similarities between immunohistochemistry and immunocytochemistry
- Describe the different purposes of direct and indirect ELISA

Similar to the western blot, **enzyme immunoassays (EIAs)** use antibodies to detect the presence of antigens. However, EIAs differ from western blots in that the assays are conducted in microtiter plates or *in vivo* rather than on an absorbent membrane. There are many different types of EIAs, but they all involve an antibody molecule whose constant region binds an enzyme, leaving the variable region free to bind its specific antigen. The addition of a substrate for the enzyme allows the antigen to be visualized or quantified (Figure 16.22).

In EIAs, the substrate for the enzyme is most often a chromogen, a colorless molecule that is converted into a colored end product. The most widely used enzymes are alkaline phosphatase and horseradish peroxidase for which appropriate substrates are readily available. In some EIAs, the substrate is a **fluorogen**, a nonfluorescent molecule that the enzyme converts into a fluorescent form. EIAs that utilize a fluorogen are called **fluorescent enzyme immunoassays (FEIAs)**. Fluorescence can be detected by either a fluorescence microscope or a spectrophotometer.

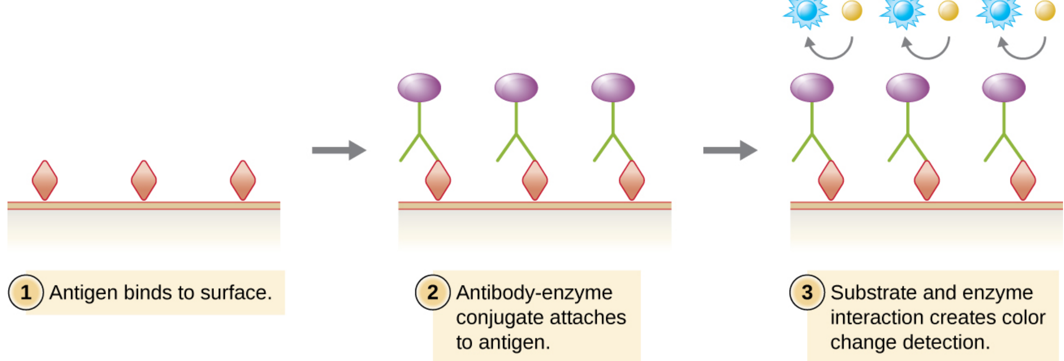


Figure 16.22 Enzyme immunoassays, such as the direct ELISA shown here, use an enzyme-antibody conjugate to deliver a detectable substrate to the site of an antigen. The substrate may be a colorless molecule that is converted into a colored end product or an inactive fluorescent molecule that fluoresces after enzyme activation. (credit: modification of work by "Cavitri"/Wikimedia Commons)

The MMR Titer

The MMR vaccine is a combination vaccine that provides protection against measles, mumps, and rubella (German measles). Most people receive the MMR vaccine as children and thus have antibodies against these diseases. However, for various reasons, even vaccinated individuals may become susceptible to these diseases again later in life. For example, some children may receive only one round of the MMR vaccine instead of the recommended two. In addition, the titer of protective antibodies in an individual's body may begin to decline with age or as the result of some medical conditions.

To determine whether the titer of antibody in an individual's bloodstream is sufficient to provide protection, an MMR titer test can be performed. The test is a simple immunoassay that can be done quickly with a blood sample. The results of the test will indicate whether the individual still has immunity or needs another dose of the MMR vaccine.

Submitting to an MMR titer is often a pre-employment requirement for healthcare workers, especially those who will frequently be in contact with young children or immunocompromised patients. Were a healthcare worker to become infected with measles, mumps, or rubella, the individual could easily pass these diseases on to susceptible patients, leading to an outbreak. Depending on the results of the MMR titer, healthcare workers might need to be revaccinated prior to beginning work.

Immunostaining

One powerful use of EIA is **immunostaining**, in which antibody-enzyme conjugates enhance microscopy. **Immunohistochemistry (IHC)** is used for examining whole tissues. As seen in **Figure 16.23**, a section of tissue can be stained to visualize the various cell types. In this example, a mAb against CD8 was used to stain CD8 cells in a section of tonsil tissue. It is now possible to count the number of CD8 cells, determine their relative numbers versus the other cell types present, and determine the location of these cells within this tissue. Such data would be useful for studying diseases such as AIDS, in which the normal function of CD8 cells is crucial for slowing disease progression.

Immunocytochemistry (ICC) is another valuable form of immunostaining. While similar to IHC, in ICC, extracellular matrix material is stripped away, and the cell membrane is etched with alcohol to make it permeable to antibodies. This allows antibodies to pass through the cell membrane and bind to specific targets inside the cell. Organelles, cytoskeletal components, and other intracellular structures can be visualized in this way. While some ICC techniques use EIA, the enzyme can be replaced with a fluorescent molecule, making it a fluorescent immunoassay.

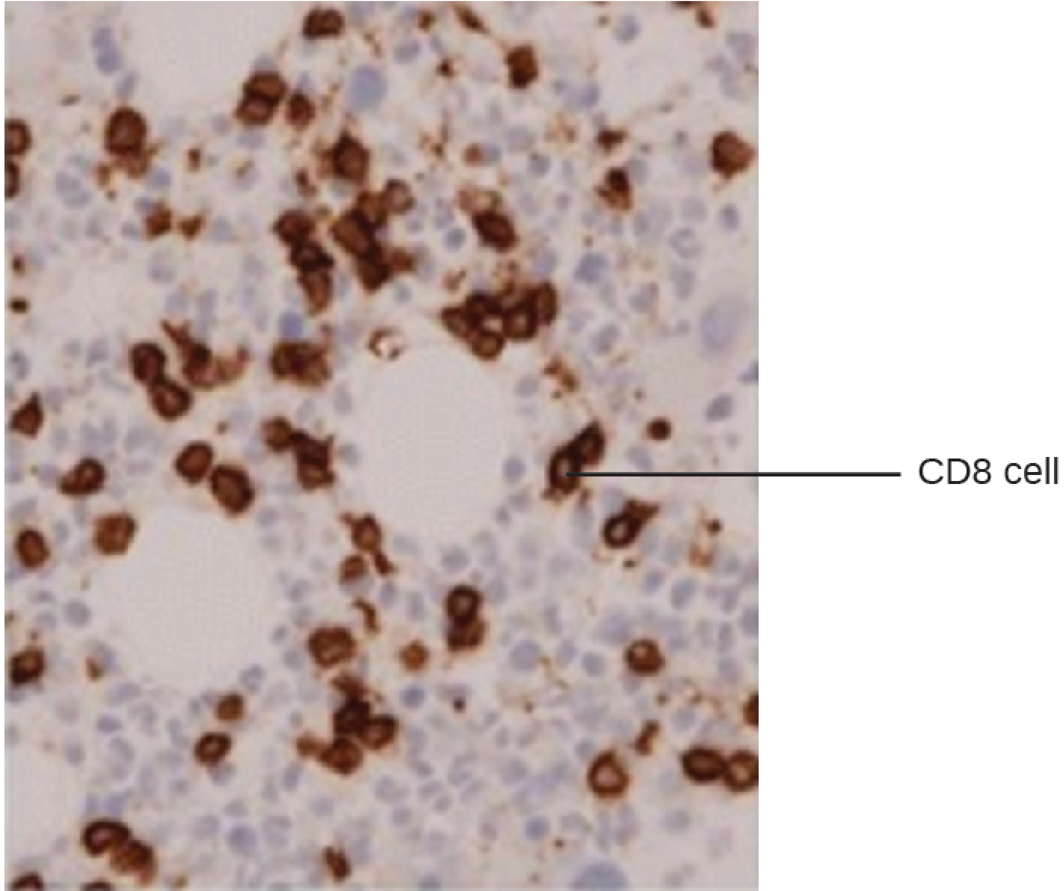


Figure 16.23 Enzyme-linked antibodies against CD8 were used to stain the CD8 cells in this preparation of bone marrow using a chromogen. (credit: modification of work by Yamashita M, Fujii Y, Ozaki K, Urano Y, Iwasa M, Nakamura S, Fujii S, Abe M, Sato Y, Yoshino T)

- What is the difference between immunohistochemistry and immunocytochemistry?
- What must be true of the product of the enzymatic reaction used in immunohistochemistry?

Enzyme-linked Immunosorbent Assays (ELISAs)

The **enzyme-linked immunosorbent assays (ELISAs)** are widely used EIAs. In the **direct ELISA**, antigens are immobilized in the well of a microtiter plate. An antibody that is specific for a particular antigen and is conjugated to an enzyme is added to each well. If the antigen is present, then the antibody will bind. After washing to remove any unbound antibodies, a colorless substrate (chromogen) is added. The presence of the enzyme converts the substrate into a colored end product (Figure 16.22). While this technique is faster because it only requires the use of one antibody, it has the disadvantage that the signal from a direct ELISA is lower (lower sensitivity).

In a **sandwich ELISA**, the goal is to use antibodies to precisely quantify specific antigen present in a solution, such as antigen from a pathogen, a serum protein, or a hormone from the blood or urine to list just a few examples. The first step of a sandwich ELISA is to add the **primary antibody** to all the wells of a microtiter plate (Figure 16.24). The antibody sticks to the plastic by hydrophobic interactions. After an appropriate incubation time, any unbound antibody is washed away. Comparable washes are used between each of the subsequent steps to ensure that only specifically bound molecules remain attached to the plate. A blocking protein is then added (e.g., albumin or the milk protein casein) to bind the remaining nonspecific protein-binding sites in the well. Some of the wells will receive known amounts of antigen to allow the construction of a standard curve, and unknown antigen solutions are added to the other wells. The primary antibody captures the antigen and, following a wash, the **secondary antibody** is added, which is a polyclonal antibody that is conjugated to an enzyme. After a final wash, a colorless substrate (chromogen) is added, and the enzyme converts it into a colored end product. The color intensity of the sample caused by the end product is measured with a spectrophotometer. The amount of color produced (measured as absorbance) is directly proportional to the

amount of enzyme, which in turn is directly proportional to the captured antigen. ELISAs are extremely sensitive, allowing antigen to be quantified in the nanogram (10^{-9} g) per mL range.

In an **indirect ELISA**, we quantify antigen-specific antibody rather than antigen. We can use indirect ELISA to detect antibodies against many types of pathogens, including *Borrelia burgdorferi* (Lyme disease) and HIV. There are three important differences between indirect and direct ELISAs as shown in **Figure 16.25**. Rather than using antibody to capture antigen, the indirect ELISA starts with attaching known antigen (e.g., peptides from HIV) to the bottom of the microtiter plate wells. After blocking the unbound sites on the plate, patient serum is added; if antibodies are present (primary antibody), they will bind the antigen. After washing away any unbound proteins, the secondary antibody with its conjugated enzyme is directed against the primary antibody (e.g., antihuman immunoglobulin). The secondary antibody allows us to quantify how much antigen-specific antibody is present in the patient's serum by the intensity of the color produced from the conjugated enzyme-chromogen reaction.

As with several other tests for antibodies discussed in this chapter, there is always concern about cross-reactivity with antibodies directed against some other antigen, which can lead to false-positive results. Thus, we cannot definitively diagnose an HIV infection (or any other type of infection) based on a single indirect ELISA assay. We must confirm any suspected positive test, which is most often done using either an immunoblot that actually identifies the presence of specific peptides from the pathogen or a test to identify the nucleic acids associated with the pathogen, such as reverse transcriptase PCR (RT-PCR) or a nucleic acid antigen test.

