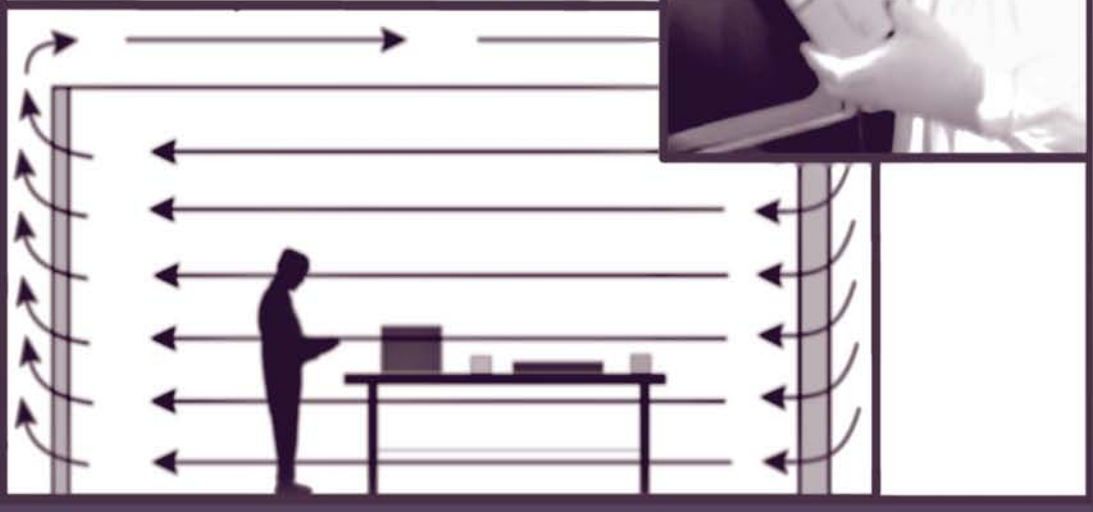
Cleanroom Microbiology

for the Non-Microbiologist

SECOND EDITION



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DEDICATION

To my wife, Margaret, sons Marvin and Howard, daughter-in-law Shirley, and grandsons Bryce and Dana.

PREFACE TO THE SECOND EDITION

There have been significant developments in the art and science of biological contamination control since the publication of the first edition of this volume in 1995. Some examples are:

1. Issuance of a series of key international cleanroom standards
2. Heightened interest in applying rapid, automated methods for detecting and identifying microbial contaminants
3. Greater understanding of the role of biofilms in pure water systems
4. Increased activity in the production of therapeutic products derived from live tissues and cells

The purpose of the present edition has not changed. It is to provide background information for supervisors, production and maintenance personnel, and others who are involved in cleanroom facilities that must include the control of microbiological contamination. One need not be a microbiologist to understand the whats and whys of the responsibilities of cleanroom microbiologists. It is hoped that this volume will provide that understanding.

PREFACE TO THE FIRST EDITION

Managers, supervisors, plant engineers, maintenance personnel, machine operators, and other workers in biotechnology and bioengineering areas including pharmaceutical and medical device manufacturers and the food industries are the principal audience for this book. The volume introduces important concepts and principles of microbiology to people who may have little or no training in microbiology but who must deal with problems involving microbial contamination and its control or who regularly interact with microbiologists. While not intended to be a comprehensive textbook on microbiology, the book may also be of some value to trained microbiologists for its overview of cleanroom microbiology.

The objectives of this book are as follows:

1. To introduce people who work in cleanrooms to the basic vocabulary of microbiology.
2. To describe some of the more common types of microorganisms that may be encountered in the cleanroom, their roles in human activities, and some of the techniques used to study them.
3. To explain the techniques used to control microorganisms in the cleanroom.
4. To describe some common types of equipment and techniques used to assess levels of microbiological contamination in the cleanroom.
5. To show the rationales behind many of the regulations and operational procedures associated with biocleanrooms.
6. To help establish an understanding relationship between the reader and microbiologist coworkers.

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THE AUTHOR

David Carlberg, Ph.D., is professor emeritus at California State University, Long Beach. He received a doctorate in microbiology in 1963 from the University of California at Los Angeles and in the years following has been involved in research in both industrial and academic settings covering such areas as biological warfare defense, aerobiology, planetary life detection, biocleanroom operation, and biotechnology. He has been a technical advisor for major Hollywood films, an industrial consultant to pharmaceutical and medical device companies and has given lectures and workshops on contamination control and biological warfare defense. Until his retirement he taught university courses in microbiology, bioethics, microbial genetics, and physiology. He is the author of two books on microbiology and chapters and papers on instrumentation, biosafety, and aerobiology. Dr. Carlberg has been president of the Southern California Branch of the American Society for Microbiology, president of the Western Regional Branch of the American Association for Contamination Control, and charter president of the Contamination Control Society (Southern California).

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1

THE SCOPE OF MICROBIOLOGY

I. INTRODUCTION

Microbiology is the branch of biology that deals with the smallest living things, microorganisms. These organisms include bacteria, algae, fungi, and protozoa. Viruses, while not living organisms in a strict sense, are also included in the study of microorganisms, as are subviral infectious particles such as viroids and prions.

Microorganisms are universal. They nearly always occur wherever other forms of life are present, but they also thrive in places where environmental conditions are too harsh for other life. Microorganisms are frequently the only living things found at the bottoms of oil wells, for example, or in the hot springs of Yellowstone National Park, ice-covered lakes in Antarctica, and the extremely dry desert soils of Asia and South America. Bacteria that grow in environments at temperatures higher than the boiling point of water have been isolated near volcanic vents deep in the Pacific Ocean.

Natural microbial populations can reach unbelievable levels. Common garden soil is particularly rich in microorganisms. A cubic centimeter (about 1/5 tsp.) may contain as many as 10^9 bacteria, possibly consisting of up to 7000 different species, most of which have not been named. This mass represents 0.3% of the total bulk of the soil. Stated another way, an acre of soil down to a depth of 3 ft contains about 3000 lb of bacteria. Scientists have estimated that bacteria make up about half of all the living biological mass on Earth.

The bodies of all animals, including humans, are also rich sources of microorganisms. As many as a million bacteria may live on each square centimeter of our skin, and saliva may contain 10^9 microorganisms per milliliter. Nasal washings from normal, healthy adults have been shown

to yield as many as 10^5 microorganisms per milliliter. Fecal matter contains about 10^{11} bacteria per gram, which represents about half its dry weight.

During a normal working day, a person sheds millions of microorganisms into the surrounding environment through breathing, talking, sneezing, coughing, or just moving about. For instance, it has been estimated that a single sneeze can spray as many as 200,000 microorganisms into the surrounding air. Normal body movement and friction of clothing against the skin cause profuse shedding of dead skin cells, which contain millions of bacteria. Depending on the intensity of activity, a person might release 100,000 to as many as 10 million particles every minute. Thus, it is not surprising that soil and humans are the two most common sources of microbiological contamination in the cleanroom. Most efforts to control microbial contamination in the cleanroom are directed toward these two sources.

Since microorganisms are so common in such large numbers, one might ask why infectious diseases are not more widespread. Besides the fact that animals (and plants) have evolved very effective defense mechanisms against infection, the main reason is that of the tens of thousands of species of microorganisms that share the planet Earth with humans and other plants and animals, only a few hundred are known to cause disease; the others are generally harmless, and in many instances beneficial. For example, many microorganisms are responsible for maintaining soil fertility or are part of a food chain. Through competition, their presence on our bodies actually protects us from infection by harmful organisms, and microorganisms in our intestines supply us with vitamins and assist us in the digestion of food. In addition, microorganisms aid in the manufacture of hundreds of products such as pharmaceuticals, including vitamins, antibiotics and hormones, as well as food products like wine, bread, and cheese. However, microorganisms in cleanrooms, no matter how beneficial they may be elsewhere, can cause serious problems.

Consumers place great trust in the belief that products they put on or in their bodies will do what they are designed to do and not cause harm in any way. In response to that trust, producers of cosmetics, foods, medical devices, and drugs strive to deliver products that are free of harmful chemicals and microorganisms. Choice of raw materials, design of suitable manufacturing facilities and processes, and effective packaging are a few examples of how manufacturers are able to turn out consistently safe products.

The metabolic activities or the mere presence of microorganisms in a product can create a wide range of undesirable consequences. Examples are:

1. Deterioration of organoleptic qualities of a product such as taste, smell, and appearance

2. Destruction of potency
3. Potential for causing infection and disease

Upper limits for the numbers of microorganisms allowed in products depend on the type of product and the types of organisms. Fresh produce may be considered safe even when there are moderate numbers of microorganisms present as long as the organisms are not disease-producing types. At the other extreme are those products that come in direct contact with those areas of the body that are most sensitive to infectious agents, like the bloodstream and the eyes. These products must be totally free of live microorganisms; that is, sterile. Between the two extremes fall most personal care and drug products like ointments, cosmetics, soaps, and medications taken by mouth. Control of microorganisms in products such as these has been possible through development of cleanrooms and other facilities that control contamination. But before we get into the subject of cleanroom microbiology, let us look at some basic principles of biology.

II. CELLS

A basic principle of biology is that every living organism, from the simplest to the most complex, is made up of one or more functional units called cells. While the body of an adult human consists of billions of cells, it starts out as a single cell, a fertilized ovum or zygote. Within 24 h of its formation, the zygote divides into two cells to become an embryo. During its subsequent development, the embryo's cells undergo a process known as differentiation, in which cells are programmed to carry out specific functions: a blood cell carries oxygen, a muscle cell contracts, a nerve cell conducts impulses, and a gland cell manufactures and secretes hormones. Microorganisms, on the other hand, such as bacteria, yeasts, protozoa, and algae, exist for the most part as single cells. Each individual microbial cell must be able to carry out all the necessary functions for life: respiration, reproduction, movement, and so forth. Molds, a type of fungus, are multi-celled but are still considerably simpler than plants and animals and must function independently.

III. BINOMIAL NOMENCLATURE

The term nomenclature refers to the naming of things. In biology, organisms are named according to a system called binomial nomenclature; that is, names consisting of two words. The first word represents the genus (plural "genera"); the second word represents the species. Everyone is probably familiar with the scientific name for humans, *Homo sapiens;* for the domestic dog, *Canis familiaris;* or for the horse, *Equus caballus*. A

genus represents a group of related species. Thus, the genus *Canis* consists of all species closely related to the dog: *Canis lupus*, wolf; *Canis latrans*, coyote; and so on. Scientific names are often printed in italics (or underlined) because they are foreign words. The basic language of binomial nomenclature is Latin or latinized words from other languages. Latin is an international language that can be used and understood throughout the world. A French biologist visiting China would have no trouble explaining what organism has been the subject of her research. Every known plant, animal, and microorganism is identified by the same scientific name around the world, even though it may have very different local names. A sparrow is known as a *passero* in Italy, a *sperling* in Germany, and a *moineau* in France, but the genus name for sparrows is *Passer*, a name recognized by every ornithologist and bird watcher from Missouri to Mongolia. *Escherichia coli* and *Staphylococcus aureus* are two examples of binomial names of some common bacterial species.

The roots of microorganism names are frequently descriptive, derived from Latin, Greek, or other languages (*Staphylococcus*: "grape-like"; *Bacillus*: "rod-like") or are based on names of people (*Escherichia*, *Neisseria*, *Pasteurella*), places (*Pseudomonas fairmontensis*: Fairmount Park, PA; *Pasteurella tularensis*: Tulare County, CA), and even organizations (*Legionella*: American Legion; *Afipia*: Air Force Institute of Pathology).

Obviously, some sort of worldwide organizations must be in place to coordinate the naming of organisms, since new ones are being discovered on a regular basis. Three major branches of biology, that is, zoology, botany, and microbiology, have international organizations that oversee the naming of newly discovered organisms. When a new species appears to have been found, its complete description along with a proposed name is forwarded to the appropriate organization for its review and approval. Then, to make the name official, news of the discovery is published and distributed worldwide.

Occasionally one may see the term "strain" used with a particular species of microorganism. The term refers to an organism that differs only slightly from other members of the same species, but not enough to justify placing it into a separate species. For example, there are many strains of the bacterium *Staphylococcus aureus* that are resistant to penicillin, whereas most members of that species are not. If a strain exhibits many differences from other members of the species, but still not enough to justify placing it into another species, it may be referred to as a subspecies.

IV. MORPHOLOGY

Morphology refers to the size, shape, and other physical features of an organism. Listing its morphological characteristics is the first step employed

in the identification of an unknown microorganism. The unit of length used by microbiologists to measure and report the size of microorganisms is the micrometer (μm), a millionth of a meter, which is equivalent to 1/25,400 in. The remainder of this chapter will be concerned with morphological and other characteristics of various microorganisms.

V. SPECIFIC MICROBIAL GROUPS

A. Bacteria

Bacteria constitute a large group of thousands of species of small, single-celled organisms. Since they appear to be the most numerous type of microorganism in soil and on the body, they are probably the most common microbial contaminant in cleanrooms. Bacteria occur in three basic shapes (Figure 1.1 and Figure 1.18): rods, or bacilli (singular bacillus); spheres, or cocci (singular coccus); and spirals, or spirilla (singular spir-

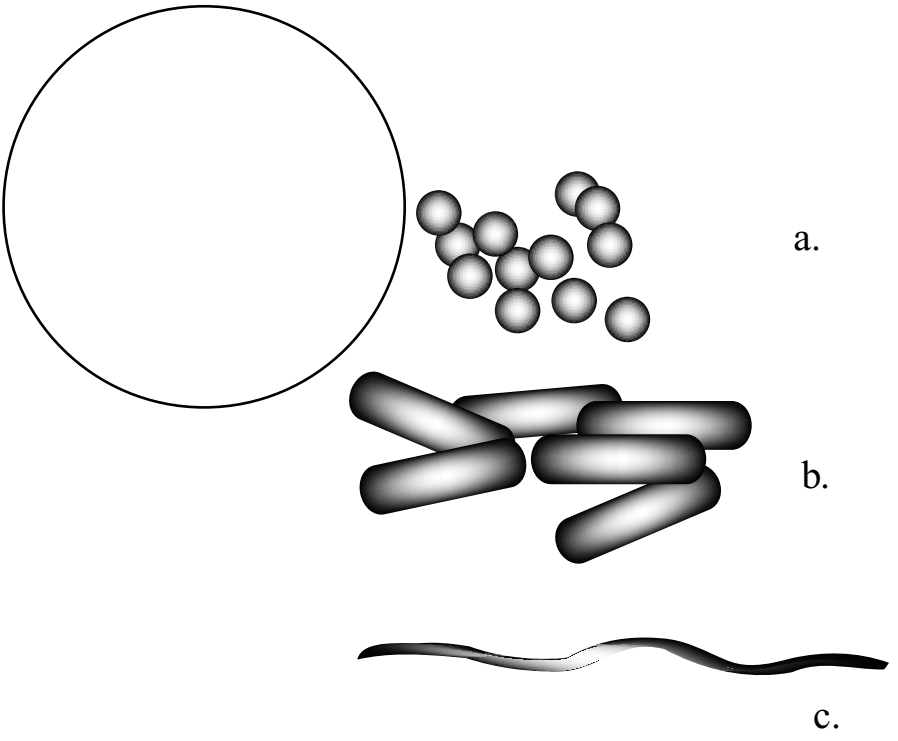

Figure 1.1 The three basic shapes of bacteria. (a) Coccus, (b) bacillus, (c) spirillum. Typical cocci would be about 1 to 2 μm in diameter. Large circle shows relative size of a human red blood cell.

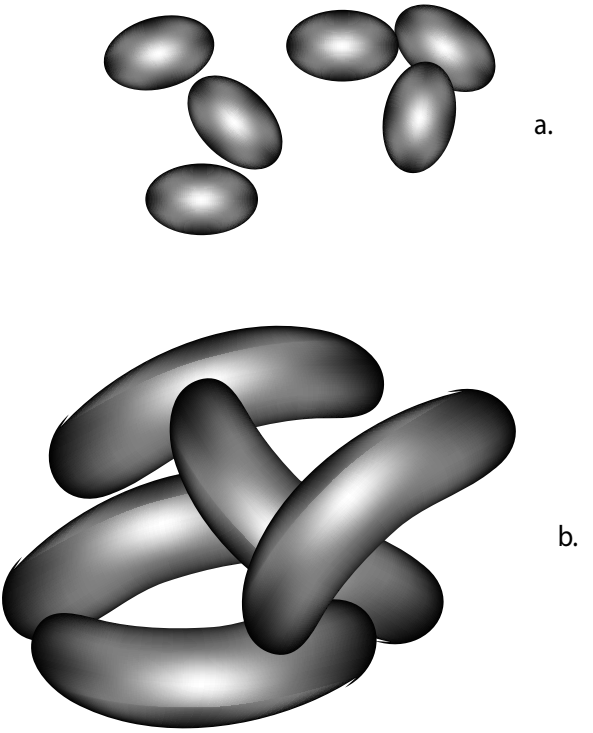

Figure 1.2 Variations on bacterial shapes. (a) Coccobacillus, (b) vibrio.

illum). Numerous variations on these three shapes are common (Figure 1.2), such as ovoid spheres, known as coccobacilli, and curved rods, called vibrios, some of which may even form a complete circle.

The arrangements that cocci exhibit in relation to one another also have special names (Figure 1.3). If the cells occur predominantly in random clusters, they are referred to as staphylococci (from *staphyle*, Greek for bunches of grapes). They are labeled streptococci if they are mostly seen in chains. "Streptococcus" comes from the Greek word *streptos* for pliant, referring to the flexible chains they form. If they occur in pairs, they are called diplococci (from the Greek *diplous* for double).

Nearly all morphological descriptors just introduced have also been used in the formal names of some bacteria: *Bacillus*, *Staphylococcus*, *Streptococcus*, and *Diplococcus* are all names of bacterial genera. To determine if a name that appears in print is a specific taxonomic name or merely a morphological descriptor, remember that genus names are always capitalized and italicized; descriptors and general references to a genus are not.

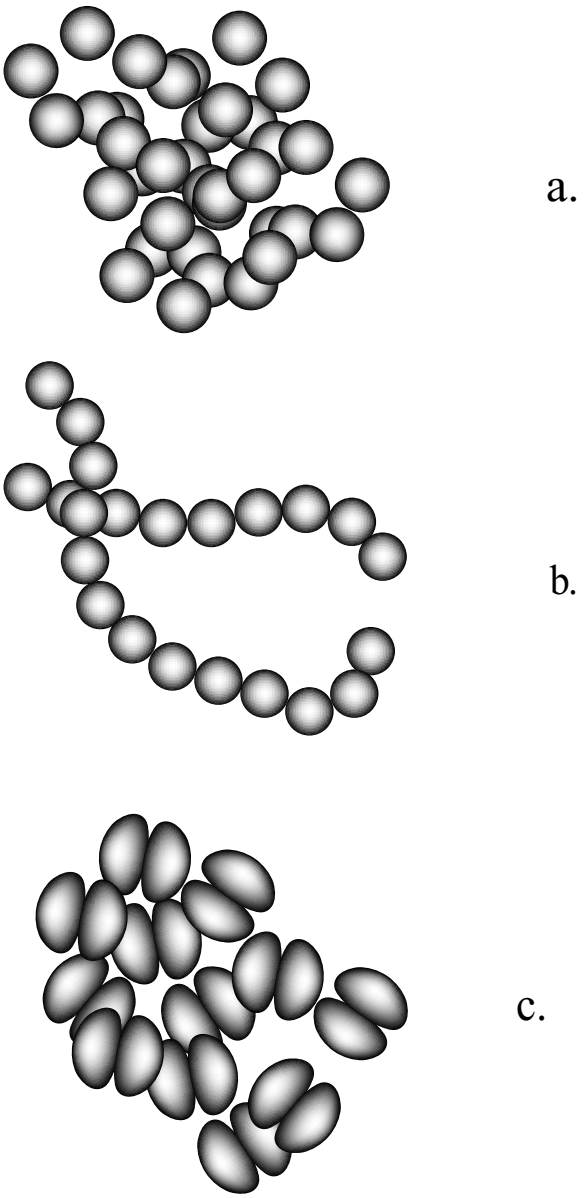

Figure 1.3 Arrangements of cocci. (a) Staphylococcus, (b) streptococcus, (c) diplococcus.

The size of bacteria depends on the species and the age of the cells. Sizes of bacterial cells vary over an enormous range. The cells of at least one extraordinary species of bacterium that was discovered in 1993 reach 500 μm in length. At the other extremity of the size scale are the so-called nanobacteria, measuring typically 0.2 to 0.5 μm in diameter. Most common bacterial cells, however, ones that are encountered as contaminants in cleanrooms, are about 1 to 3 μm in size.

1. The Gram Stain

In 1884 the Danish biologist Hans Christian Gram discovered that certain bacteria, once stained with a particular dye and treated with a dye-fixing mordant, would retain the dye even after being subjected to a strong solvent such as acetone or alcohol. Other bacteria would become decolorized; that is, the dye would be extracted from the cells upon exposure to the solvent and they would appear colorless. Following treatment with solvent, the cells would then be exposed to a second dye, a "counterstain" of a contrasting color to the first dye. Those species of bacteria that retain the first dye, usually crystal violet, would appear violet and are referred to as "Gram-positive"; those that do not retain the first dye and then appear in the color of the counterstain are "Gram-negative." The counterstain that is used in this process is normally a red dye called safranin. The Gram stain, as this procedure is now known, is the most important benchmark in microbiology in the identification of bacteria. Although microbiologists are not entirely sure what the mechanism of the Gram stain involves, reactions of cells in the Gram stain appear to be caused primarily by fundamental differences in physical structures of the two types of cells, differences that will be described in this text.

Figure 1.4 shows some of the features of a bacterial cell. A typical cell is enclosed within one or two thin membranes and a rigid cell wall. The membranes generally control what enters and leaves the cell, and the cell wall both controls the shape of the cell and protects it from external physical forces. The cell walls of Gram-positive bacteria are quite thick, composed of many layers of a substance called peptidoglycan. The layers are held together by polypeptide cross-bridges and the entire structure gives the appearance of a stack of fences (Figure 1.5).

One major group of bacteria lacks a peptidoglycan cell wall. They are called mycoplasmas, and many are pathogens of animals, including humans. Genetically they appear to be closely related to Gram-positive bacteria. In spite of the lack of a cell wall, mycoplasmas are able to survive in nature due at least in part to an especially tough cytoplasmic membrane. Many mycoplasmas are not more than 0.2 to 0.3 μm in size, making them the smallest free-living organisms. Mycoplasmas are rarely problems as

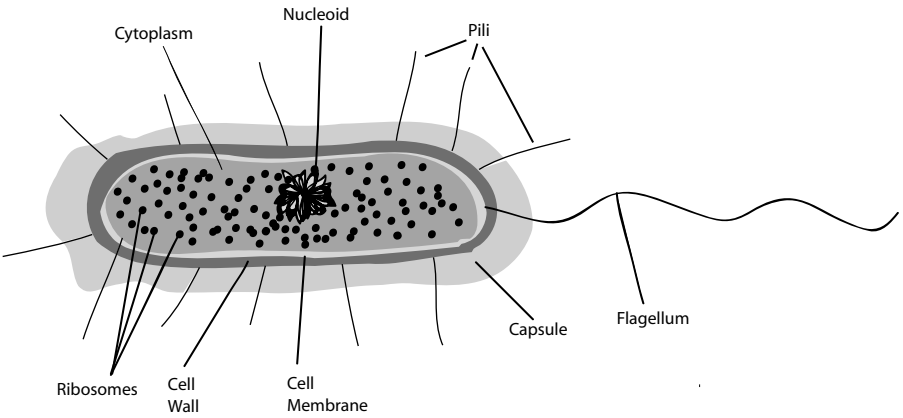

Figure 1.4 A typical bacterial cell showing major structures.

contaminants in cleanrooms except in those facilities that deal with vaccine production, organ and tissue transplants, and other activities involving live animal cultures.

The cell walls of Gram-negative bacteria, while still made of peptidoglycan, are considerably thinner than the cell walls of Gram-positive bacteria. This dissimilarity has been proposed to explain the difference in Gram stain reactions between the two kinds of cells. Because of the greater thickness of the cell walls of Gram-positive bacteria, it is thought that once the cells are treated with the mordant it is simply more difficult to remove the dye from these bacteria than it is from Gram-negative cells. Interestingly, some bacteria that normally stain Gram positive in time

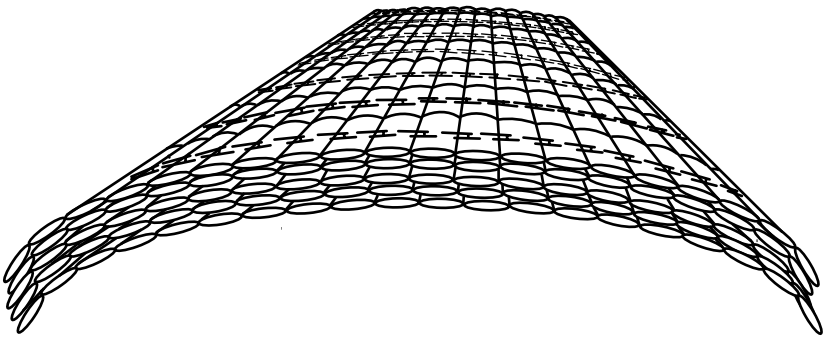

Figure 1.5 Sketch of peptidoglycan structure of bacterial cell wall. Peptidoglycan consists of many layers (only four are shown) of long chains of alternating rod-like molecules of acetylglucosamine and muramic acid held together with polypeptide cross-bridges.

become Gram negative. It is thought that as Gram-positive cells age, changes in the structure of their cell wall occur and the cells no longer react positive with the Gram stain. It is thus imperative that the Gram stain be carried out on cells that are less than 24 h old.

The outer surface of the peptidoglycan layer of Gram-negative bacteria is covered by a second membrane consisting of lipopolysaccharides, phospholipids, and lipoproteins. The membrane provides the cells with additional protection from harmful substances such as antibiotics and disinfectants. Gram-positive bacteria do not have this outermost protective layer and are therefore generally more susceptible to these substances. Knowledge of the Gram reaction of a bacterium that may be the cause of an infection is thus helpful in the choice of the type and amount of an antibiotic needed for treatment of the infection.

2. External Features of Bacterial Cells

Most species of bacteria are capable of producing large quantities of polysaccharides or polypeptides, which frequently accumulate on the outer surface of the cell as a thick, slimy layer called a capsule (Figure 1.6). For those species that infect animal hosts, a thick capsule plays an important role in protecting the cells from the host's natural defense mechanisms and thus may result in a more serious infection. In other species, a thicker, fibrous polysaccharide material, called a glycocalyx, may form on the surface of the cell. This substance can act as a cement to anchor cells to hard surfaces such as underwater rocks; the inner surfaces of pipes and vessels of water purification systems; teeth; or medical devices implanted in the body, such as catheters and artificial joints. Other substances, collectively called adhesins, also play a role in the attachment of microbes to surfaces.

The layer of microbial cells that forms on a surface is known as a biofilm (Figure 1.7). A biofilm may eventually build up to several layers of cells. There are a number of advantages for the organisms that are members of a biofilm. The biofilm is very much like a sponge, with open spaces running through the polysaccharide matrix. The open spaces act as channels to trap nutrients and conduct them to the member organisms and also carry away waste matter. It is thought that most infections involve the formation of biofilms. When formed in the body during the establishment of an infection, biofilms protect their members from the effects of antibiotics and the host's defense mechanisms. The significance of biofilms in pure water systems will be discussed further in Chapter 3.