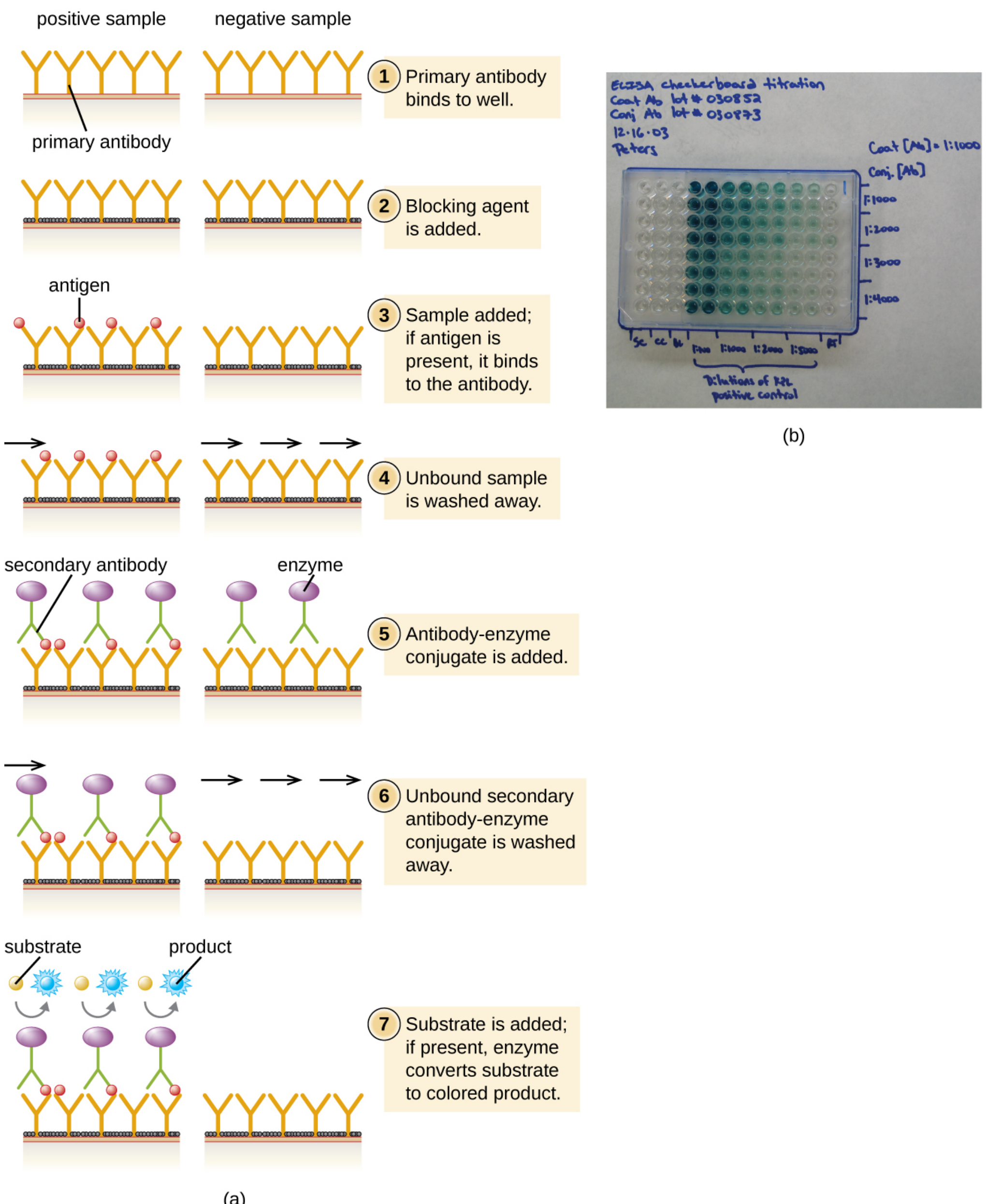


Figure 16.24 (a) In a sandwich ELISA, a primary antibody is used to first capture an antigen with the primary antibody. A secondary antibody conjugated to an enzyme that also recognizes epitopes on the antigen is added. After the addition of the chromogen, a spectrophotometer measures the absorbance of end product, which is directly proportional to the amount of captured antigen. (b) An ELISA plate shows dilutions of antibodies (left) and antigens (bottom). Higher concentrations result in a darker final color. (credit b: modification of work by U.S. Fish and Wildlife Service Pacific Region)

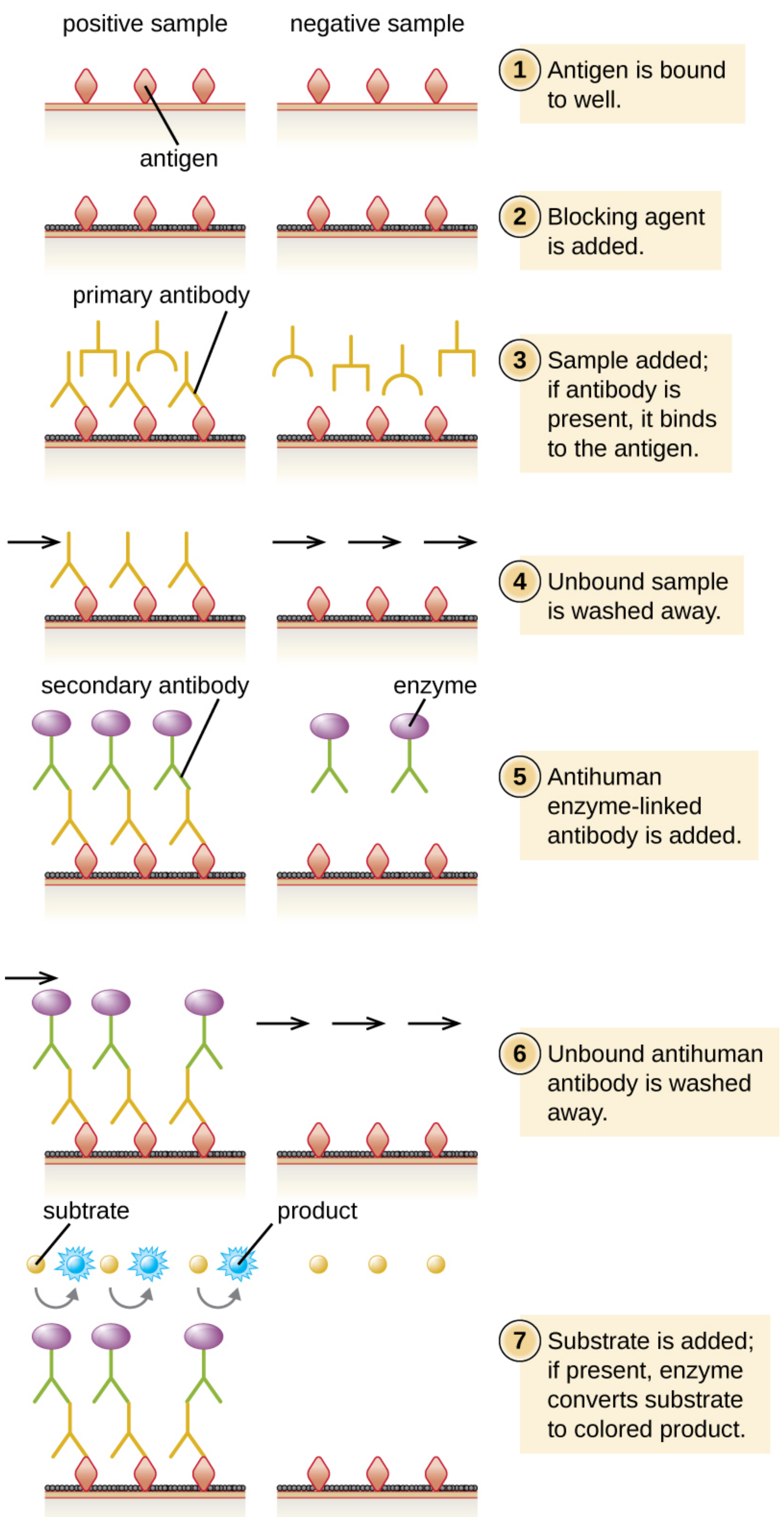

positive sample
negative sample
antigen
1 Antigen is bound to well.
2 Blocking agent is added.
primary antibody
3 Sample added; if antibody is present, it binds to the antigen.
4 Unbound sample is washed away.
secondary antibody
enzyme
5 Antihuman enzyme-linked antibody is added.
6 Unbound antihuman antibody is washed away.
subtrate
product
7 Substrate is added; if present, enzyme converts substrate to colored product.

Figure 16.25 The indirect ELISA is used to quantify antigen-specific antibodies in patient serum for disease diagnosis. Antigen from the suspected disease agent is attached to microtiter plates. The primary antibody comes from the patient's serum, which is subsequently bound by the enzyme-conjugated secondary antibody. Measuring the production of end product allows us to detect or quantify the amount of antigen-specific antibody present in the patient's serum.

- What is the purpose of the secondary antibody in a direct ELISA?
- What do the direct and indirect ELISAs quantify?

Part 2

Although contacting and testing the 1300 patients for HIV would be time consuming and expensive, administrators hoped to minimize the hospital's liability by proactively seeking out and treating potential victims of the rogue employee's crime. Early detection of HIV is important, and prompt treatment can slow the progression of the disease.

There are a variety of screening tests for HIV, but the most widely used is the indirect ELISA. As with other indirect ELISAs, the test works by attaching antigen (in this case, HIV peptides) to a well in a 96-well plate. If the patient is HIV positive, anti-HIV antibodies will bind to the antigen and be identified by the second antibody-enzyme conjugate.

- How accurate is an indirect ELISA test for HIV, and what factors could impact the test's accuracy?
- Should the hospital use any other tests to confirm the results of the indirect ELISA?

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Immunofiltration and Immunochromatographic Assays

For some situations, it may be necessary to detect or quantify antigens or antibodies that are present at very low concentration in solution. Immunofiltration techniques have been developed to make this possible. In **immunofiltration**, a large volume of fluid is passed through a porous membrane into an absorbent pad. An antigen attached to the porous membrane will capture antibody as it passes; alternatively, we can also attach an antibody to the membrane to capture antigen.

The method of immunofiltration has been adapted in the development of **immunochromatographic assays**, commonly known as **lateral flow tests** or strip tests. These tests are quick and easy to perform, making them popular for point-of-care use (i.e., in the doctor's office) or in-home use. One example is the TORCH test that allows doctors to screen pregnant women or newborns for infection by an array of viruses and other pathogens (*Toxoplasma*, other viruses, rubella, cytomegalovirus, herpes simplex). In-home pregnancy tests are another widely used example of a lateral flow test (**Figure 16.26**). Immunofiltration tests are also popular in developing countries, because they are inexpensive and do not require constant refrigeration of the dried reagents. However, the technology is also built into some sophisticated laboratory equipment.

In lateral flow tests (**Figure 16.27**), fluids such as urine are applied to an absorbent pad on the test strip. The fluid flows by capillary action and moves through a stripe of beads with antibodies attached to their surfaces. The fluid in the sample actually hydrates the reagents, which are present in a dried state in the stripe. Antibody-coated beads made of latex or tiny gold particles will bind antigens in the test fluid. The antibody-antigen complexes then flow over a second stripe that has immobilized antibody against the antigen; this stripe will retain the beads that have bound antigen. A third control stripe binds any beads. A red color (from gold particles) or blue (from latex beads) developing at the test line indicates a positive test. If the color only develops at the control line, the test is negative.

Like ELISA techniques, lateral flow tests take advantage of antibody sandwiches, providing sensitivity and specificity. While not as quantitative as ELISA, these tests have the advantage of being fast, inexpensive, and not dependent on special equipment. Thus, they can be performed anywhere by anyone. There are some concerns about putting such powerful diagnostic tests into the hands of people who may not understand the tests' limitations, such as the possibility of false-positive results. While home pregnancy tests have become widely accepted, at-home antibody-detection tests for diseases like HIV have raised some concerns in the medical community. Some have questioned whether self-administration of such tests should be allowed in the absence of medical personnel who can explain the test results and order appropriate confirmatory tests. However, with growing numbers of lateral flow tests becoming available, and the rapid development of lab-on-a-

chip technology (**Figure 16.1**), home medical tests are likely to become even more commonplace in the future.

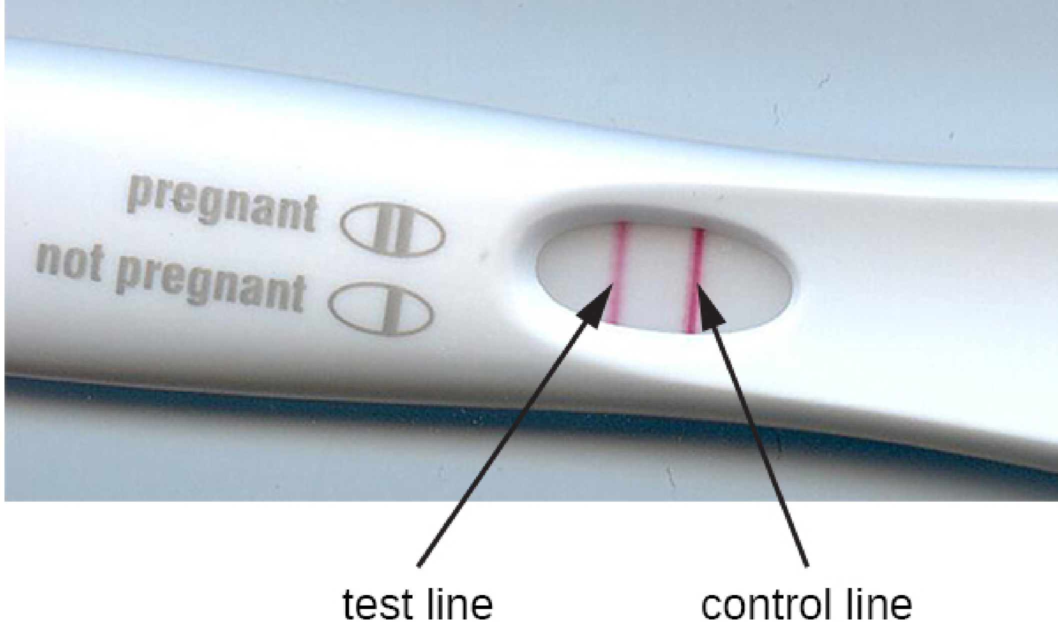


Figure 16.26 A lateral flow test detecting pregnancy-related hormones in urine. The control stripe verifies the validity of the test and the test line determines the presence of pregnancy-related hormones in the urine. (credit: modification of work by Klaus Hoffmeier)

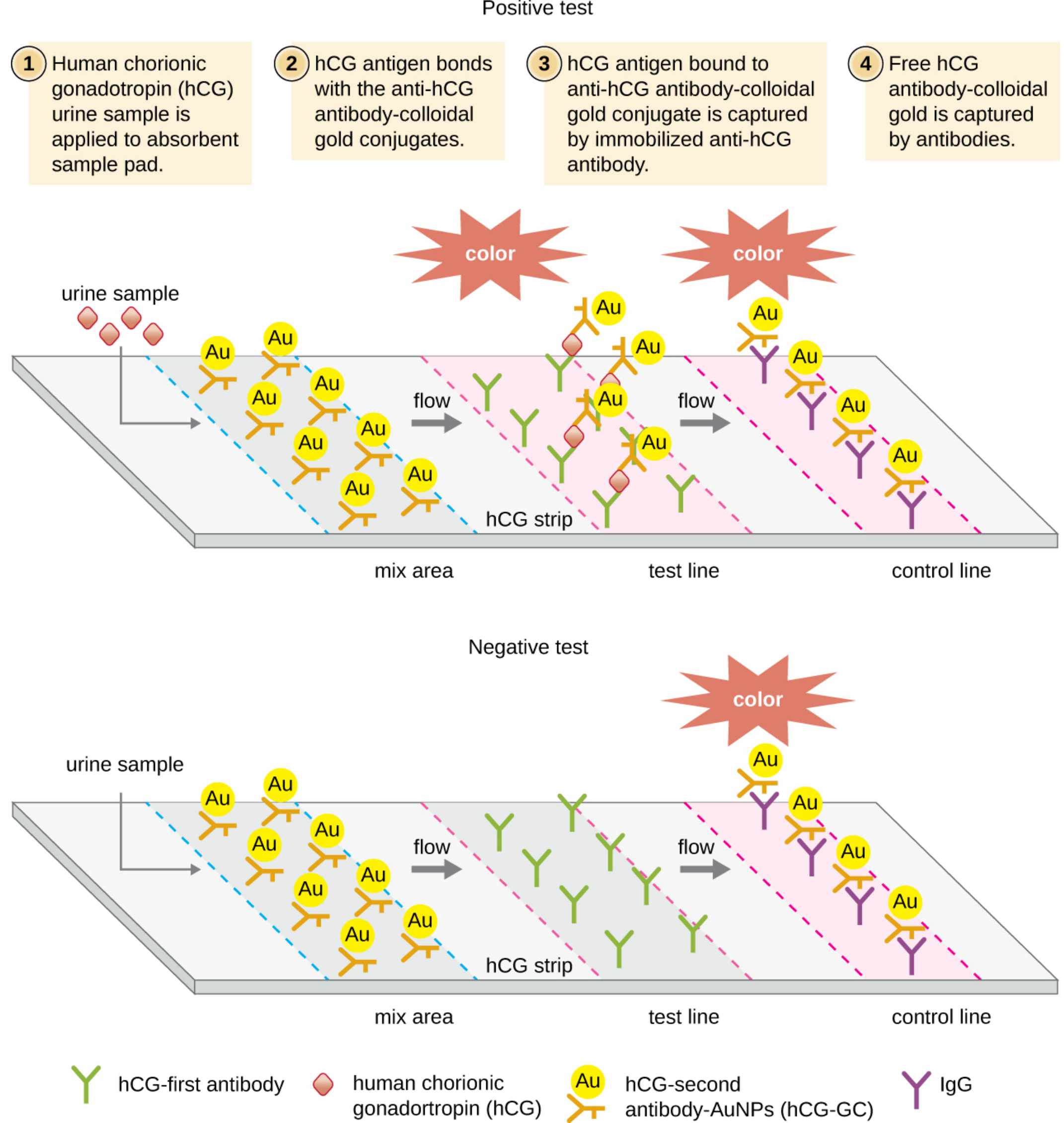


Figure 16.27 Immunochromatographic assays, or lateral flow tests, allow the testing of antigen in a dilute solution. As the fluid flows through the test strip, it rehydrates the reagents. Antibodies conjugated to small particles bind the antigen in the first stripe and then flow onto the second stripe where they are bound by a second, fixed antibody. This produces a line of color, depending on the color of the beads. The third, control stripe binds beads as well to indicate that the test is working properly. (credit: modification of work by Yeh CH, Zhao ZQ, Shen PL, Lin YC)

- What physical process does the lateral flow method require to function?
- Explain the purpose of the third strip in a lateral flow assay.

Table 16.4 compares some of the key mechanisms and examples of some of the EIAs discussed in this section as well as immunoblots, which were discussed in **Detecting Antigen-Antibody Complexes**.

Immunoblots & Enzyme Immunoassays

Type of Assay	Mechanism	Specific Procedures	Examples
Immunoblots	Uses enzyme-antibody conjugates to identify specific proteins that have been transferred to an absorbent membrane	Western blot: Detects the presence of a particular protein	Detecting the presence of HIV peptides (or peptides from other infectious agents) in patient sera
Immunostaining	Uses enzyme-antibody conjugates to stain specific molecules on or in cells	Immunohistochemistry: Used to stain specific cells in a tissue	Stain for presence of CD8 cells in host tissue
Enzyme-linked immunosorbent assay (ELISA)	Uses enzyme-antibody conjugates to quantify target molecules	Direct ELISA: Uses a single antibody to detect the presence of an antigen	Detection of HIV antigen p24 up to one month after being infected
		Indirect ELISA: Measures the amount of antibody produced against an antigen	Detection of HIV antibodies in serum
Immunochromatographic (lateral flow) assays	Techniques use the capture of flowing, color-labeled antigen-antibody complexes by fixed antibody for disease diagnosis	Sandwich ELISA: Measures the amount of antigen bound by the antibody	Detection of antibodies for various pathogens in patient sera (e.g., rapid strep, malaria dipstick)
			Pregnancy test detecting human chorionic gonadotrophin in urine

Table 16.4

Part 3

Although the indirect ELISA for HIV is a sensitive assay, there are several complicating considerations. First, if an infected person is tested too soon after becoming infected, the test can yield false-negative results. The seroconversion window is generally about three weeks, but in some cases, it can be more than two months.

In addition to false negatives, false positives can also occur, usually due to previous infections with other viruses that induce cross-reacting antibodies. The false-positive rate depends on the particular brand of test used, but 0.5% is not unusual.[10] Because of the possibility of a false positive, all positive tests are followed up with a confirmatory test. This confirmatory test is often an immunoblot (western blot) in which HIV peptides from the patient's blood are identified using an HIV-specific mAb-enzyme conjugate. A positive western blot would confirm an HIV infection and a negative blot would confirm the absence of HIV despite the positive ELISA.

Unfortunately, western blots for HIV antigens often yield indeterminant results, in which case, they neither confirm nor invalidate the results of the indirect ELISA. In fact, the rate of indeterminants can be 10–49% (which is why, combined with their cost, western blots are not used for screening). Similar to the indirect ELISA, an indeterminant western blot can occur because of cross-reactivity or previous viral infections, vaccinations, or autoimmune diseases.

- Of the 1300 patients being tested, how many false-positive ELISA tests would be expected?
- Of the false positives, how many indeterminant western blots could be expected?

10. Thomas, Justin G., Victor Jaffe, Judith Shaffer, and Jose Abreu, "HIV Testing: US Recommendations 2014," *Osteopathic Family Physician* 6, no. 6 (2014).

- How would the hospital address any cases in which a patient's western blot was indeterminant?

*Jump to the **previous** Clinical Focus box. Jump to the **next** Clinical Focus box.*

16.5 | Fluorescent Antibody Techniques

Learning Objectives

By the end of this section, you will be able to:

- Describe the benefits of immunofluorescent antibody assays in comparison to nonfluorescent assays
- Compare direct and indirect fluorescent antibody assays
- Explain how a flow cytometer can be used to quantify specific subsets of cells present in a complex mixture of cell types
- Explain how a fluorescence-activated cell sorter can be used to separate unique types of cells

Rapid visualization of bacteria from a clinical sample such as a throat swab or sputum can be achieved through **fluorescent antibody (FA) techniques** that attach a fluorescent marker (fluorogen) to the constant region of an antibody, resulting in a reporter molecule that is quick to use, easy to see or measure, and able to bind to target markers with high specificity. We can also label cells, allowing us to precisely quantify particular subsets of cells or even purify these subsets for further research.

As with the enzyme assays, FA methods may be direct, in which a labeled mAb binds an antigen, or indirect, in which secondary polyclonal antibodies bind patient antibodies that react to a prepared antigen. Applications of these two methods were demonstrated in **Figure 9.16**. FA methods are also used in automated cell counting and sorting systems to enumerate or segregate labeled subpopulations of cells in a sample.

Direct Fluorescent Antibody Techniques

Direct fluorescent antibody (DFA) tests use a fluorescently labeled mAb to bind and illuminate a target antigen. DFA tests are particularly useful for the rapid diagnosis of bacterial diseases. For example, fluorescence-labeled antibodies against *Streptococcus pyogenes* (group A strep) can be used to obtain a diagnosis of strep throat from a throat swab. The diagnosis is ready in a matter of minutes, and the patient can be started on antibiotics before even leaving the clinic. DFA techniques may also be used to diagnose pneumonia caused by *Mycoplasma pneumoniae* or *Legionella pneumophila* from sputum samples (**Figure 16.28**). The fluorescent antibodies bind to the bacteria on a microscope slide, allowing ready detection of the bacteria using a fluorescence microscope. Thus, the DFA technique is valuable for visualizing certain bacteria that are difficult to isolate or culture from patient samples.

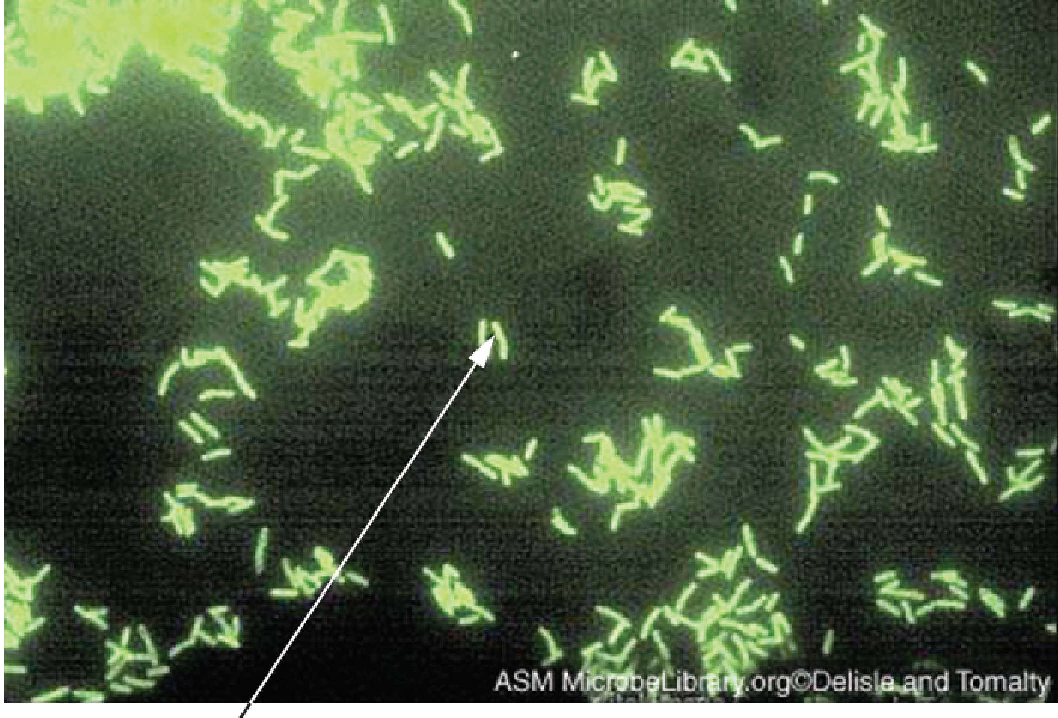


Figure 16.28 A green fluorescent mAb against *L. pneumophila* is used here to visualize and identify bacteria from a smear of a sample from the respiratory tract of a pneumonia patient. (credit: modification of work by American Society for Microbiology)

Watch the **animation (https://openstax.org/l/22dirfluorant)** on this page to review the procedures of the direct fluorescent antibody test.