Some bacteria possess one or more very long, corkscrew-like appendages called flagella (singular flagellum) (Figure 1.8), which give a cell the ability to move through liquid. The movement, or motility, is caused by

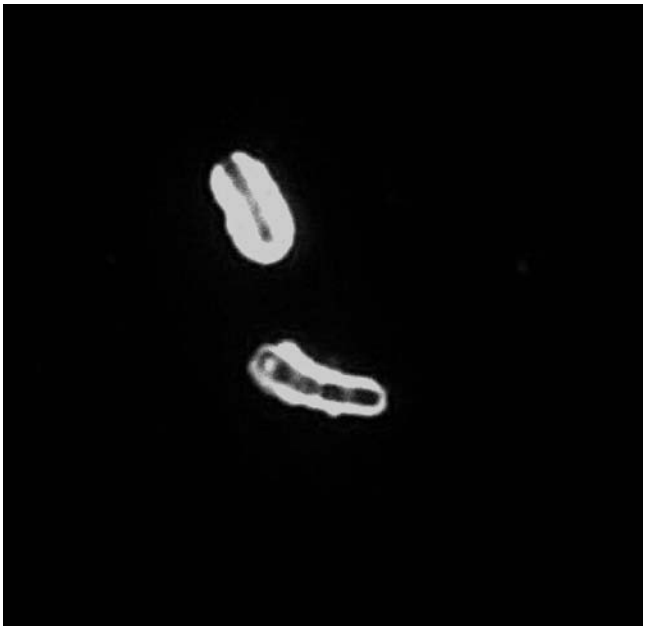

Figure 1.6 Anthrax bacteria stained with a fluorescent capsule stain. Capsules surrounding cells appear light against dark background. (Photo: CDC Public Health Image Iibrary ID #1888.)

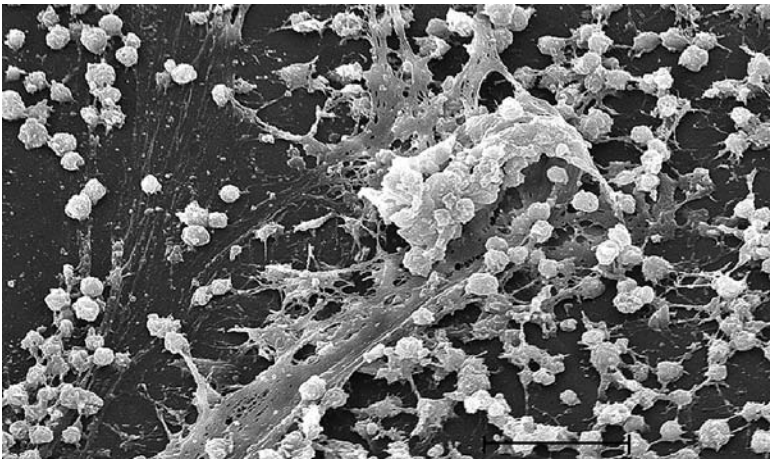

Figure 1.7 Scanning electron micrograph of a biofilm of Staphylococcal cells that has begun to form on the inner surface of a needleless connector. Note glycocalyx strands. Several layers of cells would eventually accumulate. (Photo: CDC/Janice Carr. CDC Public Health Image Library ID #2264.)

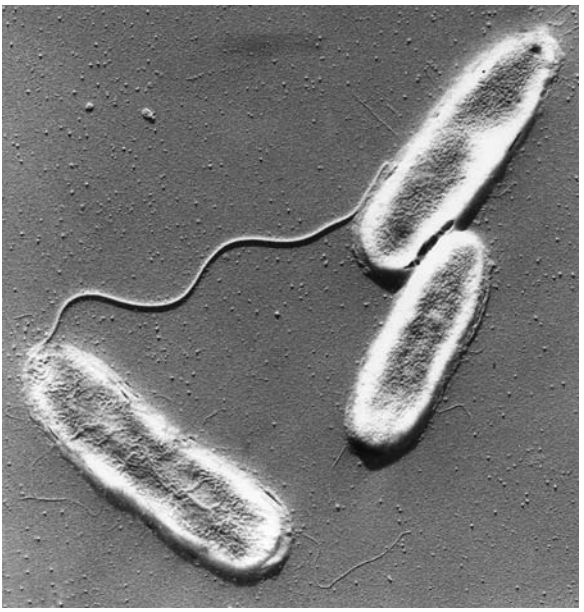

Figure 1.8 Appendages of bacterial cells. Transmission electron micrograph showing a flagellum (large curly structure) and pili (finer filamentous structures). See text for description of the shadowing process used in this micrograph. (Micrograph courtesy of Frederick Eiserling and Robert Romig, University of California at Los Angeles.)

the propeller action of the rotating flagella, which is generated by molecule-sized motors at the base of each flagellum. The motors have components much like bushings, rotors, and stators found in an electric motor, but these motors are powered by a current of protons rather than electrons. Flagellar movement is controlled by sensing molecules near the surface of the cells. If the sensors detect nutrients, the flagella are directed to propel the cell forward. If toxic chemicals are sensed, the flagella reverse their rotation, causing the cells to change direction. The axial filament, which is somewhat similar in structure and function to flagella, is embedded in the cell wall of the spiral bacteria. Other external appendages, seen only in Gram-negative bacteria, are pili (Figure 1.8). These play a role in the attachment of cells to membrane surfaces of animal hosts and to other bacterial cells.

a. Pyrogens

As just mentioned, the outermost membrane of Gram-negative bacteria contains lipopolysaccharides, substances that are highly toxic to many

animals, including humans. This toxicity is due to the presence of a molecule known as lipid A, or endotoxin. Besides affecting a wide variety of physiological functions in animals, endotoxin is a pyrogen (fever inducer). As they grow, Gram-negative bacteria often release small quantities of endotoxin into the surrounding medium. And when Gram-negative bacterial cells die, they usually disintegrate due to the action of enzymes within the cells, causing the release of large amounts of endotoxin. If the cells happen to be in the bloodstream of an animal host, the effect can be severe and even fatal. If pharmaceutical fluids intended for parenteral use, that is, for injection, or medical devices that come into contact with the bloodstream contain Gram-negative cells or endotoxins, the effect may be equally dangerous. Therefore, all solutions, containers, instruments, or devices intended to come in contact with the bloodstream, spinal fluid, or regions of the eye must be essentially free of endotoxin; that is, pyrogen free. How to produce pyrogen-free products will be covered in Chapter 3.

How do the endotoxins get into parenteral fluids in the first place? Remember, microorganisms are everywhere, including air, soil, and the water and raw products used to make up parenteral solutions and other pharmaceutical liquids. Generally, the processes used to purify water, such as distillation, reverse osmosis, and ultrafiltration, rid the water of most impurities, including bacteria and endotoxins. However, unless the water is protected from subsequent contamination, which is extremely difficult if not impossible, bacteria reenter the water through cracks and openings in pipes and storage containers. Although only a few bacteria may gain access to the water supply, certain species can derive enough nutrients from so-called pure water to multiply to surprisingly large populations. There is usually enough organic matter leaching from plastic tanks and piping, valve lubricants, and dust to feed the few bacteria present until they reach populations large enough to produce measurable quantities of endotoxin. The problems caused by microorganisms in purified water systems is covered further in Chapters 3 and 5.

i. Testing for Pyrogens As revealed earlier, one of the characteristics of the lipid A endotoxin is its pyrogenicity. This feature lent itself to a test that was designed to detect the presence of endotoxin in solutions or medical devices that come in contact with the bloodstream. In the rabbit pyrogen assay, test samples are injected into rabbits and the animals are closely observed for the development of fever.

Later, a more sensitive test, the limulus amoebocyte lysate (LAL) assay, based on an entirely different principle, was developed. This test takes advantage of the fact that the endotoxin causes the gelling of a substance found in the amoebocytes or blood cells of the horseshoe crab *Limulus*. The LAL assay is capable of detecting considerably lower concentrations

of endotoxin than the rabbit pyrogen assay, and of course it avoids the need for live test animals. The crabs can be captured, partially bled, and returned to the sea without harm.

However, due to variations in the quality of the product, labor costs, and the possible risk to crab populations, a new test for pyrogens has been developed. The test uses a factor from the horseshoe crab that is synthesized through genetic engineering, thus avoiding the continuing need to harvest crabs. The gene for the factor was cloned into bacteria and now the bacteria produce the horseshoe crab factor. Thus, horseshoe crabs are no longer needed to carry out the LAL test for pyrogens. Other tests for endotoxin depend on the substance's effect on certain cells of the immune system.

3. The Bacterial Interior

The cytoplasm is the aqueous fluid that fills the bacterial cell. Found within the cytoplasm are numerous structures and dissolved substances necessary for the growth and reproduction of the cell. The most prominent object in the cytoplasm is the nucleoid, the depository of most of the genetic information of the cell. The bacterial nucleoid consists of a single molecule of deoxyribonucleic acid (DNA) (see next section for more details). In the cells of plants, animals, and most other microorganisms like fungi and algae, the comparable structure (the nucleus) contains several molecules of DNA and proteins that are part of very large and complex structures known as chromosomes. The nucleus is surrounded by the nuclear membrane. The much simpler nucleoid of bacteria has no surrounding membrane, and therefore its DNA is in direct contact with the cytoplasm. This is the reason for the difference in names (nucleoid versus nucleus). Consequently, bacteria with their simpler nucleoids are designated as procaryotes, whereas the cells of nearly all other microorganisms, as well as all plants and animals, having a true nucleus, are called eucaryotes. The principal features that distinguish procaryotes from eucaryotes are listed in Table 1.1. There are actually over 40 differences between eucaryotic and procaryotic cells.

In addition to the nucleoid, many bacteria also possess small fragments of self-replicating DNA, called plasmids, which also carry genetic information. While not necessary for the survival of a cell, a plasmid can frequently provide the cell with some unique or useful capacity, such as the ability to digest hydrocarbons, to better penetrate or dismantle host tissue, or to resist the effects of certain antibiotics.

Plasmids can be found as independent, self-replicating entities in the bacterial cytoplasm or as integral parts of the nucleoid. Plasmids can be transferred from cell to cell through various genetic mechanisms, allowing

Table 1.1 Comparative features of procaryotes and eucaryotes

Features	*Procaryotes*	*Eucaryotes*
Cell wall	Present	Usually absent
Interior membrane systems	Absent	Present
Vacuoles	Absent or rare	Usually present
DNA	Usually as a single, circular molecule	Usually in multiple, linear molecules
Histones	Absent	Present
Nuclear membrane	Absent	Present
Mitotic apparatus	Absent	Present

bacteria to "share" their biochemical capabilities with other bacteria. One mechanism by which a bacterial cell can transfer a plasmid to another is conjugation, where the donor cell comes in direct contact with the recipient. In some situations, conjugation may involve the transfer of nucleoid DNA in addition to plasmid DNA. Conjugation is discussed further in the next section.

Closer examination of the cytoplasm of bacteria reveals the presence of small particles called ribosomes, which are the protein factories of the cell (Figure 1.4). A typical bacterial cell may contain several thousand ribosomes. Copies of nuclear genetic information are made in the form of messenger ribonucleic acid (mRNA); the mRNA, amino acids, sources of chemical energy, and other cofactors all congregate at the ribosomes to contribute to the formation of enzymes and other proteins. Over 90% of the energy consumed by a bacterial cell goes into the synthesis of proteins.

Endospores are found in a few species of bacteria. The endospore (frequently referred to simply as a spore) is an extremely resistant, dormant body produced primarily by members of the genera *Bacillus* and *Clostridium*. Bacterial spores can withstand extreme environmental conditions such as heat and drying; their great resistance appears to be due to their chemical makeup. Spores are covered with several layers of very tough materials, one of which is similar to the keratin in human fingernails and teeth. Their water content (less than 15%) is also lower than that of vegetative cells (around 90%). Species within the spore-forming bacteria produce spores (one per cell) when the cells face unfavorable conditions such as heat or desiccation (drying). Some bacterial spores can survive boiling water for several hours and are unaffected by many common disinfectants like alcohol. In the dry state, spores may remain viable for centuries, ready to germinate (revert to reproductive vegetative cells) whenever environmental conditions that support growth are encountered.

Because bacterial spores are extremely common in soil, any object that comes into contact with soil or dust (which includes just about everything) must be presumed to be contaminated with these resistant forms. Any procedure that is intended to sterilize the object (rid it of all live organisms) must therefore be powerful enough to destroy bacterial endospores. More on sterilization will be covered in Chapter 3.

4. Bacterial Genetics

Whenever plants or animals reproduce, the progeny always look identical or nearly identical to the parents. Camels always have baby camels, and tuna always have baby tuna. It is clear, therefore, that some form of information must be passed on to members of succeeding generations that instructs them how to look like their parents. This transfer of information is called heredity, and genetics is the study of heredity.

The genetic information of cells is stored in molecules of DNA, which consist of enormously long strands of subunits called nucleotides strung together like links of a chain. There are four types of nucleotides in DNA and they are referred to simply as G, A, C, and T. A typical bacterial DNA molecule may be made up of 4 million nucleotides, which, if stretched out, would be over a thousand times longer than the cell from which it was removed. Genetic information is written in a language consisting of only the four nucleotides, but this is more than sufficient to spell out all of the instructions that it takes to make a bacterial cell, or a human. A typical stretch of genetic information may read like:

GTGCGAGATGCTGGATGAGCGCCCGATGATCGAG

The genetic code is the basis for a cell's translation of genetic information into functional protein molecules. The genetic code is universal, in that all organisms, from viruses to humans, essentially use the same code. A universal genetic code has made genetic engineering possible. DNA from one organism, when spliced into the DNA of a different species, can usually still be understood and decoded by the recipient organism.

The complete set of genetic information of a cell is known collectively as its genome. When a cell reproduces, its genome also reproduces, resulting in the formation of one or more identical DNA copies that are distributed to the cell's progeny, ensuring that each daughter cell receives a complete and accurate copy of its parent's genetic information. Thus, DNA that we isolate today represents a continuous link back to the beginnings of life. These links to the past are not unaltered, however. DNA experiences mutations, rare changes in the sequence of nucleotides, which, if not lethal, are passed on to subsequent generations.

What causes mutations? Background radiation and chemicals in the environment are two possible causes, but the bulk of spontaneous mutations observed in organisms is due to errors cells make when they copy or repair their own DNA. Mutations may be harmful, even lethal, or they may be neutral, being neither harmful nor beneficial. On rare occasions, a mutation may be beneficial, perhaps affording a bacterium some improved function that gives it a slight advantage over its siblings. Mutations are the fuel that powers evolution.