- In a direct fluorescent antibody test, what does the fluorescent antibody bind to?

Indirect Fluorescent Antibody Techniques

Indirect fluorescent antibody (IFA) tests (**Figure 16.29**) are used to look for antibodies in patient serum. For example, an IFA test for the diagnosis of syphilis uses *T. pallidum* cells isolated from a lab animal (the bacteria cannot be grown on lab media) and a smear prepared on a glass slide. Patient serum is spread over the smear and anti-treponemal antibodies, if present, are allowed to bind. The serum is washed off and a secondary antibody added. The secondary antibody is an antihuman immunoglobulin conjugated to a fluorogen. On examination, the *T. pallidum* bacteria will only be visible if they have been bound by the antibodies from the patient's serum.

The IFA test for syphilis provides an important complement to the VDRL test discussed in **Detecting Antigen-Antibody Complexes**. The VDRL is more likely to generate false-positive reactions than the IFA test; however, the VDRL is a better test for determining whether an infection is currently active.

IFA tests are also useful for the diagnosis of autoimmune diseases. For example, systemic lupus erythematosus (SLE) (see **Autoimmune Disorders (https://legacy.cnx.org/content/m58893/latest/)**) is characterized by elevated expression levels of antinuclear antibodies (ANA). These autoantibodies can be expressed against a variety of DNA-binding proteins and even against DNA itself. Because autoimmunity is often difficult to diagnose, especially early in disease progression, testing for ANA can be a valuable clue in making a diagnosis and starting appropriate treatment.

The IFA for ANA begins by fixing cells grown in culture to a glass slide and making them permeable to antibody. The slides are then incubated with serial dilutions of serum from the patient. After incubation, the slide is washed

to remove unbound proteins, and the fluorescent antibody (antihuman IgG conjugated to a fluorogen) added. After an incubation and wash, the cells can be examined for fluorescence evident around the nucleus (**Figure 16.30**). The titer of ANA in the serum is determined by the highest dilution showing fluorescence. Because many healthy people express ANA, the American College of Rheumatology recommends that the titer must be at least 1:40 in the presence of symptoms involving two or more organ systems to be considered indicative of SLE.[11]

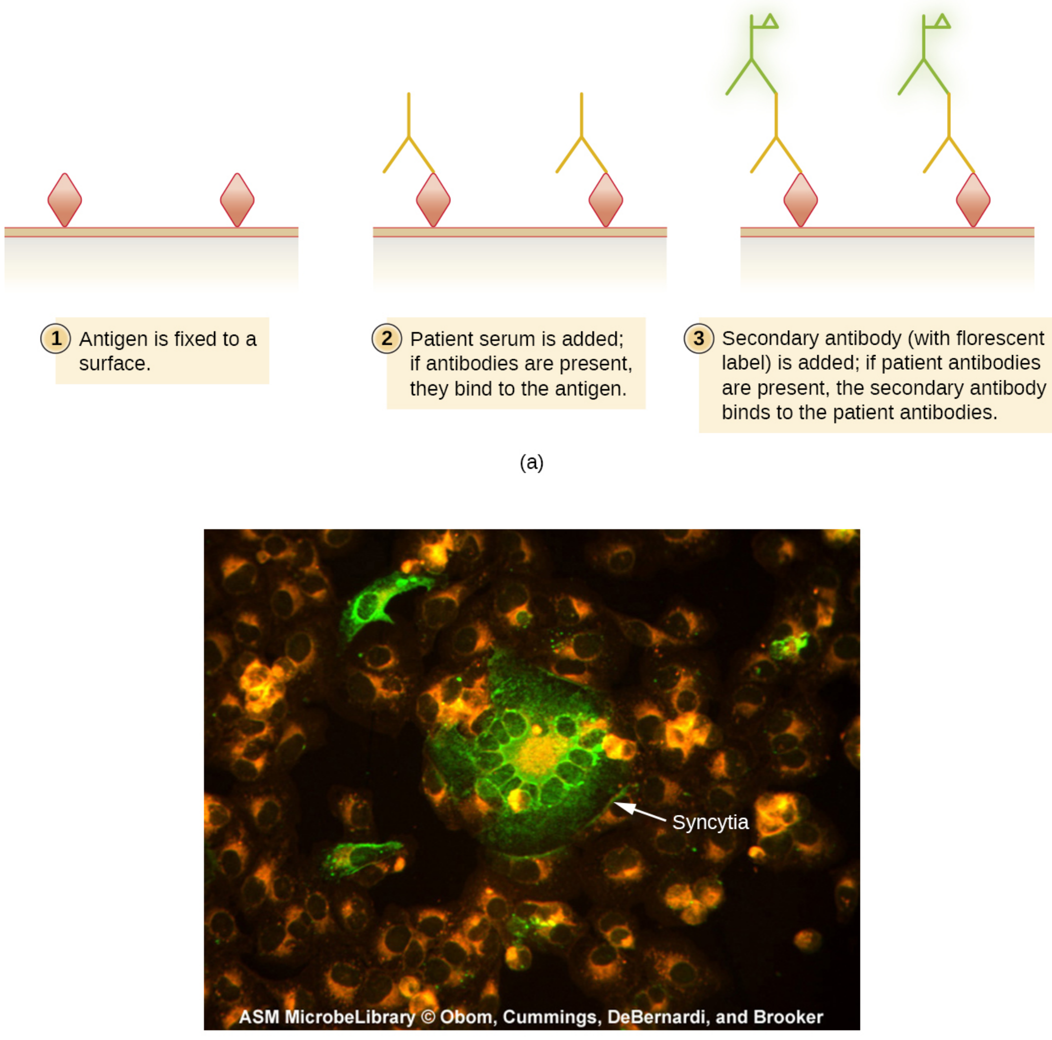
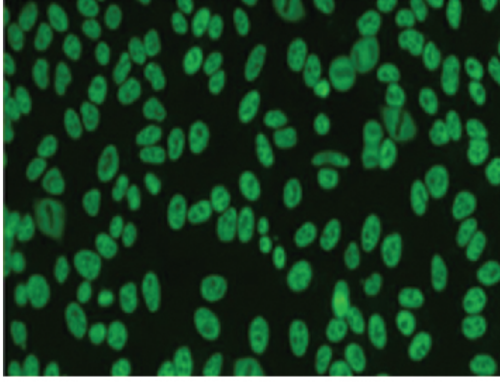


Figure 16.29 (a) The IFA test is used to detect antigen-specific antibodies by allowing them to bind to antigen fixed to a surface and then illuminating these complexes with a secondary antibody-fluorogen conjugate. (b) In this example of a micrograph of an indirect fluorescent antibody test, a patient's antibodies to the measles virus bind to viral antigens present on inactivated measles-infected cells affixed to a slide. Secondary antibodies bind the patient's antibodies and carry a fluorescent molecule. (credit b: modification of work by American Society for Microbiology)

11. Gill, James M., ANNA M. Quisel, PETER V. Rocca, and DENE T. Walters. "Diagnosis of systemic lupus erythematosus." *American family physician* 68, no. 11 (2003): 2179-2186.



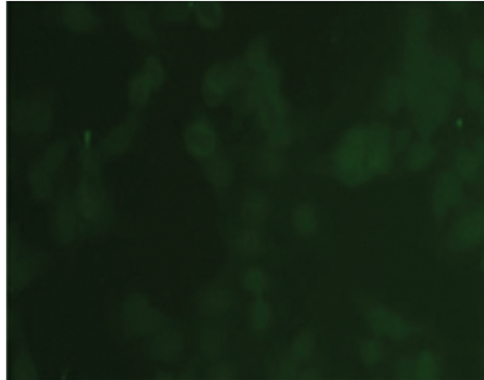


Figure 16.30 In this test for antinuclear antibodies (ANA), cells are exposed to serum from a patient suspected of making ANA and then to a fluorescent mAb specific for human immunoglobulin. As a control, serum from a healthy patient is also used. Visible fluorescence around the nucleus demonstrates the presence of ANA in the patient's serum. In the healthy control where lower levels of ANA are produced, very faint green is detected. (credit left, right: modification of work by Al-Hussaini AA, Alzahrani MD, Alenizi AS, Suliman NM, Khan MA, Alharbi SA, Chentoufi AA)

- In an indirect fluorescent antibody test, what does the fluorescent antibody bind to?
- What is the ANA test looking for?

Flow Cytometry

Fluorescently labeled antibodies can be used to quantify cells of a specific type in a complex mixture using **flow cytometry** (Figure 16.31), an automated, cell-counting system that detects fluorescing cells as they pass through a narrow tube one cell at a time. For example, in HIV infections, it is important to know the level of CD4 T cells in the patient's blood; if the numbers fall below 500 per µL of blood, the patient becomes more likely to acquire opportunistic infections; below 200 per µL, the patient can no longer mount a useful adaptive immune response at all. The analysis begins by incubating a mixed-cell population (e.g., white blood cells from a donor) with a fluorescently labeled mAb specific for a subpopulation of cells (e.g., anti-CD4). Some experiments look at two cell markers simultaneously by adding a different fluorogen to the appropriate mAb. The cells are then introduced to the flow cytometer through a narrow capillary that forces the cells to pass in single file. A laser is used to activate the fluorogen. The fluorescent light radiates out in all directions, so the fluorescence detector can be positioned at an angle from the incident laser light.

Figure 16.31 shows the obscuration bar in front of the forward-scatter detector that prevents laser light from hitting the detector. As a cell passes through the laser bar, the forward-scatter detector detects light scattered around the obscuration bar. The scattered light is transformed into a voltage pulse, and the cytometer counts a cell. The fluorescence from a labeled cell is detected by the side-scatter detectors. The light passes through various dichroic mirrors such that the light emitted from the fluorophore is received by the correct detector.

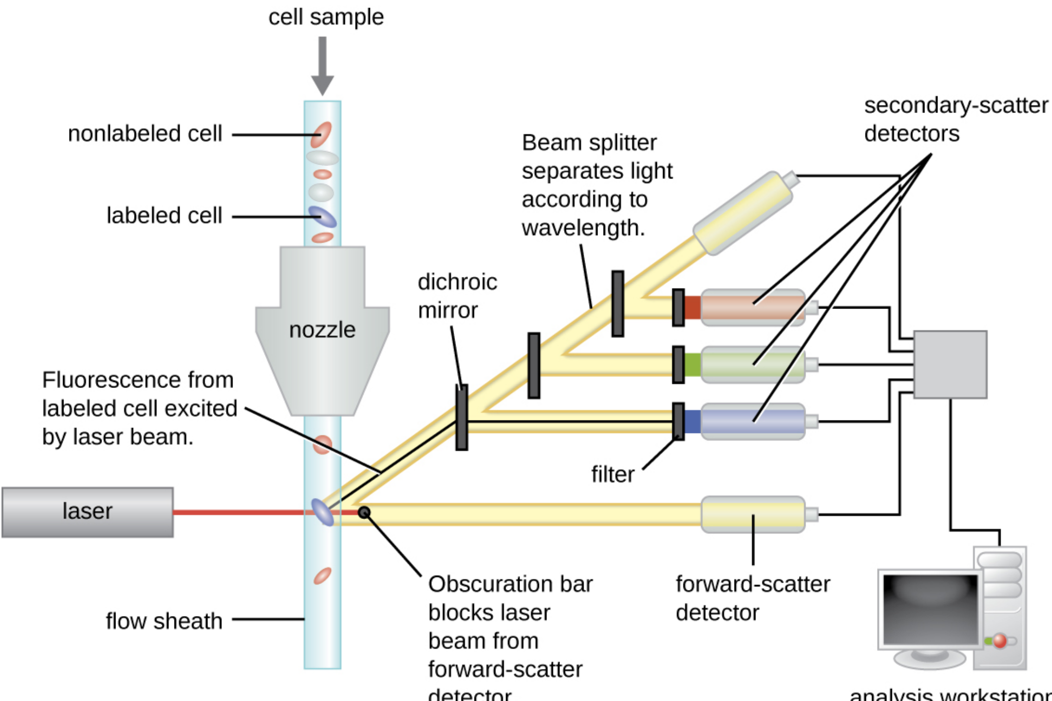


Figure 16.31 In flow cytometry, a mixture of fluorescently labeled and unlabeled cells passes through a narrow capillary. A laser excites the fluorogen, and the fluorescence intensity of each cell is measured by a detector. (credit: modification of work by “Kierano”/Wikimedia Commons)

Data are collected from both the forward- and side-scatter detectors. One way these data can be presented is in the form of a histogram. The forward scatter is placed on the *y*-axis (to represent the number of cells), and the side scatter is placed on the *x*-axis (to represent the fluoresence of each cell). The scaling for the *x*-axis is logarithmic, so fluorescence intensity increases by a factor of 10 with each unit increase along the axis. **Figure 16.32** depicts an example in which a culture of cells is combined with an antibody attached to a fluorophore to detect CD8 cells and then analyzed by flow cytometry. The histogram has two peaks. The peak on the left has lower fluorescence readings, representing the subset of the cell population (approximately 30 cells) that does not fluoresce; hence, they are not bound by antibody and therefore do not express CD8. The peak on the right has higher fluorescence readings, representing the subset of the cell population (approximately 100 cells) that show fluorescence; hence, they are bound by the antibody and therefore do express CD8.