Plants and animals share genetic information primarily through the process of sexual reproduction, in which DNA from each parent is combined in the fertilized ovum or egg, known as a zygote. The organism that develops from the zygote contains a nearly equal mixture of genetic information from its male parent and from its female parent. As simple as they are, bacteria also share genetic information among themselves, but usually in the form of small fragments. They do so by three mechanisms, called transformation, conjugation, and transduction.

Transformation is the transfer of genetic information from cell to cell by naked DNA. Transformation can be accomplished in the laboratory by extracting the DNA from a culture of bacteria and mixing it with a second suspension of live bacteria, usually of the same species. If the recipient cells are "competent," that is, physiologically capable of taking up the DNA into their cytoplasm, they may incorporate fragments of donor DNA into their own genome and soon express one or more characteristics of the bacteria from which the DNA was extracted, such as antibiotic resistance. The recipient cells are said to have "transformed."

The process of incorporating donor DNA into the recipient DNA is known as recombination. Recombination involves a piece of DNA becoming spliced seamlessly into the DNA of the recipient cell, often replacing a piece of the cell's DNA. Recombination not only occurs in bacteria but in all levels of microorganisms as well as all plants and animals. Recombination is the second fuel that powers evolution.

Recombination also is the tool that makes gene splicing or genetic engineering possible. Since all DNA from all species is essentially structurally identical, composed of the familiar double-stranded helix of Watson and Crick, it is possible, at least theoretically, to splice small fragments of any DNA into any other DNA. That is, with the help of special enzymes, human DNA can be spliced into grasshopper DNA and camel DNA can be spliced into tuna DNA, and so on. The donor DNA fragments just have to be customized to be accepted by the recipient DNA.

Conjugation requires direct cell-to-cell contact for DNA transfer. Donor ("male") cells attach to recipient ("female") cells, at which time a mechanism is triggered that causes the transfer of donor DNA to the recipient cell. Only a few strains of bacteria are capable of conjugation. The presence

of a fertility plasmid is usually necessary for a bacterial cell to act as a donor in conjugation.

Transduction involves viruses, called bacteriophages, that infect bacteria (Figure 1.14). As the bacteriophages develop in the host cells (see section on viruses), small bits of host bacterial DNA inadvertently may become packaged into one or more of the virus particles. When the infected bacterial cell bursts, the released virus particles are free to infect other bacteria. If a virus carrying some bacterial DNA infects another bacterium, it will bring bacterial DNA into the cell rather than viral DNA. The recipient bacterium will show no ill effects from this encounter with the bacteriophage, because the virus carries little or no viral DNA and is thus incapable of establishing a lethal infection. Again, through the process of recombination, the recipient bacterium may then express the new bacterial genetic information that the bacteriophage delivered to it. The recipient cell has thus "transduced."

Until recently it was not clear to what extent these various modes of bacterial genetic transfer occur in nature. Now that the sequencing of bacterial DNA is almost routine, it is becoming evident that bacteria have been swapping bits of their DNA with one another to a much greater degree than was once thought. Numerous examples of identical or near identical genes have been identified even among supposedly unrelated bacteria. Such exchange of DNA is known as horizontal gene transfer.

Transformation, transduction, and conjugation in bacteria, discovered during the period from the late 1920s to the early 1950s, have been enormously valuable tools to help biologists learn more about the basic mechanics of genetics. Of greatest significance, the discovery of transformation led to discovery of DNA as the stuff genes are made of and to the elucidation of its structure. Overall, more has been learned about heredity by studying microorganisms than all other plants and animals combined.

B. Fungi

1. Molds

The fungi occur in two forms: molds and yeasts. The appearance of molds is all too familiar to anyone who has kept food unrefrigerated for too long a time. The green or gray fuzzy mass that appears on moldy food is the mycelium, a tangle of multicellular filaments called hyphae, which is characteristic of the molds (Figure 1.9). The hyphae differentiate at their tips to produce complex reproductive structures that manufacture spores (Figure 1.10). These spores are specialized cells that serve several functions for the molds: reproduction, dissemination, and protection.

Unlike spore-forming bacteria, where only one spore is formed per cell, a single mold hypha may produce thousands of spores, each of

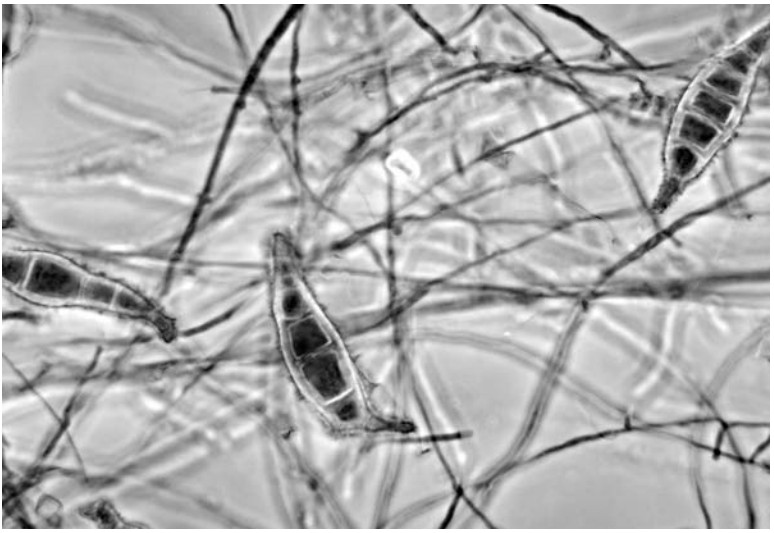

Figure 1.9 Tangled mycelium of the mold *Microsporum*. Conical structures are macroconidia or spore cases. (Photo: CDC Public Health Image Library ID #4325.)

which can be launched into the air with the slightest air movement. If the spore happens to land on a site where there are nutrients and moisture, it will germinate and eventually develop into a new mycelium.

While mold spores are somewhat more resistant to harsh conditions than the cells that make up the hyphae, mold spores generally are nowhere as resistant to adverse environmental conditions as are bacterial endospores. Thus, chemical and physical means that destroy bacterial spores easily eliminate mold spores.

People are most familiar with molds because of their association with food spoilage and the deterioration of materials and equipment through mildew and dry rot. In addition, a few species of molds are responsible for diseases in animals and plants. The destructive effects molds have on landscape plants, food crops, and consumer products cause billion dollar losses each year for farmers, manufacturers, and consumers. On the positive side, many of our most effective antibiotics, such as penicillin, griseofulvin, and gentamicin, are produced by molds. Also, many species of molds are necessary in the manufacture of important products such as corticosteroids and citric acid and dozens of foods like soy sauce, miso, and blue cheese.

Molds are the second most common microbial contaminant in the cleanroom and, under certain circumstances, they can become a serious threat. Their nutritional requirements are generally simpler than those of bacteria, and many species can grow in the absence of significant amounts

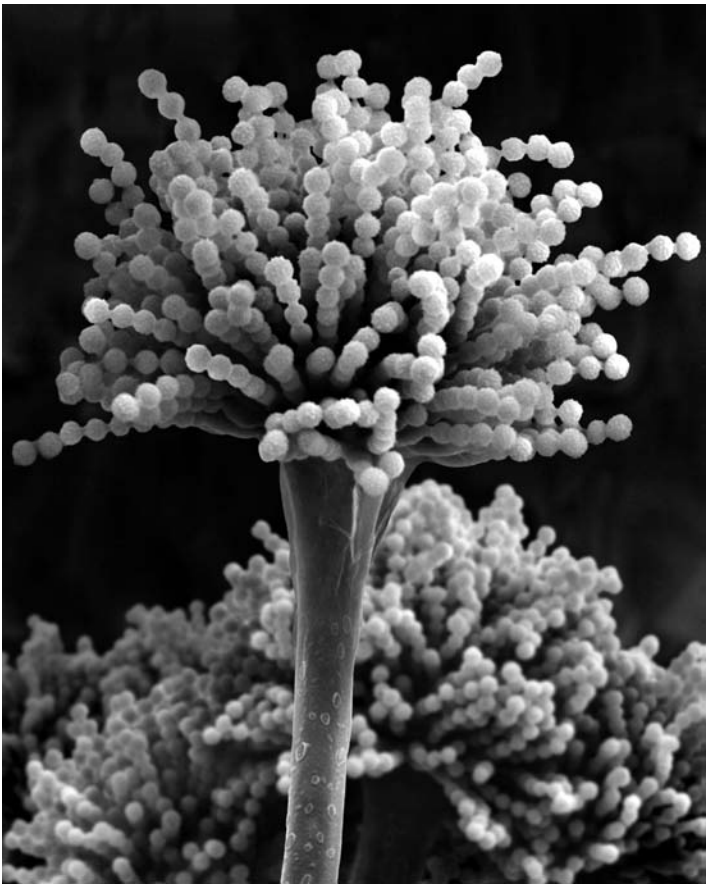

Figure 1.10 Conidia of the mold *Aspergillus* with spherical spores. (Photo: Dr. Dennis Kunkel/Visuals Unlimited.)

of moisture. Molds are capable of growing on electronic circuit boards or in HEPA filters, deriving their nutrients and water from traces of residual organic contamination and condensed moisture. There is sufficient organic residue in a single fingerprint, for instance, or a small droplet of saliva, to support a significant amount of fungal growth.

2. Yeasts

The second form of fungus is yeasts, which are single-celled organisms, usually spherical or ovoid, 5 to 8 μm in diameter. Yeasts are capable of forming four or more moderately resistant intracellular spores as one means of reproduction, but more commonly they form buds (Figure

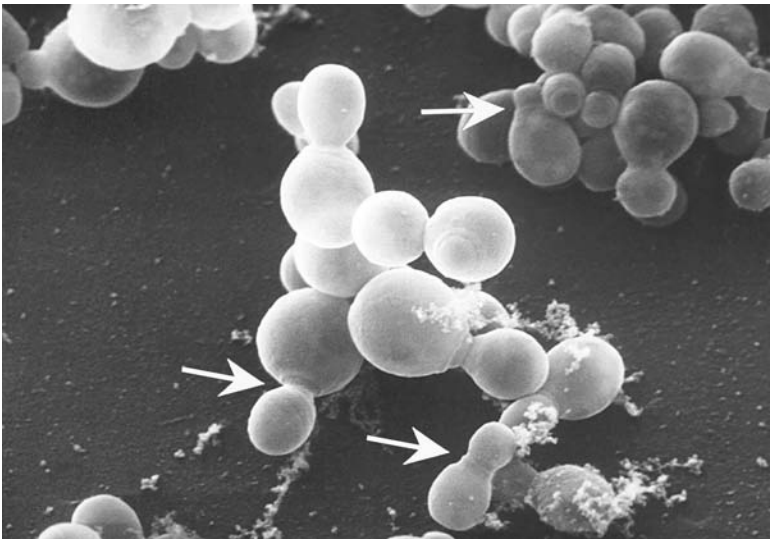

Figure 1.11 Scanning electron micrograph of the yeast *Malassezia*. Note small buds emerging from mother cells (arrows). Largest cells are about 5 µm in size. (Photo: CDC/Janice Carr. CDC Public Health Image Library ID #217.)

1.11). Certain species of yeasts play important roles in the manufacture of many foods and industrial products, such as alcoholic beverages, bread, and vitamins.

While yeasts and molds are two distinct forms of fungi, one single-celled and one multi-celled, a few species are capable of exhibiting both forms at some time during their life cycle. These unusual organisms are known as dimorphic fungi.

Because they require high levels of nutrients and water to grow, yeasts are rare contaminants in the cleanroom. They may, however, grow in certain types of pharmaceutical products that contain water and metabolizable carbohydrates.

C. Algae

Algae are somewhat distinctive among most other microorganisms in that they are capable of converting light into chemical energy. That is, they are like plants and are able to carry out photosynthesis. Photosynthesis, the ability to convert light energy into chemical energy, is also found in a few genera of bacteria. To many biologists, the classification of algae seems in chaos, because many algae closely resemble protozoa and others could almost be classified as plants. Morphologically, algae span nature's extremes of size and complexity. Some algae are nearly as small and

simple as bacteria; in fact, recently, on closer examination, the group long known as the blue-green algae was reclassified as bacteria. Other organisms classified as algae, the kelps or seaweeds, may be hundreds of feet long. A few species of algae are responsible for a variety of shellfish and fish poisonings in humans and other animals. Poisonings that are occasionally fatal occur when seafood containing the algae or their toxins is consumed.

Since algae are aquatic and photosynthetic, they are always found in environments where ample water and light are available and thus will probably never constitute a contamination problem in the cleanroom. They are, however, occasionally seen growing in sight tubes or around entry hatches in manufacturing facilities that are exposed to constant moisture and strong light.

D. Protozoa

Over 40,000 species of protozoa are known. Most are found free-living in natural water environments such as lakes, rivers, and wetlands. The importance of their role in nature as a link in the food chain cannot be overstated. These single-celled organisms (Figure 1.12) feed on bacteria and other small microorganisms and in turn become food for larger organisms. While only about a dozen genera of protozoa cause diseases in man, such as malaria and African sleeping sickness, in the millions of

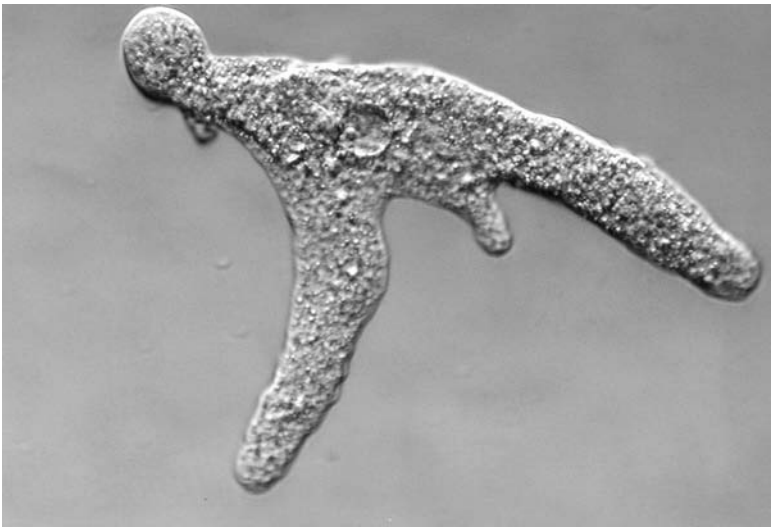

Figure 1.12 Phase contrast micrograph of an amoeba, showing multitude of cytoplasmic inclusions. (Photo: Carolina Biological/Visuals Unlimited.)

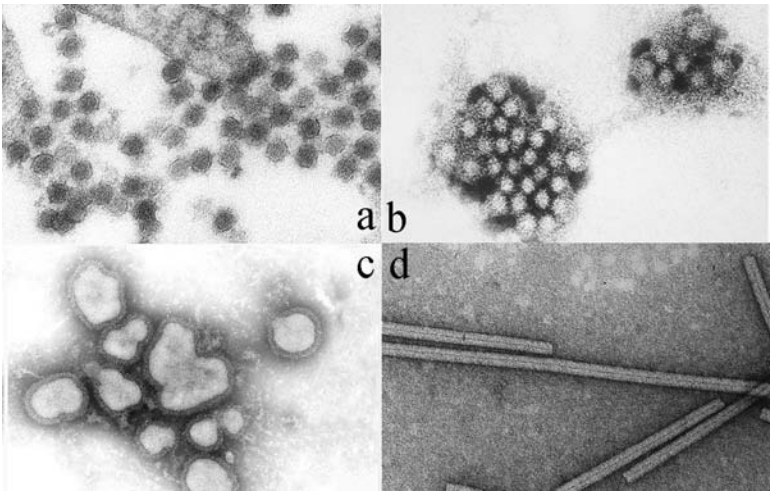

Figure 1.13 Transmission electron micrographs of some human and plant viruses. a. West Nile Virus. b. Norovirus. c. Influenza A virus. d. Tobacco Mosaic Virus. See Table 1.2 for sizes. (Micrographs: a, b, c, CDC Public Health Image Library ID #2288, 2172, 1838; d, Frederick Eiserling, UCLA.)

cases they cause annually worldwide they probably surpass all other infectious organisms in total impact. Since protozoa are nearly always associated with natural water environments, they are of little concern in cleanroom contamination control.

E. Viruses

As a group, viruses are unique among the microorganisms in that they lack any structures that we normally associate with cells. That is, they are noncellular infectious particles. Their structure is extremely simple, frequently consisting of nothing more than a single molecule of either DNA or RNA (but never both) wrapped in a protein coat, called a capsid. Certain more complex viruses (called "enveloped") may be surrounded by one or more membranes, called envelopes, which are actually remnants of their host cells. Viruses lacking envelopes are referred to as being nonenveloped. Some viruses may possess specialized structures such as spikes, tails, and fibers, and others may contain within the capsid one or more enzymes needed for the viruses to replicate in their host cells. This is necessary because the host cells do not normally make those enzymes. Figure 1.13 shows electron microscope images of some typical viruses. The complete virus particle is known as a virion. Table 1.2 lists some common viruses and their dimensions. While some viruses may be as

Table 1.2 Dimensions and nucleic acids of some viruses

Virus	*Hosts*	*Dimensions (nanometers)*[a]	*Nucleic Acids*[b]
Human parvovirus	Humans	18–25	ss DNA
Smallpox	Humans	250 × 350	ds DNA
Polio	Humans	27	ss RNA
Influenza	Humans	110	ss RNA
Ebola	Humans	50 × 1000	ss RNA
Norovirus (Norwalk)	Humans	35–40	ss RNA
Tobacco mosaic	Tobacco plants	17.5 × 300	ss RNA
Totivirus	Yeast	42	ds RNA

[a] Nanometer = 10^{-3} μm.
[b] ss, single stranded; ds, double stranded.

large as small bacteria, typically they are the smallest of the microorganisms, the smallest around 0.01 μm. As a result, viruses are not visible in ordinary laboratory light microscopes.

Viruses are obligate intracellular parasites. That is, because of their extreme simplicity, viruses are incapable of reproducing by themselves. They must find an appropriate host cell in which to multiply. Outside of a host cell, viruses are physiologically inert. To grow viruses in the laboratory, suitable host cell cultures must be maintained. The development of cell cultures for the laboratory cultivation of animal and plant viruses is one of the most important advances in microbiology in the 20th century. The cultivation of viruses will be covered in Chapter 2, and their detection in Chapter 5. It should be noted that obligate intracellular parasitism is not a unique feature of viruses alone, for many other microorganisms, such as certain species of bacteria and protozoa, must also multiply within living host cells.

There are probably no living organisms on Earth that are free of viruses. Viruses are known to infect organisms at every taxonomic level, from bacteria, protozoa, algae, and fungi, to all the higher plants and animals, including humans. Viruses frequently display rather narrow host ranges, meaning a particular virus may only infect a specific species of host, e.g., human cold viruses, or perhaps a narrow range of related species. Exceptions abound, however: West Nile virus infects both humans and birds, and the virus responsible for SARS (sudden acute respiratory syndrome) and various strains of influenza virus appear to infect a wide range of animals as well as humans.

The classification of viruses is based primarily on their biochemical properties. Major classifications depend on whether a virus contains DNA

or RNA, whether the nucleic acid is single or double stranded, and how it replicates. The presence or absence of an envelope is also considered. Presently, a system of binomial nomenclature for viruses has been established but has not been widely adopted. There are 175 genera of known viruses containing over 4000 members, but these are yet to be referred to as species by most microbiologists. In scientific reports the common names of viruses are generally used: mumps, smallpox, influenza, HIV, etc.

1. The Viral Infection Cycle

Viruses reproduce in their host cells by synthesis and assembly. Figure 1.14 depicts the steps involved when a virus (a bacteriophage in this example) infects a cell. The first step in the infection process is the attachment of the virus particle to an appropriate host cell. It is this step that controls host specificity, for the virus can only adsorb to receptor sites on the host cell surface specific for that virus. Penetration of the host cell by the virus can occur through a variety of mechanisms, depending on the type of virus, but the result is that either the entire virion or just the nucleic acid gains entrance.

If the entire virion enters the host cell, its protein coat must be removed before the next step in the infectious process can proceed. Once "uncoating," as it is called, is completed, the free viral nucleic acid immediately directs the biosynthetic mechanisms of the cell to stop making most host cell–associated molecules and begin making virus nucleic acid, enzymes, and virion structural elements based on instructions encoded in the viral nucleic acid. Soon a stockpile of virus components appears in the host cell, and eventually whole, assembled virions begin to accumulate. With certain viruses, only a handful of virions may be assembled, whereas in other instances the host cell eventually may be filled with thousands of virions. The time interval necessary to see this process to its completion may vary from less than 20 min in the case of some bacteriophages to many hours for most animal viruses.

The release of the assembled virions from the host cell is the final step in the infection process. The host cell may simply burst as a result of the destructive activity of enzymes produced from instructions in the virus genome. Such action results in the release of the entire brood of virions at one time, as demonstrated in Figure 1.14 for bacteriophages. In other instances, viruses may be extruded from the host cell one by one over a period of many hours or days. It is during this step that the exiting viruses pick up a fragment of the host cell's membrane to become the envelope that surrounds the infectious particle. In plants and animals, as more and more cells are affected by viral activity, the host organism may begin to show signs and symptoms of disease.

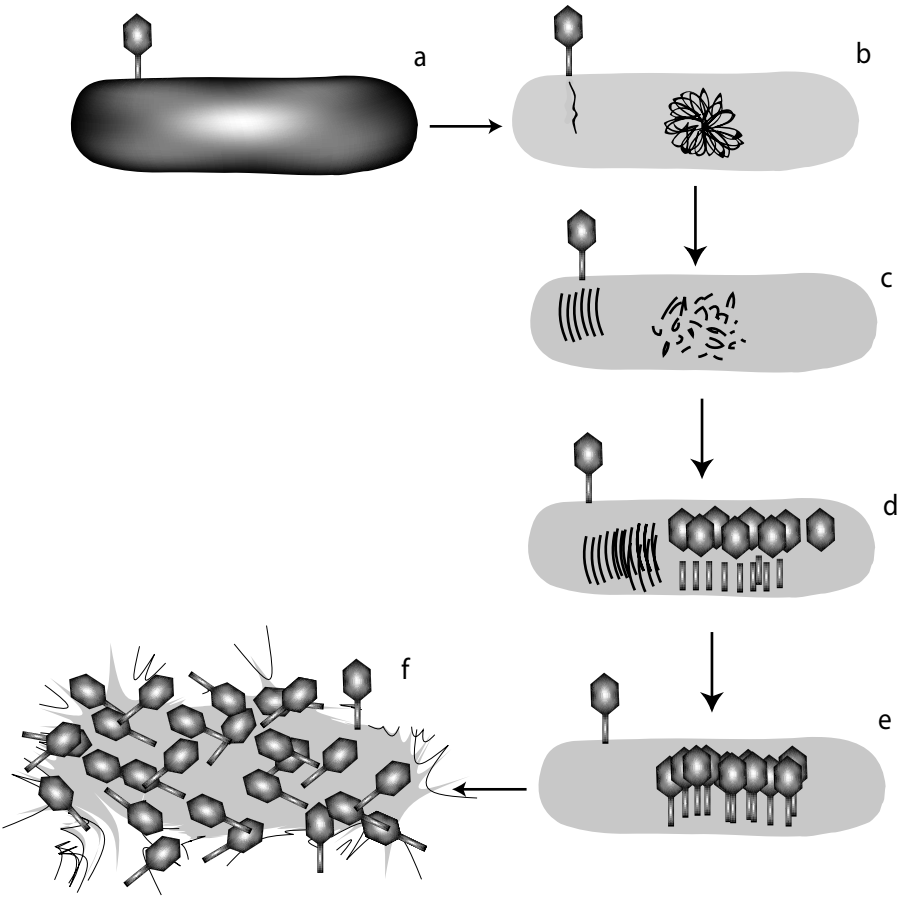

Figure 1.14 Life cycle of a bacteriophage, a virus that attacks bacteria. (a) Initial contact with host cell occurs when tip of virus tail adsorbs to bacterial cell. (b) Virus injects its DNA genome into host. Enzyme coded by virus DNA destroys host DNA. (c) Virus DNA replication begins. (d) Virus components accumulate in host cell. (e) Assembly of virus components results in hundreds of mature virus particles. (f) Within 20 minutes of initial infection, virus-coded enzyme destroys host cell, releasing viruses into medium.

In some virus infections, one may encounter a latent infection in which few if any virions are actually manufactured initially. Instead, the nucleic acid of the virus becomes integrated into the DNA of the host cell through a special kind of recombination. Herpes virus is an example of a virus that can initiate a latent infection. This virus can remain dormant in host cells for years, only to emerge without warning to initiate active disease with the resulting painful blisters and other typical symptoms. The mech-

anism that induces the dormant state of latent infections to become full-blown disease is essentially unknown in most cases.

2. Retroviruses

Viruses containing RNA may also become part of the genome of host cells. Since RNA cannot become integrated into DNA, a DNA copy of the viral RNA is made and that DNA then becomes spliced into the host DNA. Such viruses are known as retroviruses, meaning they do things backwards (*retro*, Latin for back). That is, while nearly all organisms make RNA from a DNA template, retroviruses do the reverse, making DNA from RNA. HIV, the AIDS virus, is one example of a retrovirus.

3. Oncoviruses

A special group of viruses is known to cause various forms of cancer in animals, including humans. These are known as oncoviruses. Oncoviruses can be either DNA or RNA viruses; the RNA viruses are retroviruses. A common feature of oncoviruses is their ability to establish latent infections similar to that described for herpes virus. It is believed that some oncoviruses carry oncogenes, defective mammalian genes that, when inserted into the genome of a normal cell, will trigger that cell to become a cancerous cell. Other oncoviruses appear to convert their host cells into tumor cells when the viruses or their protein products interact with their host's genome.

4. Viruses and Gene Therapy

Gene therapy involves replacing or augmenting faulty genes. One factor that has made gene therapy possible is the ability of certain viruses to become integrated into the DNA of their host cells. For example, a young boy may lack a critical protein in his immune system because a gene responsible for making the protein is not functioning, creating a life-threatening condition. A working gene can be spliced into the genome of a relatively benign virus that is known to integrate itself into host DNA. Cells taken from the boy are infected with the virus and the virus along with the gene becomes part of the host cells' genomes. The cells are then returned to the boy's body. The newly installed gene begins to function, making active protein and reversing the effects of the boy's nonperforming gene. Some limited success has been achieved in humans in this manner in treating malfunctions of the immune system. While gene therapy is not without risks, in time it may be the answer to the treatment of hundreds of genetic diseases.

Since human viruses are dependent on host cells of animal origin for their reproduction, and since such cells are unlikely contaminants in most cleanrooms, viruses are equally unlikely to be a contamination problem. However, with increased interest in products derived from human or animal cells such as active proteins, vaccines, and products produced by genetic engineering, together with the operation of tissue, organ, and bone banks, contamination controls aimed at viruses will become more common.

F. Subviral Particles

There are several types of small infectious particles that inhabit cells that almost defy definitions. Consisting of bare molecules of nucleic acid or protein, they are generally known as subviral particles. Probably the only reason they were discovered at all is due to the variety of sometimes-fatal diseases in plants and animals, including humans, that they cause. A few of these agents are described below.

1. Viroids

Approximately 15 plant diseases are caused by unique forms of infectious particles called viroids. Viroids are small, circular RNA molecules that lack the protein coat of ordinary viruses. They depend entirely on their host cells for their replication. Once established in a host cell, viroids cause the disruption of certain physiological pathways, which eventually leads to signs of disease in the host. They apparently are carried from plant to plant through grafting or through pollination.

2. Satellite RNA

Satellite RNAs are also small RNA molecules, but unlike viroids they require the presence of a helper virus to be present in the host cell for replication. The presence of satellite RNA sometimes increases the pathogenicity of the helper virus, and at other times satellite RNAs appear to lessen the impact of a virus on its host.