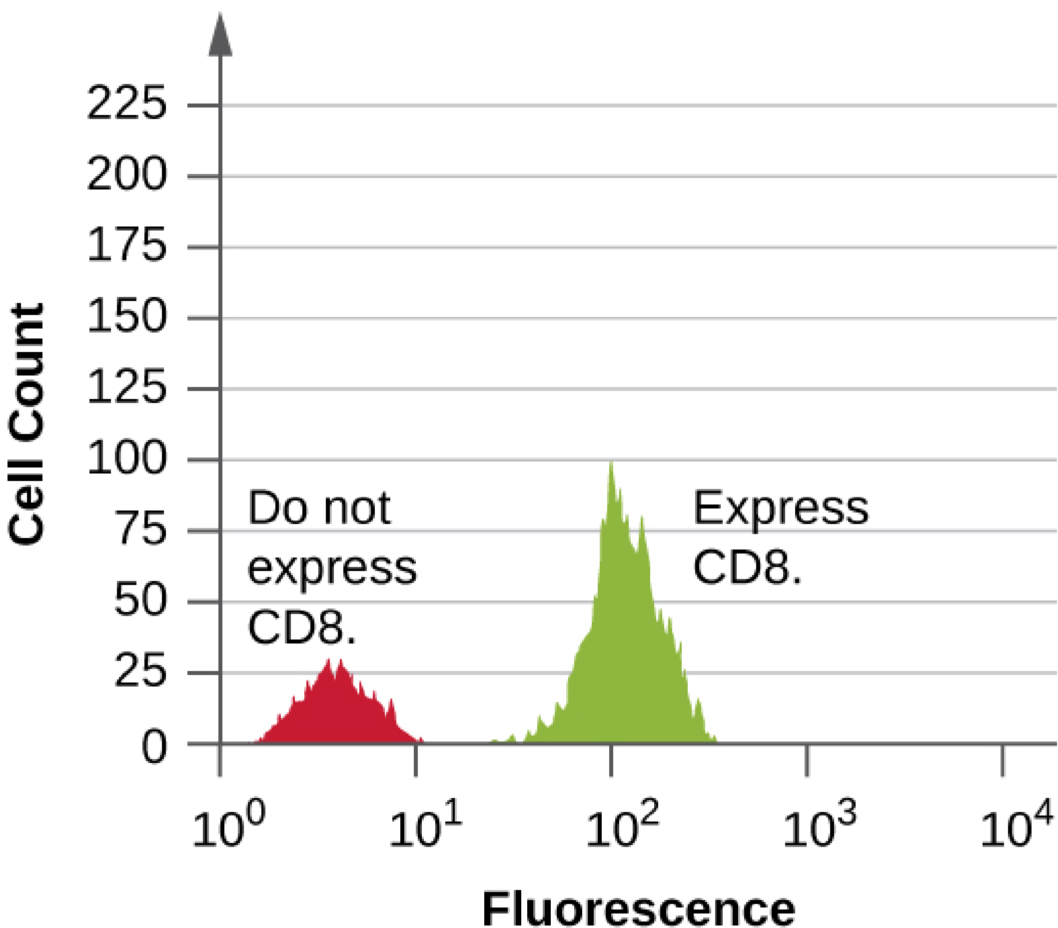


Figure 16.32 Flow cytometry data are often compiled as a histogram. In the histogram, the area under each peak is proportional to the number of cells in each population. The *x*-axis is the relative fluorescence expressed by the cells (on a log scale), and the *y*-axis represents the number of cells at a particular level of fluorescence.

- What is the purpose of the laser in a flow cytometer?
- In the output from a flow cytometer, the area under the histogram is equivalent to what?

Resolution

After notifying all 1300 patients, the hospital begins scheduling HIV screening. Appointments were scheduled a minimum of 3 weeks after the patient's last hospital visit to minimize the risk of false negatives. Because some false positives were anticipated, the public health physician set up a counseling protocol for any patient whose indirect ELISA came back positive.

Of the 1300 patients, eight tested positive using the ELISA. Five of these tests were invalidated by negative western blot tests, but one western blot came back positive, confirming that the patient had indeed contracted HIV. The two remaining western blots came back indeterminate. These individuals had to submit to a third test, a PCR, to confirm the presence or absence of HIV sequences. Luckily, both patients tested negative.

As for the lone patient confirmed to have HIV, the tests cannot prove or disprove any connection to the syringes compromised by the former hospital employee. Even so, the hospital's insurance will fully cover the patient's treatment, which began immediately.

Although we now have drugs that are typically effective at controlling the progression of HIV and AIDS, there is still no cure. If left untreated, or if the drug regimen fails, the patient will experience a gradual decline in the number of CD4 helper T cells, resulting in severe impairment of all adaptive immune functions. Even moderate declines of helper T cell numbers can result in immunodeficiency, leaving the patient susceptible to opportunistic infections. To monitor the status of the patient's helper T cells, the hospital will use flow

cytometry. This sensitive test allows physicians to precisely determine the number of helper T cells so they can adjust treatment if the number falls below 500 cells/µL.

Jump to the ***previous*** *Clinical Focus box.*

Cell Sorting Using Immunofluorescence

The flow cytometer and immunofluorescence can also be modified to sort cells from a single sample into purified subpopulations of cells for research purposes. This modification of the flow cytometer is called a **fluorescence-activated cell sorter (FACS)**. In a FACS, fluorescence by a cell induces the device to put a charge on a droplet of the transporting fluid containing that cell. The charge is specific to the wavelength of the fluorescent light, which allows for differential sorting by those different charges. The sorting is accomplished by an electrostatic deflector that moves the charged droplet containing the cell into one collecting vessel or another. The process results in highly purified subpopulations of cells.

One limitation of a FACS is that it only works on isolated cells. Thus, the method would work in sorting white blood cells, since they exist as isolated cells. But for cells in a tissue, flow cytometry can only be applied if we can excise the tissue and separate it into single cells (using proteases to cleave cell-cell adhesion molecules) without disrupting cell integrity. This method may be used on tumors, but more often, immunohistochemistry and immunocytochemistry are used to study cells in tissues.

Watch videos to learn more about how **flow cytometry (https://openstax.org/l/22flowcytometry)** and a **FACS (https://openstax.org/l/22FACSwork)** work.

- In fluorescence activated cell sorting, what characteristic of the target cells allows them to be separated?

Table 16.5 compares the mechanisms of the fluorescent antibody techniques discussed in this section.

Fluorescent Antibody Techniques

Type of Assay	Mechanism	Examples
Direct fluorescent antibody (DFA)	Uses fluorogen-antibody conjugates to label bacteria from patient samples	Visualizing *Legionella pneumophila* from a throat swab
Indirect fluorescent antibody (IFA)	Detects disease-specific antibodies in patent serum	Diagnosing syphilis; detecting antinuclear antibodies (ANA) for lupus and other autoimmune diseases
Flow cytometry	Labels cell membranes with fluorogen-antibody conjugate markers excited by a laser; machine counts the cell and records the relative fluorescence	Counting the number of fluorescently labeled CD4 or CD8 cells in a sample
Fluorescence activated cell sorter (FACS)	Form of flow cytometry that both counts cells and physically separates them into pools of high and low fluorescence cells	Sorting cancer cells

Table 16.5

CHAPTER SUMMARY

16.1 Polyclonal and Monoclonal Antibody Production

- Antibodies bind with high **specificity** to antigens used to challenge the immune system, but they may also show **cross-reactivity** by binding to other antigens that share chemical properties with the original antigen.
- Injection of an antigen into an animal will result in a **polyclonal antibody** response in which different antibodies are produced that react with the various epitopes on the antigen.
- **Polyclonal antisera** are useful for some types of laboratory assays, but other assays require more specificity. Diagnostic tests that use polyclonal antisera are typically only used for screening because of the possibility of **false-positive** and **false-negative** results.
- **Monoclonal antibodies** provide higher specificity than polyclonal antisera because they bind to a single epitope and usually have high **affinity**.
- Monoclonal antibodies are typically produced by culturing antibody-secreting **hybridomas** derived from mice. mAbs are currently used to treat cancer, but their exorbitant cost has prevented them from being used more widely to treat infectious diseases. Still, their potential for laboratory and clinical use is driving the development of new, cost-effective solutions such as **plantibodies**.

16.2 Detecting Antigen-Antibody Complexes

- When present in the correct ratio, antibody and antigen will form a **precipitin**, or lattice that precipitates out of solution.
- A **precipitin ring test** can be used to visualize lattice formation in solution. The **Ouchterlony assay** demonstrates lattice formation in a gel. The **radial immunodiffusion** assay is used to quantify antigen by measuring the size of a precipitation zone in a gel infused with antibodies.
- Insoluble antigens in suspension will form **flocculants** when bound by antibodies. This is the basis of the VDRL test for syphilis in which anti-treponemal antibodies bind to cardiolipin in suspension.
- Viral infections can be detected by quantifying virus-neutralizing antibodies in a patient’s serum.
- Different antibody classes in plasma or serum are identified by using **immunoelectrophoresis**.
- The presence of specific antigens (e.g., bacterial or viral proteins) in serum can be demonstrated by **western blot** assays, in which the proteins are transferred to a nitrocellulose membrane and identified using labeled antibodies.
- In the complement fixation test, complement is used to detect antibodies against various pathogens.

16.3 Agglutination Assays

- Antibodies can agglutinate cells or large particles into a visible matrix. **Agglutination** tests are often done on cards or in **microtiter plates** that allow multiple reactions to take place side by side using small volumes of reagents.
- Using antisera against certain proteins allows identification of **serovars** within species of bacteria.
- Detecting antibodies against a pathogen can be a powerful tool for diagnosing disease, but there is a period of time before patients go through **seroconversion** and the level of antibodies becomes detectable.
- Agglutination of latex beads in **indirect agglutination assays** can be used to detect the presence of specific antigens or specific antibodies in patient serum.
- The presence of some antibacterial and antiviral antibodies can be confirmed by the use of the direct **Coombs’ test**, which uses Coombs’ reagent to cross-link antibodies bound to red blood cells and facilitate **hemagglutination**.

- Some viruses and bacteria will bind and agglutinate red blood cells; this interaction is the basis of the **direct hemagglutination assay**, most often used to determine the titer of virus in solution.
- **Neutralization assays** quantify the level of virus-specific antibody by measuring the decrease in hemagglutination observed after mixing patient serum with a standardized amount of virus.
- Hemagglutination assays are also used to screen and **cross-match** donor and recipient blood to ensure that the transfusion recipient does not have antibodies to antigens in the donated blood.

16.4 EIAs and ELISAs

- **Enzyme immunoassays (EIA)** are used to visualize and quantify antigens. They use an antibody conjugated to an enzyme to bind the antigen, and the enzyme converts a substrate into an observable end product. The substrate may be either a chromogen or a fluorogen.
- **Immunostaining** is an EIA technique for visualizing cells in a tissue (**immunohistochemistry**) or examining intracellular structures (**immunocytochemistry**).
- **Direct ELISA** is used to quantify an antigen in solution. The primary antibody captures the antigen, and the secondary antibody delivers an enzyme. Production of end product from the chromogenic substrate is directly proportional to the amount of captured antigen.
- **Indirect ELISA** is used to detect antibodies in patient serum by attaching antigen to the well of a microtiter plate, allowing the patient (primary) antibody to bind the antigen and an enzyme-conjugated secondary antibody to detect the primary antibody.
- **Immunofiltration and immunochromatographic assays** are used in **lateral flow tests**, which can be used to diagnose pregnancy and various diseases by detecting color-labeled antigen-antibody complexes in urine or other fluid samples

16.5 Fluorescent Antibody Techniques

- **Immunofluorescence** assays use antibody-fluorogen conjugates to illuminate antigens for easy, rapid detection.
- **Direct immunofluorescence** can be used to detect the presence of bacteria in clinical samples such as sputum.
- **Indirect immunofluorescence** detects the presence of antigen-specific antibodies in patient sera. The fluorescent antibody binds to the antigen-specific antibody rather than the antigen.
- The use of indirect immunofluorescence assays to detect **antinuclear antibodies** is an important tool in the diagnosis of several autoimmune diseases.
- **Flow cytometry** uses fluorescent mAbs against cell-membrane proteins to quantify specific subsets of cells in complex mixtures.
- **Fluorescence-activated cell sorters** are an extension of flow cytometry in which fluorescence intensity is used to physically separate cells into high and low fluorescence populations.