3. Prions

Prions are renegade infectious proteins that produce a number of invariably fatal diseases in animals. The diseases are known generally as spongiform encephalopathies, which translated means the brain is turned into a sponge. Bovine spongiform encephalopathy (BSE or mad cow disease), scrapie in sheep, and kuru and Creutzfeldt–Jakob disease in humans are a few examples of the diseases these mysterious molecules

cause. Prions appear to be transmitted primarily when animals ingest tissue, mostly from the central nervous system, that contains the prions. They also appear to originate from mutations in the animal's genome or they can be transmitted through contaminated surgical instruments or infected transplant tissue. Once they enter cells of the nervous system such as the brain, the prions cause an alteration of the structures of similar proteins. As these abnormal proteins accumulate, they induce pathogenic changes that lead to destruction of brain function and eventual death. Prions are exceptionally resistant to many common chemical sterilants and heat. While prions may be transmitted through the consumption of infected food, they do not appear to be destroyed when exposed to typical cooking temperatures. Prions can be destroyed by autoclaving at 135°C for 4.5 h or by treatment with a 4% solution of sodium hydroxide.

Like viruses, subviral particles normally are associated with animal or plant tissue and generally would be of interest only in those cleanroom activities that deal with such materials.

VI. TAXONOMY OF MICROORGANISMS

Historically, nomenclature has been related to taxonomy, the way organisms are related to one another. As discussed earlier, a genus is a group of related species, and a family is a group of related genera. The genera to which bighorn sheep, American bison, domestic cattle, Indian water buffalo, and the musk ox belong are all distantly related and are thus placed in the same family. The next higher taxonomic levels above family are order, class, phylum, and, finally, kingdom. Implied in the classification scheme is the idea that organisms placed within a given taxonomic grouping evolved from a common ancestor and are therefore related. More recent developments in DNA sequencing have shed a completely new light on tracing the origins of organisms and their relatedness with one another. This approach is based on the assumption that the more closely organisms are related to one another, the more their DNA will show similar or identical sequences. A number of bacteria have recently had their niche in the taxonomy of bacteria questioned when DNA analysis revealed that their classification, based almost entirely on traditional biochemical characteristics, may be in error. In some instances the mislabeled organisms have required a name change.

The present biological taxonomic system consists of five kingdoms, three of which include the cellular microorganisms. The five kingdoms are Animalia, Plantae, Monera (containing the bacteria), Protista (containing the algae and the protozoa), and Fungi (containing the yeasts and molds). Since viruses are noncellular, they are not included in the biological classification system. It has been proposed that the kingdom Monera

be split into two domains, Eubacteria ("true bacteria") and Aarchaea ("ancient ones"). Members of the domain known as Archaea have characteristics of both bacteria and animal cells and appear to represent a very early branching in the evolution of life. Because they appear superficially like bacteria, the Archaea were previously called Archaebacteria, but since biochemically they do not seem to be any more closely related to bacteria than they are to typical cells of more complex eucaryotic forms, the name was changed. Most Archaea so far discovered are found in extremely harsh habitats, such as high temperatures, high salt concentrations, and extremes in acidity or alkalinity, all conditions that are supposed to have been present in the very early periods of the Earth's development. Thus, members of the Archaea are thought to be more closely related to the first life on Earth.

Taxonomic classifications are based for the most part on physical characteristics, and for nearly all higher plants and animals, with their thousands of characteristics, this approach has generally been satisfactory. When it comes to the taxonomy of microorganisms, particularly bacteria, there are relatively few observable characteristics. This has provided microbiologists with a less than satisfactory taxonomic system. It is anticipated that with increasing DNA sequence data, unprecedented progress will be made in bacterial taxonomy.

VII. MICROSCOPY

Under normal conditions, the unaided human eye is probably not capable of seeing objects smaller than about 100 μm. Thus, most individual microbial cells are invisible to us without the aid of some type of optical device. Such a device, invented some time in the 17th century, is the microscope. The familiar magnifying glass is referred to as a simple microscope, consisting of a single lens, whereas a compound microscope consists of two or more lenses. The compound microscope is the type most often used in microbiology laboratories.

Magnification is defined as the degree to which an optical system can increase the size of an object's image. While ordinary laboratory microscopes theoretically could be designed to enlarge images millions of times, the practical limit is only around 1000 to 2000×. This is because of another limiting characteristic of optical devices: resolution. Resolution is defined as the ability of an optical device to separate two very closely spaced objects; that is, to render fine detail.

While resolution of a microscope depends heavily on the quality of its optical components, the wavelength of the light used to illuminate the object under examination also plays a major role. This is due to a fundamental law of physics that one cannot see an object that is equal to

or smaller than the wavelength of the light that is illuminating it. Common laboratory microscopes use visible light as a source of illumination, the wavelengths of which are only slightly smaller than bacterial cells.

A. Light Microscopy

Since common microscopes used in a microbiology laboratory use visible light, they are referred to as light microscopes (Figure 1.15). As mentioned above, the wavelengths of visible light, that is, light that the human eye is sensitive toward, range from about 0.4 micrometers (μm, 400 nanometers [nm], deep violet) to 0.7 μm (700 nm, deep red). Most bacteria measure around 1–2 μm or 1000–2000 nm, and their fine structural features such as cell walls, flagella, ribosomes, and so on, are considerably smaller. Thus, because the dimensions of bacterial cells are so close to the wavelengths of visible light, the cells are on the edge of the resolving power of the light microscope, and their fine structure is for the most part beyond its resolving power. Larger microorganisms such as fungi and protozoa are somewhat more clearly seen in the light microscope, but, again, most of their cell components are not easily observable. As pointed out earlier, because they are even smaller, most viruses are completely invisible in the light microscope.

1. Staining

Making matters worse, because microbial cells are composed of over 60% water, they appear transparent and thus are barely visible in the light microscope. Considerable improvement in the visibility (but not resolution) of cells in the light microscope can be achieved by staining, which is the application of dyes to impart color to the cells. Staining has the added advantage of giving one the ability to distinguish taxonomic groups of cells, such as with the previously described Gram stain in the case of bacteria. By providing contrast, staining also improves the visibility of larger structural features such as nucleoids, endospores, and flagella. For example, in the case of flagella, which are far too narrow to be visible in the light microscope, special procedures can make them visible. Multiple layers of dye are applied to the flagella until they become thick enough to be seen in the microscope.

To stain bacteria, a smear is first made by applying a film of cells upon a glass microscope slide. The cells are then fixed by applying gentle heat to the slide, causing the bacteria to stick to the glass surface. The final step is staining, the application of one or more dyes to the smear. If a single dye solution is applied, the process is referred to as a simple stain. Differential stains consist of two or more dye solutions applied one after

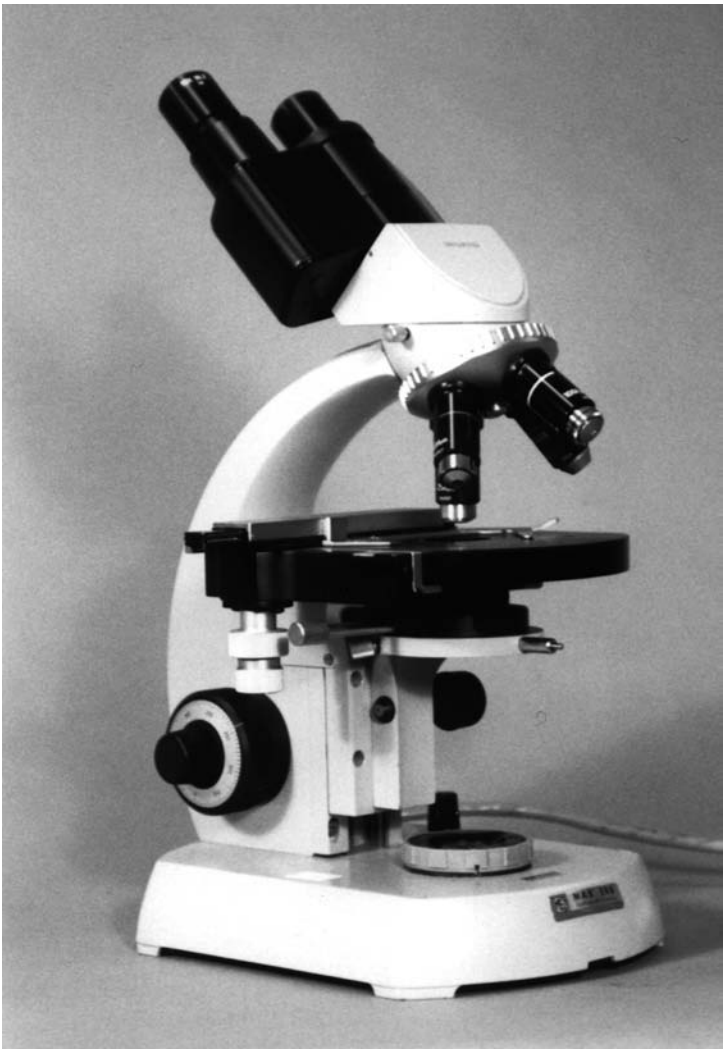

Figure 1.15 A light microscope, capable of enlarging images up to 1000 to 1500×.

another, with various washes and other chemical treatments in between the staining steps. The Gram stain is a differential stain. Another technique that uses fluorescent dyes is described in Chapter 2. Table 1.3 lists the most common differential stains used in microbiology. One of the major disadvantages of staining microorganisms is that the conditions of the fixing and staining steps tend to cause distortions and artifacts in the cells, often creating difficulties in determining true size and shape.

Table 1.3 Some common differential stains

Stain Technique	*Applications*
Gram	Divides most bacteria into two groups: Gram positive and Gram negative
Acid fast	Helps identify members of the genus *Mycobacterium*
Spore	Causes bacterial endospores to stand out in contrasting color
Nuclear	Allows visualization of nuclear region
Flagella	Coats flagella with thick layer of dye so they are visible in light microscope

2. Types of Light Microscopes

Many design improvements in light microscopes have helped to increase the visibility of whole microorganisms and some of their internal structures. This is especially useful when one needs to observe live organisms. One improvement is phase contrast microscopy, which takes advantage of the differences in index of refraction between the cytoplasm, its internal structures, and the surrounding medium. Index of refraction is a measure of how a substance causes a beam of light to bend and is the basis for the sparkle in diamonds. Figure 1.12 of an amoeba was made by phase contrast microscopy. The cells and some of their internal structures such as nuclei and food vacuoles are visible because they bend the light at angles different from the cytoplasm and the surrounding medium. Another advance is dark-field microscopy, which is based on the ability of microbial cells to scatter light (see Chapter 3). Here, cells are illuminated against a dark background in a manner similar to how dust particles appear in a strong light beam in an otherwise unlighted room.

In examining thick specimens such as biofilms or muscle tissue with a conventional light microscope, the operator focuses on a particular plane of the specimen. Because the entire specimen is fully illuminated, layers of the specimen above and below the plane of focus are out of focus but still visible. The resulting image is cluttered with blurs of irrelevant particles that may obscure the area of interest. It is like trying to observe an animal through a stand of trees. In confocal microscopy, only the plane that is of interest is fully illuminated; the other levels receive less light. This renders the out-of-focus levels less visible and less likely to interfere with receiving a clear image of the area of interest. Lasers are usually the sources of illumination in confocal microscopes.

Figure 1.16 A scanning electron microscope, capable of enlarging images by over 100,000×. It also can chemically analyze specimens as they are being observed.

B. Electron Microscopy

Resolution can be improved enormously by illuminating objects with a beam of electrons rather than with ordinary light. That is what is achieved with the electron microscope (Figure 1.16). However, a beam of electrons, which possesses a wavelength considerably shorter than ordinary light, has certain characteristics that make electron microscopy technically much more difficult to carry out than light microscopy.

Because of collisions with air molecules, a beam of electrons cannot pass through air without severely reducing the intensity of the beam. Consequently, the path of the electrons and the specimen under observation must be in a high vacuum, requiring the specimen to be dried, a procedure that can lead to shrinkage and distortion of its fine structure. If electrons cannot penetrate air to any extent, then it follows that they cannot penetrate whole cells either. For transmission electron microscopy, specimens must be prepared in thin sections or slices 1 μm or smaller to allow passage of the electron beam. To observe whole bacterial cells in an electron microscope, the cells must be cut into thin sections. Since there is no way one can hold an individual bacterial cell while cutting it into several slices, the cell must be embedded in a plastic material, which

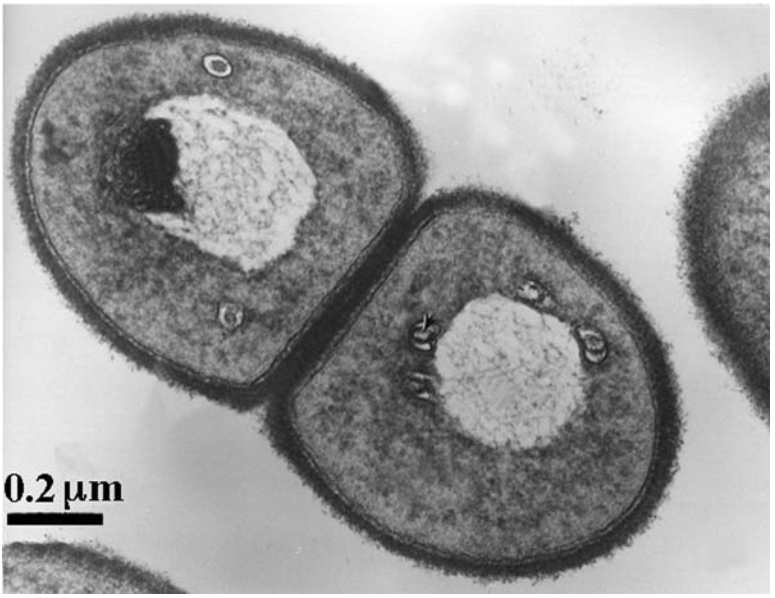

Figure 1.17 Transmission electron micrograph of a staphylococcus cell that has just divided into two daughter cells. Thin sections of the cells were stained with uranium to increase contrast. Dark outer layer is the thick cell wall typical of Gram-positive bacteria. The lighter staining cell membrane also can be seen. The large, lighter area within the cytoplasm is the nucleoid. (Micrograph courtesy Captain Max Wu, U.S. Army.)

is then subjected to sectioning by a glass or diamond knife on an apparatus called a microtome.

To improve the contrast of the image produced in an electron microscope, various staining procedures involving heavy metals like uranium are frequently applied to the specimens (Figure 1.13 and Figure 1.17). In another technique called shadowing (Figure 1.8), a three-dimensional effect is produced. Shadowing involves spray-coating the specimen with a thin layer of vaporized metal such as platinum. The coating is applied at a low angle, leaving shadow areas around objects uncoated. When the resulting image is printed in reverse, uncoated areas appear as black shadows, producing a three-dimensional effect.

Although somewhat lacking the high resolution of transmission electron microscopy, scanning electron microscopy gives one a three-dimensional impression of objects (Figure 1.7 and Figure 1.11). In this instrument, as the electron beam scans the object it causes a shower of secondary radiation to be released from its surface. A detector collects the secondary

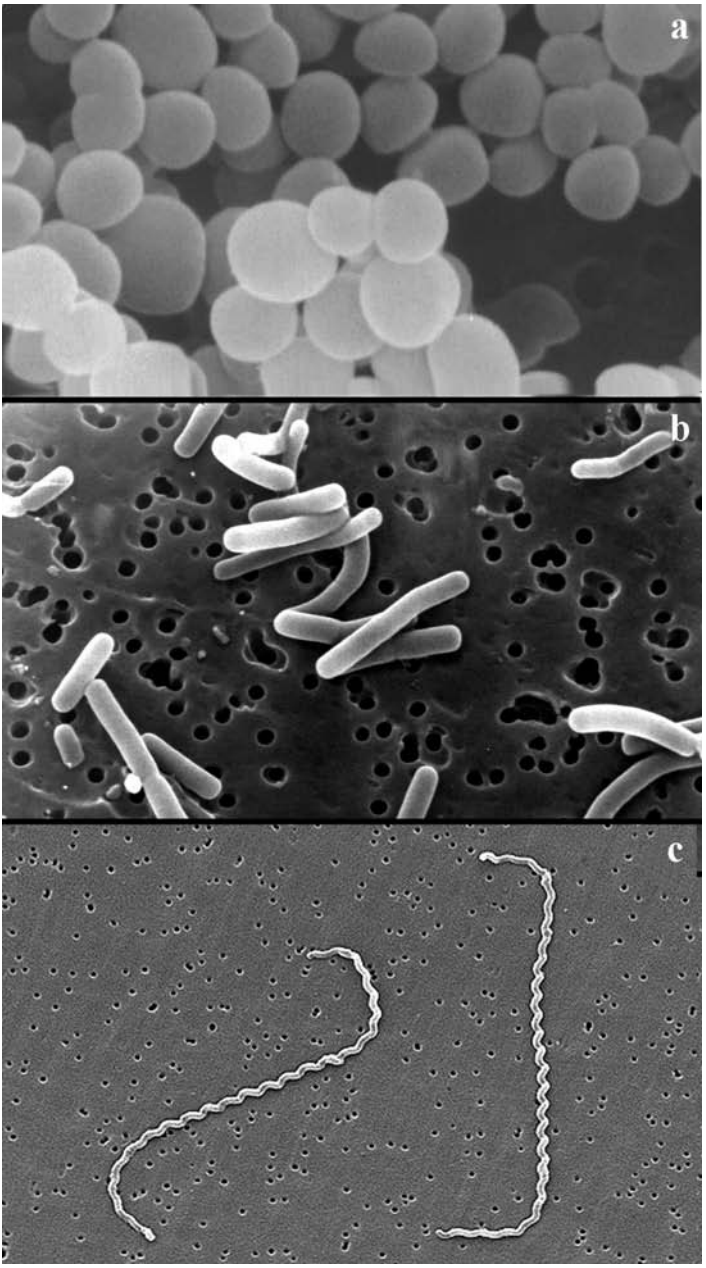

Figure 1.18 Scanning electron micrographs of the three basic shapes of bacteria. (a) Coccus, (b) bacillus, (c) spirillum. (Micrographs courtesy Captain Max Wu, U.S. Army.)

radiation and converts it into an image that can be observed on a monitor, photographed, or stored in digital form for later analysis.

A more recent development, the tunneling electron microscope scans the surface of an object with a super-sharp probe and traces the object's contours with incredible precision. The movement of the probe is then converted into an image on a monitor. Shapes of objects as small as DNA molecules have been visualized with this instrument.

Electron microscopes are useful in determining sources of particulate cleanroom contamination. For example, traces of tobacco smoke, cosmetics, or pollen can easily be identified by their characteristic appearance. As a further aid in the identification of sources of contamination, instruments such as the one depicted in Figure 1.16 are able to carry out a chemical analysis of an object while it is being observed. Electron microscopes are expensive pieces of equipment, requiring highly trained operators and significant maintenance costs. It is not unusual to spend over $100,000 for such a microscope, but the volume of information that can be gained from these instruments is enormous.

VIII. SUMMARY

Microbiology is the study of the smallest of living cells, the microorganisms. These include bacteria, yeasts, molds, algae, and protozoa. Viruses, while not living cells, are nevertheless included in the study of microorganisms. Because of their small size, microorganisms cannot be seen with the unaided eye, but must be observed with a microscope, either the type that uses visible light or one that uses an electron beam as its source of illumination.

Most microorganisms are either beneficial to other forms of life on Earth, including humans, or are neutral, but a few are known to be the cause of food spoilage, deterioration of materials, and infectious diseases. Microorganisms can create problems in cleanrooms through a variety of activities. Bacteria and molds are the most common microorganisms associated with contamination in cleanrooms. Because they can conduct an electrical current, their presence in electronic circuitry could cause a short circuit and lead to a malfunction. If microorganisms or their products gain entrance into a pharmaceutical product or medical device, they could alter characteristics of the product or produce illness or death.

2

GROWTH OF MICROORGANISMS

I. INTRODUCTION

The word "growth" means different things to different people. To most, growth implies increase in size, as in the growth of a tree or a child. To a microbiologist, growth may also mean the increase in the size of a microbial cell, but more often it refers to an increase in the numbers of cells in a microbial population. In this chapter and for the remainder of this book, growth will usually refer to the latter meaning: an increase in the numbers of organisms in a microbial population.

Most of our knowledge of microorganisms has been gained by observing their activities while growing them in the laboratory, which microbiologists learned to do only a little over 120 years ago. Growing microorganisms in the laboratory is a necessary and important activity for the cleanroom microbiologist. Raw materials, process water, finished products, and the air and surfaces of the manufacturing facility must be sampled for the possible presence of excessive numbers of microorganisms. The presence of microorganisms in materials and environmental samples is almost always detected by observing their growth through certain laboratory procedures. In this chapter we will describe the most common techniques used by microbiologists for growing microorganisms as well as some of the methods for monitoring their growth. Then, in Chapter 5 we will describe how these techniques are used to determine levels of microbial contamination in the cleanroom.

A. Scientific Notation

Because of their rapid growth rate, microbial populations frequently reach enormous levels. It is not unusual, for example, to have 1 billion bacterial cells in a milliliter of growth fluid. Dealing with such large numbers can be difficult without a useful tool known as scientific notation, which uses exponents to denote zeros or decimal places. That is, we write the number 1,000,000,000 as 1×10^9 (one times ten to the ninth power), 7200 as 7.2×10^3, and 301 as 3.01×10^2, and so on. Scientific notation will be used throughout this book to express large numbers. Incidentally, scientific notation can also be used to express very small numbers. For instance, 1/1,000,000 (0.000001) can be written in scientific notation with a negative exponent: 1×10^{-6}.

II. GROWTH OF BACTERIA

A. Nutritional Requirements

In order to grow bacteria in the laboratory, a medium (plural "media") must be prepared. A proper bacteriological medium contains all the nutrients necessary to sustain the growth of a population of bacteria. A microbial population growing in a medium is frequently referred to as a culture. Like all other living organisms, bacteria need the basic elements carbon, nitrogen, phosphorus, sulfur, and other inorganic elements, plus a source of energy. The range of nutritional requirements among the many species of bacteria encountered in nature is unbelievably broad. Some species can grow in a medium that contains nothing more than a few mineral salts, like ammonium phosphate and magnesium sulfate, plus glucose as a carbon and energy source. Other species go a step further in nutritional independence in being able to obtain their carbon from carbon dioxide (CO_2). From these simple media the bacteria can synthesize all of their complex nutritional needs such as amino acids, vitamins, purines, and pyrimidines that are used to assemble living cells. At the other end of the nutrition spectrum there are species that require dozens of preformed nutrients in order to grow. The kinds of bacteria most commonly found in the cleanroom can synthesize some of their complex requirements but must have other organic nutrients supplied to them in the medium.

Hundreds of media recipes are available to microbiologists and no one medium will support even a fraction of all known bacterial species, not to mention the possible thousands of unknown species that are yet to be identified. However, since the great majority of bacteria encountered as contaminants in cleanrooms generally have common nutritional needs, two or three media are usually sufficient to grow those bacteria that would

be of interest to the cleanroom microbiologist. The one bacteriological medium most often recommended is one containing enzymatic digests of casein (a milk protein) and soybean protein, known as soybean casein digest or tryptic soy medium (TSA). This medium will support the growth of many bacteria that originate from soil contamination, as well as many that reside on the human body. Bacteria found growing in purified water systems require special media. These and other media will be described in more detail in Chapter 5.

Agar, a polysaccharide derived from certain types of seaweed, is frequently added to microbiological media as a solidifying agent. Concentrations of one or two percent are usually sufficient to provide a firm gel-like consistency to the medium. Microbiologists also use the word "agar" to refer to a medium that contains the solidifying agent plus the nutrient components. The purpose of growing bacteria on agar-containing media is explained below.

Before a medium can be used it must be sterilized; that is, it must be rid of all living organisms. The reason for that is clear: one does not want contaminants (unwanted organisms) growing in the medium when trying to grow a specific organism. Methods of sterilization are covered in Chapter 3. Once sterilized, an agar-containing medium is melted at 100°C, allowed to cool until it is just about to solidify (around 45°C) and then poured into sterile Petri dishes (Figure 2.1) or culture tubes. The agar will solidify when it cools to about 40°C and will become a semirigid medium. When bacteria are inoculated (deposited) on the agar surface and if the nutritional and environmental conditions are appropriate and the bacteria are culturable, they will grow. When growth occurs, visible accumulations of bacterial cells will eventually form. These are called colonies (Figure 2.2). Each colony may start out as a single cell but it eventually may contain 10^8 or more cells. Due to the very rapid growth rate of typical bacterial species, this population can easily be reached in less than 16 h. As explained later in this chapter, the ability to grow bacteria in colonies often aids one in identifying species and it is also a tool in determining bacterial populations.

If agar is not included in the recipe, a liquid medium known as broth is achieved. Colony formation is not possible in broth; instead, as the population increases, a uniformly dispersed suspension of cells usually results. Once growth reaches a certain level (usually around 10^7 to 10^8 cells per milliliter [ml]), the growth is noted as a distinct cloudiness, or turbidity. The degree of turbidity can be used to estimate the number of cells in the culture. This procedure will be covered in greater detail later in this chapter.

In order to obtain large numbers of cells in laboratory cultures in a relatively short time, most commonly used bacteriological growth media

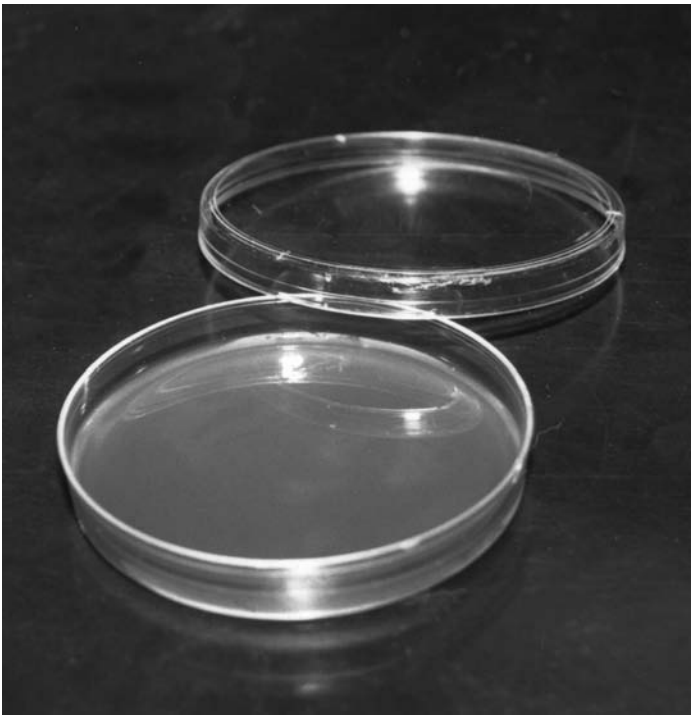

Figure 2.1 Petri dish containing agar medium for cultivating bacteria.

are intentionally prepared relatively rich in nutrients. For example, organic carbon in these media may exceed 2000 mg/l. Such concentrations of nutrients seldom occur in nature. For instance, natural waters in unpolluted lakes and rivers may contain around 10 to 20 mg/l of organic carbon. Bacteria are capable of growing in water with even less available carbon. It is not unusual to detect levels of organic carbon in distilled water as low as 0.5 mg/l, a concentration quite sufficient to support a moderate amount of bacterial growth.