REVIEW QUESTIONS

1. For many uses in the laboratory, polyclonal antibodies work well, but for some types of assays, they lack sufficient ________ because they cross-react with inappropriate antigens.

a. specificity
b. sensitivity
c. accuracy
d. reactivity

2. How are monoclonal antibodies produced?

a. Antibody-producing B cells from a mouse are fused with myeloma cells and then the cells are grown in tissue culture.
b. A mouse is injected with an antigen and then antibodies are harvested from its serum.
c. They are produced by the human immune system as a natural response to an infection.
d. They are produced by a mouse's immune system as a natural response to an infection.

3. The formation of ________ is a positive result in the VDRL test.
a. flocculant
b. precipitin
c. coagulation
d. a bright pink color

4. The titer of a virus neutralization test is the highest dilution of patient serum
a. in which there is no detectable viral DNA.
b. in which there is no detectable viral protein.
c. that completely blocks plaque formation.
d. that reduces plaque formation by at least 50%.

5. In the Ouchterlony assay, we see a sharp precipitin arc form between antigen and antiserum. Why does this arc remain visible for a long time?

a. The antibody molecules are too large to diffuse through the agar.
b. The precipitin lattice is too large to diffuse through the agar.
c. Methanol, added once the arc forms, denatures the protein and blocks diffusion.
d. The antigen molecules are chemically coupled to the gel matrix.

6. We use antisera to distinguish between various ________ within a species of bacteria.
a. isotypes
b. serovars
c. subspecies
d. lines

7. When using antisera to characterize bacteria, we will often link the antibodies to ________ to better visualize the agglutination.
a. latex beads
b. red blood cells
c. other bacteria
d. white blood cells

8. The antibody screening test that is done along with pretransfusion blood typing is used to ensure that the recipient
a. does not have a previously undetected bacterial or viral infection.
b. is not immunocompromised.
c. actually does have the blood type stated in the online chart.
d. is not making antibodies against antigens outside the ABO or Rh systems.

9. The direct Coombs' test is designed to detect when people have a disease that causes them to

a. have an excessively high fever.
b. quit making antibodies.
c. make too many red blood cells.
d. produce antibodies that bind to their own red blood cells.

10. Viral hemagglutination assays only work with certain types of viruses because
a. the virus must be able to cross-link red blood cells directly.
b. the virus must be able to lyse red blood cells.
c. the virus must not be able to lyse red blood cells.
d. other viruses are too dangerous to work with in a clinical lab setting.

11. In an enzyme immunoassay, the enzyme

a. is bound by the antibody's antigen-binding site.
b. is attached to the well of a microtiter plate.
c. is conjugated to the suspect antigen.
d. is bound to the constant region of the secondary antibody.

12. When using an EIA to study microtubules or other structures inside a cell, we first chemically fix the cell and then treat the cells with alcohol. What is the purpose of this alcohol treatment?
a. It makes holes in the cell membrane large enough for antibodies to pass.
b. It makes the membrane sticky so antibodies will bind and be taken up by receptor-mediated endocytosis.
c. It removes negative charges from the membrane, which would otherwise repulse the antibodies.
d. It prevents nonspecific binding of the antibodies to the cell membrane.

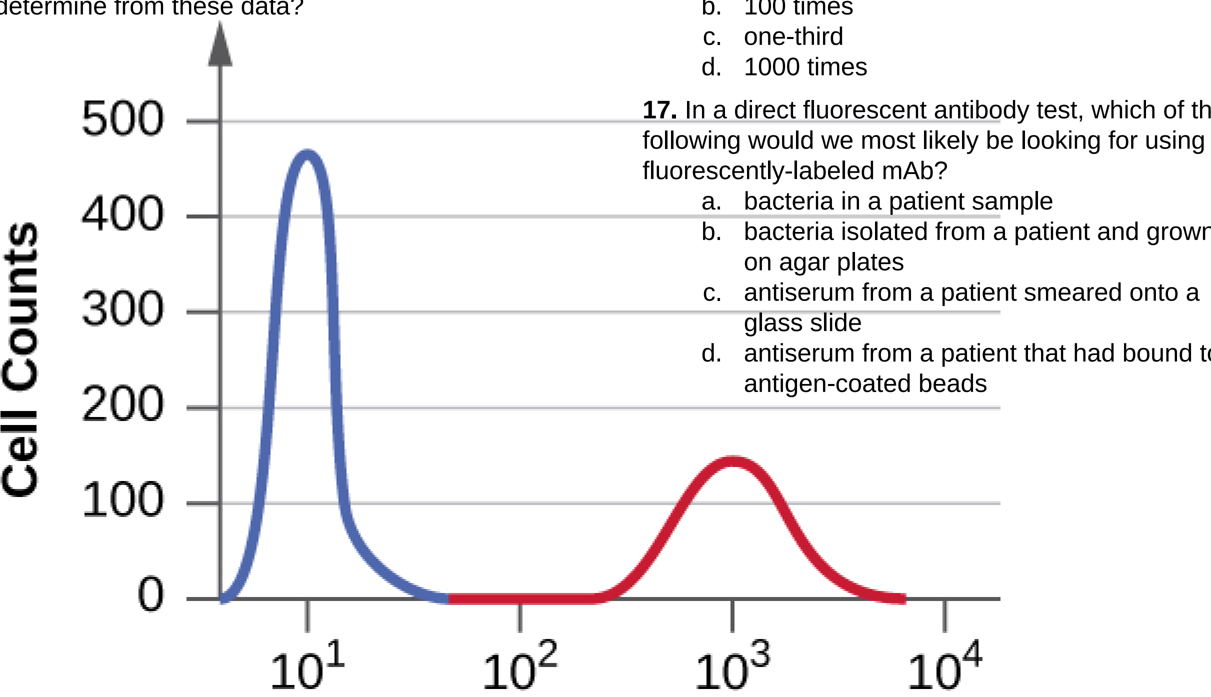
13. In a lateral-flow pregnancy test, you see a blue band form on the control line and no band form on the test line. This is probably a ________ test for pregnancy.
a. positive
b. false-positive
c. false-negative
d. negative

14. When performing an FEIA, the fluorogen replaces

the ________ that is used in an EIA.

a. antigen
b. chromogenic substrate
c. enzyme
d. secondary antibody

15. Suppose you need to quantify the level of CD8 T cells in the blood of a patient recovering from influenza. You treat a sample of the patient's white blood cells using a fluorescent mAb against CD8, pass the cells through a flow cytometer, and produce the histogram shown below. The area under the peak to the left (blue) is three times greater than the area of the peak on the right (red). What can you determine from these data?



a. There are no detectable CD8 cells.
b. There are three times as many CD4 cells than CD8 cells.
c. There are three times as many CD8 cells than CD4 cells.
d. CD8 cells make up about one-fourth of the total number of cells.

16. In the data described in the previous question, the average fluorescence intensity of cells in the second (red) peak is about ________ that in the first (blue) peak.

a. three times
b. 100 times
c. one-third
d. 1000 times

17. In a direct fluorescent antibody test, which of the following would we most likely be looking for using a fluorescently-labeled mAb?

a. bacteria in a patient sample
b. bacteria isolated from a patient and grown on agar plates
c. antiserum from a patient smeared onto a glass slide
d. antiserum from a patient that had bound to antigen-coated beads

FILL IN THE BLANK

16.1 Polyclonal and Monoclonal Antibody Production

18. When we inject an animal with the same antigen a second time a few weeks after the first, ________ takes place, which means the antibodies produced after the second injection will on average bind the antigen more tightly.

19. When using mAbs to treat disease in humans, the mAbs must first be ________ by replacing the mouse constant region DNA with human constant region DNA.

20. If we used normal mouse mAbs to treat human disease, multiple doses would cause the patient to respond with ________ against the mouse antibodies.

21. A polyclonal response to an infection occurs because most antigens have multiple ________,

16.2 Detecting Antigen-Antibody Complexes

22. When slowly adding antigen to an antiserum, the amount of precipitin would gradually increase until reaching the ________; addition of more antigen after this point would actually decrease the amount of precipitin.

23. The radial immunodiffusion test quantifies antigen by mixing ________ into a gel and then allowing antigen to diffuse out from a well cut in the gel.

16.3 Agglutination Assays

24. In the major cross-match, we mix ________ with the donor red blood cells and look for agglutination.

25. Coombs' reagent is an antiserum with antibodies that bind to human ________.

16.4 EIAs and ELISAs

26. To detect antibodies against bacteria in the bloodstream using an EIA, we would run a(n) ________, which we would start by attaching antigen from the bacteria to the wells of a microtiter plate.

16.5 Fluorescent Antibody Techniques

27. In flow cytometry, cell subsets are labeled using a fluorescent antibody to a membrane protein. The fluorogen is activated by a(n) ________ as the cells pass by the detectors.

28. Fluorescence in a flow cytometer is measured by a detector set at an angle to the light source. There is also an in-line detector that can detect cell clumps or ________.

SHORT ANSWER

16.1 Polyclonal and Monoclonal Antibody Production

29. Describe two reasons why polyclonal antibodies are more likely to exhibit cross-reactivity than monoclonal antibodies.

16.2 Detecting Antigen-Antibody Complexes

30. Explain why hemolysis in the complement fixation test is a negative test for infection.

31. What is meant by the term “neutralizing antibodies,” and how can we quantify this effect using the viral neutralization assay?

16.3 Agglutination Assays

32. Explain why the titer of a direct hemagglutination assay is the highest dilution that still causes hemagglutination, whereas in the viral hemagglutination inhibition assay, the titer is the highest dilution at which hemagglutination is not observed.

33. Why would a doctor order a direct Coombs’ test when a baby is born with jaundice?

16.4 EIAs and ELISAs

34. Why is it important in a sandwich ELISA that the antigen has multiple epitopes? And why might it be advantageous to use polyclonal antisera rather than mAb in this assay?

35. The pregnancy test strip detects the presence of human chorionic gonadotrophin in urine. This hormone is initially produced by the fetus and later by the placenta. Why is the test strip preferred for this test rather than using either a direct or indirect ELISA with their more quantifiable results?

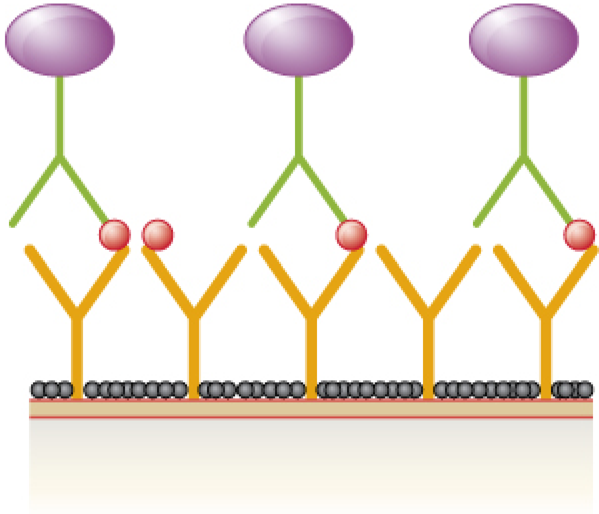
CRITICAL THINKING

36. Suppose you were screening produce in a grocery store for the presence of *E. coli* contamination. Would it be better to use a polyclonal anti-*E. coli* antiserum or a mAb against an *E. coli* membrane protein? Explain.

37. Both IgM and IgG antibodies can be used in precipitation reactions. However, one of these immunoglobulin classes will form precipitates at much lower concentrations than the other. Which class is this, and why is it so much more efficient in this regard?

38. When shortages of donated blood occur, O-negative blood may be given to patients, even if they have a different blood type. Why is this the case? If O-negative blood supplies were depleted, what would be the next-best choice for a patient with a different blood type in critical need of a transfusion? Explain your answers.

39. Label the primary and secondary antibodies, and discuss why the production of end product will be proportional to the amount of antigen.



40. A patient suspected of having syphilis is tested using both the VDRL test and IFA. The IFA test comes back positive, but the VDRL test is negative. What is the most likely reason for these results?

41. A clinician suspects that a patient with

pneumonia may be infected by *Legionella pneumophila*. Briefly describe two reasons why a DFA test might be better for detecting this pathogen than standard bacteriology techniques.

APPENDIX A | MATHEMATICAL BASICS

A1 | Squares and Other Powers

An exponent, or a power, is mathematical shorthand for repeated multiplications. For example, the exponent “2” means to multiply the base for that exponent by itself (in the example here, the base is “5”):

$$5^2 = 5 \times 5 = 25$$

The exponent is “2” and the base is the number “5.” This expression (multiplying a number by itself) is also called a square. Any number raised to the power of 2 is being squared. Any number raised to the power of 3 is being cubed:

$$5^3 = 5 \times 5 \times 5 = 125$$

A number raised to the fourth power is equal to that number multiplied by itself four times, and so on for higher powers. In general:

$$n^x = n \times n^{x-1}$$

A2 | Calculating Percents

A percent is a way of expressing a fractional amount of something using a whole divided into 100 parts. A percent is a ratio whose denominator is 100. We use the percent symbol, %, to show percent. Thus, 25% means a ratio of $\frac{25}{100}$, 3% means a ratio of $\frac{3}{100}$, and 100 % percent means $\frac{100}{100}$, or a whole.

Converting Percents

A percent can be converted to a fraction by writing the value of the percent as a fraction with a denominator of 100 and simplifying the fraction if possible.

$$25\% = \frac{25}{100} = \frac{1}{4}$$

A percent can be converted to a decimal by writing the value of the percent as a fraction with a denominator of 100 and dividing the numerator by the denominator.

$$10\% = \frac{10}{100} = 0.10$$

To convert a decimal to a percent, write the decimal as a fraction. If the denominator of the fraction is not 100, convert it to a fraction with a denominator of 100, and then write the fraction as a percent.

$$0.833 = \frac{833}{1000} = \frac{83.3}{100} = 83.3\%$$

To convert a fraction to a percent, first convert the fraction to a decimal, and then convert the decimal to a percent.

$$\frac{3}{4} = 0.75 = \frac{75}{100} = 75\%$$

Suppose a researcher finds that 15 out of 23 students in a class are carriers of *Neisseria meningitides*. What percentage of students are carriers? To find this value, first express the numbers as a fraction.

$$\frac{\text{carriers}}{\text{total students}} = \frac{15}{23}$$

Then divide the numerator by the denominator.

$$\frac{15}{23} = 15 \div 23 \approx 0.65$$

Finally, to convert a decimal to a percent, multiply by 100.

$$0.65 \times 100 = 65\%$$

The percent of students who are carriers is 65%.

You might also get data on occurrence and non-occurrence; for example, in a sample of students, 9 tested positive for *Toxoplasma* antibodies, while 28 tested negative. What is the percentage of seropositive students? The first step is to determine the "whole," of which the positive students are a part. To do this, sum the positive and negative tests.

$$\text{positive} + \text{negative} = 9 + 28 = 37$$

The whole sample consisted of 37 students. The fraction of positives is:

$$\frac{\text{positive}}{\text{total students}} = \frac{9}{37}$$

To find the percent of students who are carriers, divide the numerator by the denominator and multiply by 100.

$$\frac{9}{37} = 9 \div 37 \approx 0.24$$
$$0.24 \times 100 = 24\%$$

The percent of positive students is about 24%.

Another way to think about calculating a percent is to set up equivalent fractions, one of which is a fraction with 100 as the denominator, and cross-multiply. The previous example would be expressed as:

$$\frac{9}{37} = \frac{x}{100}$$

Now, cross multiply and solve for the unknown:

$$\begin{aligned} 9 \times 100 &= 37x & \\ \frac{9 \times 100}{37} &= x & \text{Divide both sides by 37} \\ \frac{900}{37} &= x & \text{Multiply} \\ 24 &\approx x & \text{Divide} \end{aligned}$$

The answer, rounded, is the same.

A3 | Multiplying and Dividing by Tens

In many fields, especially in the sciences, it is common to multiply decimals by powers of 10. Let's see what happens when we multiply 1.9436 by some powers of 10.

$$\begin{aligned} 1.9436(10) &= 19.436 \\ 1.9436(100) &= 194.36 \\ 1.9436(1000) &= 1943.6 \end{aligned}$$

The number of places that the decimal point moves is the same as the number of zeros in the power of ten. **Table A1** summarizes the results.

Multiply by	Zeros	Decimal point moves . . .
10	1	1 place to the right
100	2	2 places to the right
1,000	3	3 places to the right
10,000	4	4 places to the right

Table A1

We can use this pattern as a shortcut to multiply by powers of ten instead of multiplying using the vertical format.

We can count the zeros in the power of 10 and then move the decimal point that same number of places to the right.

So, for example, to multiply 45.86 by 100, move the decimal point 2 places to the right.

$$45.86 \times 100 = 4586.$$

Sometimes when we need to move the decimal point, there are not enough decimal places. In that case, we use zeros as placeholders. For example, let’s multiply 2.4 by 100. We need to move the decimal point 2 places to the right. Since there is only one digit to the right of the decimal point, we must write a 0 in the hundredths place.

$$2.4 \times 100 = 240.$$

When dividing by powers of 10, simply take the opposite approach and move the decimal to the left by the number of zeros in the power of ten.

Let’s see what happens when we divide 1.9436 by some powers of 10.

$$\begin{aligned} 1.9436 \div 10 &= 0.19436 \\ 1.9436 \div 100 &= 0.019436 \\ 1.9436 \div 1000 &= 0.0019436 \end{aligned}$$

If there are insufficient digits to move the decimal, add zeroes to create places.

A4 | Scientific Notation

Scientific notation is used to express very large and very small numbers as a product of two numbers. The first number of the product, the digit term, is usually a number not less than 1 and not greater than 10. The second number of the product, the exponential term, is written as 10 with an exponent. Some examples of scientific notation are given in **Table A2**.

Standard Notation	Scientific Notation
1000	1×10^3
100	1×10^2
10	1×10^1
1	1×10^0
0.1	1×10^{-1}
0.01	1×10^{-2}

Table A2

Scientific notation is particularly useful notation for very large and very small numbers, such as 1,230,000,000 = 1.23×10^9, and 0.00000000036 = 3.6×10^{-10}.

A5 | Expressing Numbers in Scientific Notation

Converting any number to scientific notation is straightforward. Count the number of places needed to move the decimal next to the left-most non-zero digit: that is, to make the number between 1 and 10. Then multiply that number by 10 raised to the number of places you moved the decimal. The exponent is positive if you moved the

decimal to the left and negative if you moved the decimal to the right. So

$$2386 = 2.386 \times 1000 = 2.386 \times 10^3$$

and

$$0.123 = 1.23 \times 0.1 = 1.23 \times 10^{-1}$$

The power (exponent) of 10 is equal to the number of places the decimal is shifted.

A6 | Logarithms

The common logarithm (log) of a number is the power to which 10 must be raised to equal that number. For example, the common logarithm of 100 is 2, because 10 must be raised to the second power to equal 100. Additional examples are in **Table A3**.

Number	Exponential Form	Common Logarithm
1000	10^3	3
10	10^1	1
1	10^0	0
0.1	10^{-1}	−1
0.001	10^{-3}	−3

Table A3

To find the common logarithm of most numbers, you will need to use the LOG button on a calculator.

A7 | Rounding and Significant Digits

In reporting numerical data obtained via measurements, we use only as many significant figures as the accuracy of the measurement warrants. For example, suppose a microbiologist using an automated cell counter determines that there are 525,341 bacterial cells in a one-liter sample of river water. However, she records the concentration as 525,000 cells per liter and uses this rounded number to estimate the number of cells that would likely be found in 10 liters of river water. In this instance, the last three digits of the measured quantity are not considered *significant.* They are rounded to account for variations in the number of cells that would likely occur if more samples were measured.

The importance of significant figures lies in their application to fundamental computation. In addition and subtraction, the sum or difference should contain as many digits to the right of the decimal as that in the *least* certain (indicated by underscoring in the following example) of the numbers used in the computation.

Suppose a microbiologist wishes to calculate the total mass of two samples of agar.

$$\begin{array}{l} 4.38\underline{3}\ \text{g} \\ \underline{3.002\underline{1}}\ \text{g} \\ 7.38\underline{5}\ \text{g} \end{array}$$

The least certain of the two masses has three decimal places, so the sum must have three decimal places.

In multiplication and division, the product or quotient should contain no more digits than than in the factor containing the *least* number of significant figures. Suppose the microbiologist would like to calculate how much of a reagent would be present in 6.6 mL if the concentration is 0.638 g/mL.

$$0.63\underline{8}\ \frac{\text{g}}{\text{mL}} \times 6.\underline{6}\ \text{mL} = 4.1\ \text{g}$$

Again, the answer has only one decimal place because this is the accuracy of the least accurate number in the calculation.

When rounding numbers, increase the retained digit by 1 if it is followed by a number larger than 5 (“round up”). Do not change the retained digit if the digits that follow are less than 5 (“round down”). If the retained digit is followed by 5, round up if the retained digit is odd, or round down if it is even (after rounding, the retained digit will thus always be even).

A8 | Generation Time

It is possible to write an equation to calculate the cell numbers at any time if the number of starting cells and doubling time are known, as long as the cells are dividing at a constant rate. We define N_0 as the starting number of bacteria, the number at time $t = 0$. N_i is the number of bacteria at time $t = i$, an arbitrary time in the future. Finally we will set j equal to the number of generations, or the number of times the cell population doubles during the time interval. Then we have,

$$N_i = N_0 \times 2^j$$

This equation is an expression of growth by binary fission.

In our example, $N_0 = 4$, the number of generations, j, is equal to 3 after 90 minutes because the generation time is 30 minutes. The number of cells can be estimated from the following equation:

$$\begin{aligned} N_i &= N_0 \times 2^j \\ N_{90} &= 4 \times 2^3 \\ N_{90} &= 4 \times 8 = 32 \end{aligned}$$

The number of cells after 90 minutes is 32.

A9 | Most Probable Number

The table in **Figure A1** contains values used to calculate the most probable number example given in **How Microbes Grow**.

Most Probable Number Table					
Number of tubes giving a positive reaction for a 5-tube set			MPN (per 100 ml)	95% Confidence Limits	
10 ml	*1 ml*	*0.1 ml*		*Low*	*High*
0	0	0	<2	<1	7
0	1	0	2	<1	7
0	2	0	4	<1	11
1	0	0	2	<1	7
1	0	1	4	<1	11
1	1	0	4	<1	11
1	1	1	6	<1	15
2	0	0	5	<1	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46
3	3	0	17	5	46
4	0	0	13	3	31
4	0	1	17	5	46
4	1	0	17	5	46
4	1	1	21	7	63
4	1	2	26	9	78
4	2	0	22	7	67
4	2	1	26	9	78
4	3	0	27	9	80
4	3	1	33	11	93
4	4	0	34	12	93
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	110
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	150
5	2	0	49	17	130
5	2	1	70	23	170
5	2	2	94	28	220
5	3	0	79	25	190
5	3	1	110	31	250
5	3	2	140	37	340
5	3	3	180	44	500

Figure A1

APPENDIX B | MEASUREMENTS AND THE METRIC SYSTEM

B1 | Measurements and the Metric System

Measurements and the Metric System

Measurement	Unit	Abbreviation	Metric Equivalent	Approximate Standard Equivalent
Length	nanometer	nm	1 nm = 10^{-9} m	1 mm = 0.039 inch 1 cm = 0.394 inch 1 m = 39.37 inches 1 m = 3.28 feet 1 m = 1.093 yards 1 km = 0.621 miles
	micrometer	µm	1 µm = 10^{-6} m	
	millimeter	mm	1 mm = 0.001 m	
	centimeter	cm	1 cm = 0.01 m	
	meter	m	1 m = 100 cm 1 m = 1000 mm	
	kilometer	km	1 km = 1000 m	
Mass	microgram	µg	1 µg = 10^{-6} g	1 g = 0.035 ounce 1 kg = 2.205 pounds
	milligram	mg	1 mg = 10^{-3} g	
	gram	g	1 g = 1000 mg	
	kilogram	kg	1 kg = 1000 g	
Volume	microliter	µl	1 µl = 10^{-6} l	1 ml = 0.034 fluid ounce 1 l = 1.057 quarts 1 kl = 264.172 gallons
	milliliter	ml	1 ml = 10^{-3} l	
	liter	l	1 l = 1000 ml	
	kiloliter	kl	1 kl = 1000 l	
Area	square centimeter	cm^2	1 cm^2 = 100 mm^2	1 cm^2 = 0.155 square inch 1 m^2 = 10.764 square feet 1 m^2 = 1.196 square yards 1 ha = 2.471 acres
	square meter	m^2	1 m^2 = 10,000 cm^2	
	hectare	ha	1 ha = 10,000 m^2	
Temperature	Celsius	°C	—	1 °C = 5/9 × (°F − 32)

Table B1

APPENDIX C | THE PERIODIC TABLE OF ELEMENTS

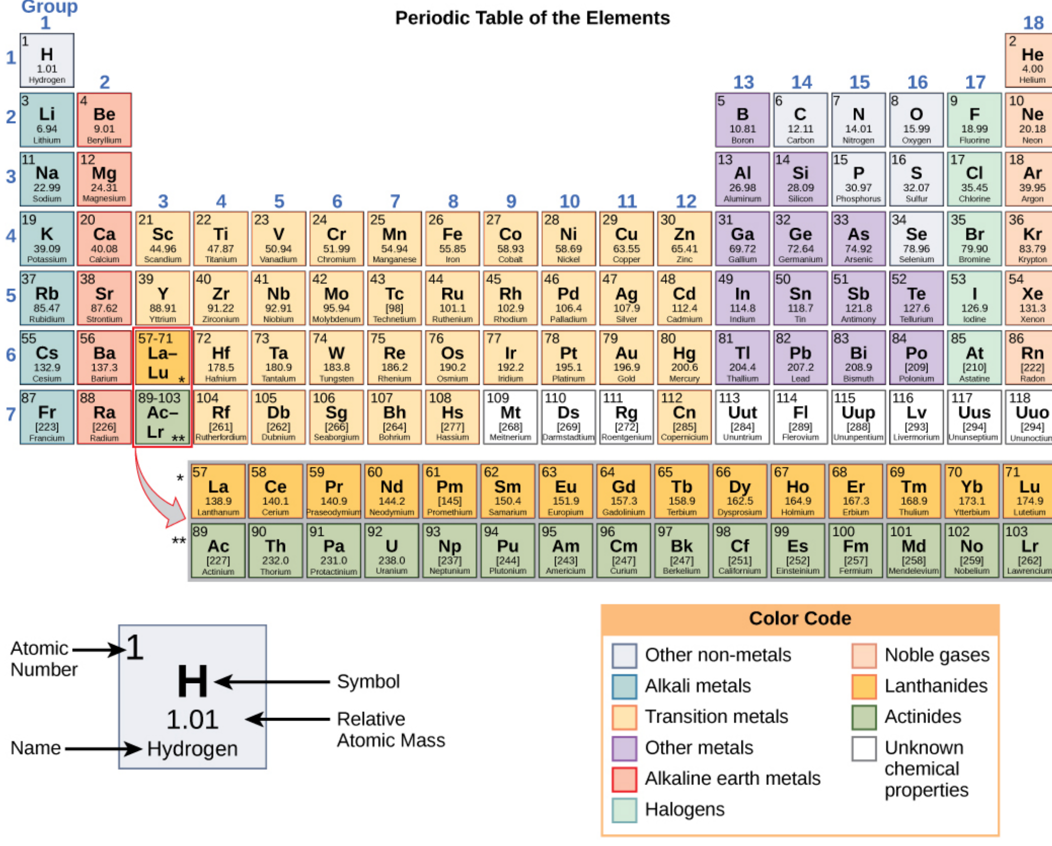


Figure C1

ANSWER KEY

Chapter 1

Chapter 2

1 Figure 2.3 Potassium-39 has twenty neutrons. Potassium-40 has twenty one neutrons.
3 A **5** A **7** C **9** A **11** D **13** D **15** C **16** Hydrogen bonds and van der Waals interactions form weak associations between different molecules. They provide the structure and shape necessary for proteins and DNA within cells so that they function properly. Hydrogen bonds also give water its unique properties, which are necessary for life. **18** Water molecules are polar, meaning they have separated partial positive and negative charges. Because of these charges, water molecules are able to surround charged particles created when a substance dissociates. The surrounding layer of water molecules stabilizes the ion and keeps differently charged ions from reassociating, so the substance stays dissolved. **20** Carbon monoxide has a higher affinity for hemoglobin than oxygen. This means that carbon monoxide will preferentially bind to hemoglobin over oxygen. Administration of 100 percent oxygen is an effective therapy because at that concentration, oxygen will displace the carbon monoxide from the hemoglobin. **22** A change in gene sequence can lead to a different amino acid being added to a polypeptide chain instead of the normal one. This causes a change in protein structure and function. For example, in sickle cell anemia, the hemoglobin β chain has a single amino acid substitution. Because of this change, the disc-shaped red blood cells assume a crescent shape, which can result in serious health problems.

Chapter 3

1 Figure 3.7 Because even though the original cell came from a Scottish Blackface sheep and the surrogate mother was a Scottish Blackface, the DNA came from a Finn-Dorset.
2 B **4** A **6** C **7** The polymerase chain reaction is used to quickly produce many copies of a specific segment of DNA when only one or a very few copies are originally present. The benefit of PCR is that there are many instances in which we would like to know something about a sample of DNA when only very small amounts are available. PCR allows us to increase the number of DNA molecules so that other tests, such as sequencing, can be performed with it.

Chapter 4

Chapter 5

1(a) 12.01 amu; (b) 12.01 amu; (c) 144.12 amu; (d) 60.05 amu **5**(a) 56.107 amu; (b) 54.091 amu; (c) 199.9976 amu; (d) 97.9950 amu **9**Formic acid. Its formula has twice as many oxygen atoms as the other two compounds (one each). Therefore, 0.60 mol of formic acid would be equivalent to 1.20 mol of a compound containing a single oxygen atom. **13**(a) 256.48 g/mol; (b) 72.150 g mol^{-1}; (c) 378.103 g mol^{-1}; (d) 58.080 g mol^{-1}; (e) 180.158 g mol^{-1} **17**(a) 0.819 g; (b) 307 g; (c) 0.23 g; (d) 1.235×10^6 g (1235 kg); (e) 765 g **21**(a) 9.60 g; (b) 19.2 g; (c) 28.8 g **25**$AlPO_4$: 1.000 mol, or 26.98 g Al; Al_2Cl_6: 1.994 mol, or 53.74 g Al; Al_2S_3: 3.00 mol, or 80.94 g Al; The Al_2S_3 sample thus contains the greatest mass of Al. **29**0.865 servings, or about 1 serving. **33**We need to know the number of moles of sulfuric acid dissolved in the solution and the volume of the solution. **37**(a) determine the number of moles of glucose in 0.500 L of solution; determine the molar mass of glucose; determine the mass of glucose from the number of moles and its molar mass; (b) 27 g **41**(a) Determine the molar mass of $KMnO_4$; determine the number of moles of $KMnO_4$ in the solution; from the number of moles and the volume of solution, determine the molarity; (b) 1.15×10^{-3} *M* **45**0.025 *M* **49**1.9 mL **53**11.9 *M* **57**(a) The dilution equation can be used, appropriately modified to accommodate mass-based concentration units: $\%\text{mass}_1 \times \text{mass}_1 = \%\text{mass}_2 \times \text{mass}_2$. This equation can be rearranged to isolate mass_1 and the given quantities substituted into this equation. (b) 58.8 g **61**1.75×10^{-3} *M* **65**2.38×10^{-4} mol

Chapter 7

1 Figure 7.4 Plant cells have plasmodesmata, a cell wall, a large central vacuole, chloroplasts, and plastids. Animal cells have lysosomes and centrosomes. **3** Figure 7.19 No, it must have been hypotonic, as a hypotonic solution would cause water to enter the cells, thereby making them burst.
4 D **6** D **8** A **10** C **13** Prokaryotic cells are surrounded by a plasma membrane and have DNA, cytoplasm, and ribosomes, like eukaryotic cells. They also have cell walls and may have a cell capsule. Prokaryotes have a single large chromosome that is not surrounded by a nuclear membrane. Prokaryotes may have flagella or motility, pili for

conjugation, and fimbriae for adhesion to surfaces. **15** The fluidity of the cell membrane is necessary for the operation of some enzymes and transport mechanisms within the membrane. **17** The cell harvests energy from ATP produced by its own metabolism to power active transport processes, such as pumps.

Chapter 8

1D **3**A **5**C **7**D **9**B **11**C **13**D **15** D **17** B
19scientific history **21**fermentation **23**Protista and Monera **25**Viruses **27**helminths **29**nucleus
46 Remind them of the important roles prokaryotes play in decomposition and freeing up nutrients in biogeochemical cycles; remind them of the many prokaryotes that are not human pathogens and that fill very specialized niches. Furthermore, our normal bacterial symbionts are crucial for our digestion and in protecting us from pathogens.

Chapter 9

1C **3**C **5**B **7**D **9**B
10refraction **12**atomic force microscope **14**acid-fast

Chapter 10

1B **3**C **5**B **7**C **9**D **11**B **13**B **15**C **17**A **19**D **21**C **23**D **25**D
27D, C, A, B **29**A, D, E, B, C
30hemocytometer, Petroff-Hausser counting chamber **32**most probable number **34**ATP, acid from fermentation **36**acidophile **38**barophiles **40**complex, differential

Chapter 11

1A **3**B **5**C **7**C **9**B **11**B **13**C
14False **16**False **18**True
19noncritical **21**steam

Chapter 12

22 **Figure 12.15** Ligase, as this enzyme joins together Okazaki fragments.
24D **26**B **28**A. **30** B **32**D **34**A **36** A **38** C **40** D **42**A **44**B **46**D **48** B **50** A **52** D **56** Telomerase has an inbuilt RNA template that extends the 3' end, so a primer is synthesized and extended. Thus, the ends are protected. **60** The cell controls which protein is expressed, and to what level that protein is expressed, in the cell. Prokaryotic cells alter the transcription rate to turn genes on or off. This method will increase or decrease protein levels in response to what is needed by the cell. Eukaryotic cells change the accessibility (epigenetic), transcription, or translation of a gene. This will alter the amount of RNA, and the lifespan of the RNA, to alter the amount of protein that exists. Eukaryotic cells also change the protein's translation to increase or decrease its overall levels. Eukaryotic organisms are much more complex and can manipulate protein levels by changing many stages in the process. **63** A human genetic map can help identify genetic markers and sequences associated with high cancer risk, which can help to screen and provide early detection of different types of cancer. **65** Genomics can provide the unique DNA sequence of an individual, which can be used for personalized medicine and treatment options.

Chapter 13

1 **Figure 13.3** Polar and charged amino acid residues (the remainder after peptide bond formation) are more likely to be found on the surface of soluble proteins where they can interact with water, and nonpolar (e.g., amino acid side chains) are more likely to be found in the interior where they are sequestered from water. In membrane proteins, nonpolar and hydrophobic amino acid side chains associate with the hydrophobic tails of phospholipids, while polar and charged amino acid side chains interact with the polar head groups or with the aqueous solution. However, there are exceptions. Sometimes, positively and negatively charged amino acid side chains interact with one another in the interior of a protein, and polar or charged amino acid side chains that interact with a ligand can be found in the ligand binding pocket.
2 C **4** A **6** A **8** D **9** A change in gene sequence can lead to a different amino acid being added to a polypeptide chain instead of the normal one. This causes a change in protein structure and function. For example, in sickle cell anemia, the hemoglobin β chain has a single amino acid substitution—the amino acid glutamic acid in position six is substituted by valine. Because of this change, hemoglobin molecules form aggregates, and the disc-shaped red blood cells assume a crescent shape, which results in serious health problems. **11** Most vitamins and minerals act as coenzymes and cofactors for enzyme action. Many enzymes require the binding of certain cofactors or coenzymes to be able to catalyze their reactions. Since enzymes catalyze many important reactions, it is critical to obtain sufficient vitamins and minerals from the diet and from supplements. Vitamin C (ascorbic acid) is a coenzyme necessary for

the action of enzymes that build collagen, an important protein component of connective tissue throughout the body. Magnesium ion (Mg++) is an important cofactor that is necessary for the enzyme pyruvate dehydrogenase to catalyze part of the pathway that breaks down sugar to produce energy. Vitamins cannot be produced in the human body and therefore must be obtained in the diet. **13** Proteomics has provided a way to detect biomarkers and protein signatures, which have been used to screen for the early detection of cancer.

Chapter 14

1D **3**C **5**B **7**C **9**B **11**C **13**A **15**C **17** B **19** C
21false **23**true **25**False
27transfection **29**reporter gene **31**northern **33**Sanger sequencing, dideoxy method, or chain termination method
50 Genome mapping helps researchers to study disease-causing genes in humans. It also helps to identify traits of organisms that can be used in applications such as cleaning up pollution.

Chapter 15

9 **Figure 15.5** D **11** **Figure 15.17** If the blood of the mother and fetus mixes, memory cells that recognize the Rh antigen of the fetus can form in the mother late in the first pregnancy. During subsequent pregnancies, these memory cells launch an immune attack on the fetal blood cells of an Rh-positive fetus. Injection of anti-Rh antibody during the first pregnancy prevents the immune response from occurring.
12 B **14** B **16** B **18** C **20** A **22**D **24**D **26** The virus cannot attach to dog cells because dog cells do not express the receptors for the virus or there is no cell within the dog that is permissive for viral replication. **28** If the MHC class I molecules expressed on donor cells differ from the MHC class I molecules expressed on recipient cells, NK cells may identify the donor cells as not normal and produce enzymes to induce the donor cells to undergo apoptosis, which would destroy the transplanted organ. **30** T cells bind antigens that have been digested and embedded in MHC molecules by APCs. In contrast, B cells function as APCs to bind intact, unprocessed antigens.

Chapter 16

1A **3**A **5**B **7**A **9**D **11**D **13**D **15**D **17**A
18affinity maturation **20**neutralizing antibodies **22**equivalence zone or zone of equivalence **24**patient serum **26**indirect ELISA **28**fragments

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