Bacteriological media are often classified into three major categories. A nonselective medium, such as TSA, will support the growth of a wide range of species. A selective medium, on the other hand, contains ingredients that allow the growth of certain types of bacteria but prevent the growth of other types. For example, there are media that are used to test for the presence of fecal contamination in drinking and recreational water. These media permit the growth of coliform bacteria, common inhabitants of the intestine, but block the growth of noncoliform types. Differential media do just that — differentiate. They contain ingredients that react when specific types of bacteria grow in or on them. They may turn color,

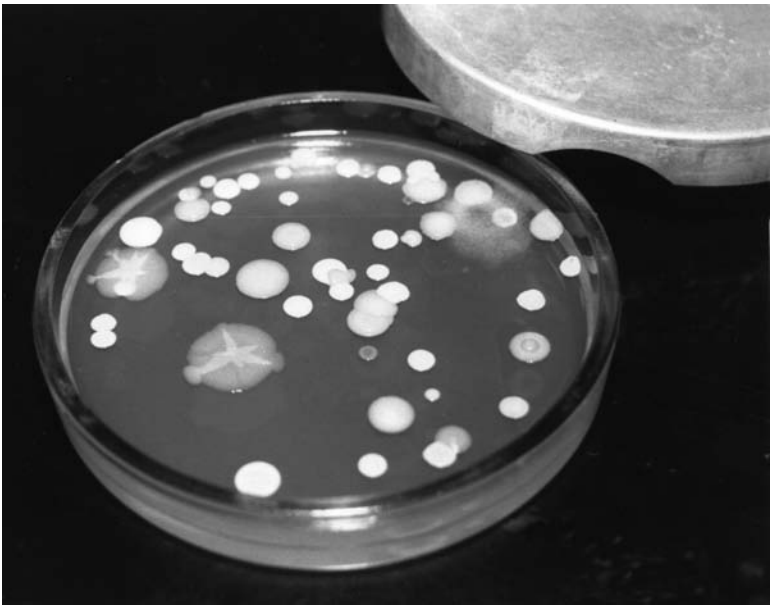

Figure 2.2 Bacterial colonies formed on surface of an agar medium from an air sample. Note variety of shapes and sizes of the colonies due to different species collected.

for example, when members of a colony utilize a certain nutrient or produce a certain metabolite. Such media are enormously useful when attempting to identify bacteria that have been isolated from clinical or environmental samples. Additional discussion of microbiological media will be found in Chapter 5.

In addition to supplying the nutritional needs of the microorganisms we wish to grow, certain environmental conditions also must be met. These include temperature, atmosphere, and pH.

B. Environmental Requirements

1. Temperature

If one were to determine the temperatures at which growth is optimum for a variety of bacterial species, one would discover that the organisms exhibit a wide range of temperature requirements (Figure 2.3). Microorganisms are usually divided into three or four groups according to their optimum (best) growth temperatures: Psychrophiles grow best below 20°C, some doing quite well below 5°C, which is the temperature of most refrigerator interiors. The optimal temperature for mesophile growth is 20

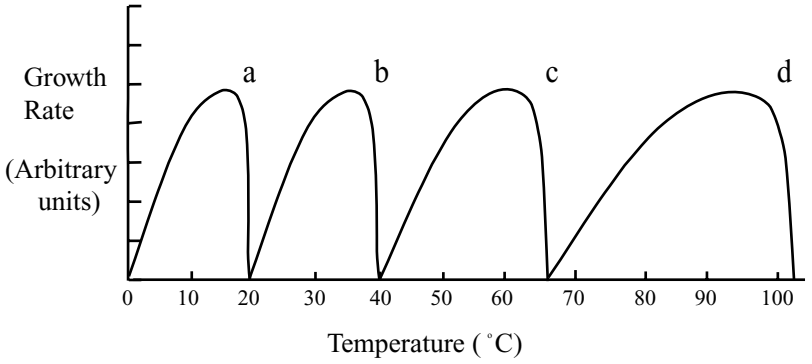

Figure 2.3 Growth responses at various temperatures for four groups of bacteria. (a) Psychrophiles, (b) mesophiles, (c) thermophiles, (d) extreme thermophiles.

to 40°C. A subgroup of mesophiles that can grow at temperatures as low as 5°C or so are known as psychrotrophs. Thermophiles prefer temperatures over 40°C. One extraordinary group of bacteria is able to grow at temperatures well over 100°C, which is hotter than boiling water. These are known as extreme thermophiles and frequently are found around volcanic vents deep in the ocean. To ensure constant growth temperatures in the laboratory, cultures are placed in an incubator (Figure 2.4), an oven-like device with a thermostatically controlled interior. Most cleanroom contaminants are mesophiles and hence are usually grown at temperatures from 30 to 35°C. An exception might occur when assessing thermophilic microbes that may inhabit water systems maintained at high temperatures to reduce bacterial growth. Thermophiles are usually incubated at 55°C.

2. Atmosphere

It was the great French chemist Louis Pasteur who first realized, while studying the microbiology of winemaking, that life was possible in the absence of gaseous oxygen. He showed that the wine yeasts were able to grow in laboratory cultures in the presence of oxygen, as well as in wine vats where there was little or no air. Organisms that can grow in the absence of free oxygen are called anaerobes. Those organisms that can grow either in the presence or absence of free oxygen (such as the wine yeasts) are referred to as facultative anaerobes, while those that must grow in the complete absence of free oxygen are called strict or obligate anaerobes. The latter types of microorganisms actually find oxygen lethal if exposed to it for an extended period of time, because they produce toxic metabolic products such as hydrogen peroxide, an

Figure 2.4 A microbiological incubator. Thermostatically controlled chamber can be maintained at a constant temperature appropriate for the species to be grown.

effective germicide, in the presence of oxygen. Anaerobes lack the enzymes that aerobic organisms have that break down the toxic substances into harmless by-products.

There are a few obligate anaerobic bacteria associated with soil and the human body that may occur in cleanroom contamination, some of which are of great medical significance. However, the vast majority of bacteria encountered in cleanrooms are aerobic, that is, requiring oxygen and thus able to grow in a normal laboratory atmosphere. This is not to say that anaerobic contaminants should be ignored; quite the contrary. For example, although relatively rare, anaerobic bacteria may be encountered in certain types of raw materials. One may also encounter microaerophiles, bacteria that grow best in atmospheres with oxygen concentrations somewhat lower than that of ordinary air, and frequently requiring higher levels of carbon dioxide.

When it is necessary to grow microaerophiles or anaerobes, various devices are available to protect these organisms from oxygen, including anaerobic jars, isolators, and incubators (Figure 2.5). Special types of media

can also be used, such as thioglycolate broth, which contains reducing agents that establish anaerobic conditions (i.e., a low oxidation–reduction potential) within a culture tube without the need for additional equipment beyond an ordinary incubator. A convenient system for culturing anaerobic bacteria involves the use of a foil pouch that contains chemicals that generate hydrogen and carbon dioxide when water is added. The pouch is placed in an airtight vessel known as an anaerobic jar (Figure 2.5). With the aid of a palladium catalyst in the jar, the hydrogen that is generated combines with residual oxygen to form water. As the oxygen in the jar is used up, anaerobic conditions are soon established. This is confirmed by observing a redox indicator strip within the jar that turns colorless in the absence of oxygen. In another ingenious system, a membrane impregnated with a culture of facultative anaerobes is sealed in the culture that has been inoculated with strict anaerobes. The facultative anaerobes use up the oxygen, allowing the strict anaerobes to grow.

Figure 2.5 Anaerobic chamber for handling strict anaerobic bacteria. All manipulations, including inoculations and incubation, are carried out in a oxygen-free atmosphere. Insert: An anaerobic jar.

Table 2.1 The pH of some familiar liquids

Liquid	*pH*
1 N Hydrochloric acid	0.1
Human gastric juice	1.0–3.0
Lime juice	1.8–2.0
Vinegar	2.4–3.4
Wine	2.8–3.8
Human urine	5.0–8.0
Human saliva	6.5–7.5
Pure water	7.0
Human blood	7.3–7.5
Fresh egg	7.6–8.0
0.1 N Sodium bicarbonate	8.4
Drain cleaner	14

3. pH

pH is a chemical term that refers to the acidity or alkalinity of a liquid and covers a scale from 0 to 14, 0 being very acid and 14 being very alkaline. A pH of 7.0 is considered neutral. Table 2.1 lists the pH of several familiar liquids. Most cleanroom contaminants grow best around neutrality — 6.5 to 7.5. Some rare species of bacteria grow optimally at pHs as low as 2.0 and can tolerate a pH as low as 0.5. At the alkaline end of the scale, some bacteria are known to require a pH of 9.5 for optimum growth, but can reproduce even when the pH is over 11. Those bacteria that require extremes in pH (and temperature for that matter) are usually found in outdoor habitats, such as hot springs, oil wells, mines, industrial waste ponds, and alkaline or saline lakes. They are sometimes referred to as extremophiles. They are unlikely to be cleanroom contaminants except where these rigorous conditions exist, such as in hot water systems or in and around brine or pickling tanks. Never assume that an environment is too harsh for microorganisms.

When a medium is prepared in the laboratory, the pH should always be tested to assure that it is within the optimum range for the desired organisms and adjusted with acid or base when necessary. During the growth of the bacteria, the pH probably will not remain constant, but will shift as a result of the organism's metabolism. It is not unusual for a culture to produce so much acid as a waste product that the pH soon shifts below the organism's pH range and growth stops. To avoid this problem, one can either periodically check the pH of the medium and

adjust it with sterile acid or alkaline or incorporate a buffer in the medium. A buffer is a substance that can counteract shifts in pH.

Checking the pH on a continuous basis is usually not practical in ordinary laboratory cultures, although apparatuses called fermenters, for growing moderately large volumes of microorganisms, have the capability of automatically monitoring and adjusting the pH. It is also common in large industrial microbial processes to continually check the pH. The addition of buffers to the medium is a convenient and inexpensive means of preventing pH shifts in small laboratory cultures, but it is probably not practical on large industrial scales. Many constituents of common bacteriological media, such as peptones (partially digested proteins), are natural buffers and are frequently adequate for most purposes. If a medium does not contain these natural buffers, or if greater buffering capacity is required, a number of chemical buffers can be used, but they must be chosen carefully; some buffers are toxic to microorganisms or metabolized by them and are not intended to be used in growth media.

C. Binary Fission and the Bacterial Growth Curve

1. Microbe Math

Bacteria grow, that is, increase their numbers, by binary fission, which means that one cell divides into two, which in turn becomes four, then eight, sixteen, and so forth (Figure 2.6). The growth of a bacterial population can thus be expressed mathematically in terms of powers of 2:

$$\text{Final Population (P)} = 2^n \tag{2.1}$$

where n is the number of times the population doubles. When a population doubles, it is said to have gone through one generation. For example, 1 cell becomes 4, 32, 1024, and 1,048,576 cells after 2, 5, 10, and 20 generations, respectively. Since some bacterial species can experience as many as four or five generations per hour under ideal conditions, it is easy to see how rapidly a bacterial population can increase.

If we start with more than one cell (which is almost always the case), the equation becomes:

$$P = P_0 * 2^n \tag{2.2}$$

where P_0 is the starting population.

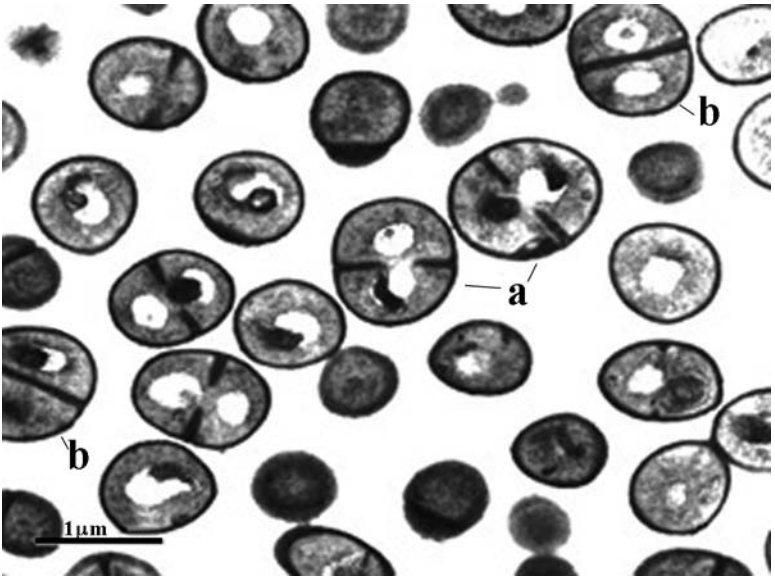

Figure 2.6 Bacteria undergoing binary fission. Thin sections of staphylococcal cells are seen in various stages of division. Some cells (a) are in early stages of cross-wall formation while others (b) appear to have completed cross-wall formation and are about to separate into two daughter cells. To improve contrast, the cells were stained with uranium. (Electron micrograph courtesy Captain Max Wu, U.S. Army.)

2. Characteristics of Bacterial Populations

Whether they are growing in a culture in the laboratory or taken from a natural aquatic or soil community or contaminated surface of a cleanroom, populations of microorganisms may consist of three subpopulations: culturable, viable but nonculturable, and nonviable. Culturable means the cells are capable of growing when placed in or on a suitable nutrient medium in an appropriate environment. Viable but nonculturable denotes cells that are still alive; that is, they are still active biochemically but are incapable of growing on any presumably suitable medium within a normal period of time. The exact causes of this condition are unknown but it has been suggested that the lack of response may be a survival mechanism that is triggered by exposure to adverse conditions. For example, incubating bacteria at low temperatures (7°C or less) for several days will cause them to become viable but nonculturable. However, in time the cells will regain the ability to form colonies when returned to their normal growth temperatures. Nonviable cells have no measurable biochemical

activity and fail to grow when placed on a suitable medium regardless of the incubation time — in a word, dead. The relative proportions of each of these subpopulations will vary depending on a variety of conditions. It is estimated, for example, that over 99% of bacteria collected from natural terrestrial or aquatic habitats are viable but nonculturable, while in a fresh bacterial culture in the laboratory, over 99% of the bacteria may be culturable.

There are frequent problems in the use of the terms culturable and viable but nonculturable. Many microbiologists use the term viable when they mean culturable. That is, when the organisms grow on media, the microbiologists say the organisms are viable when the organisms should be referred to as culturable. The technique long known as the viable plate count, described on page 55, is really a culturable plate count. Thus, if cells are viable, they may either be culturable or nonculturable depending on the conditions of recovery. This is more than an exercise in semantics but points to the importance of establishing standard and consistent sampling and culturing techniques for microbiological monitoring.

3. The Growth Curve

When we graph the culturable fraction of a growing population of bacteria against time, a curve such as the one shown in Figure 2.7a would result. Notice that because the population increases so rapidly, we soon run out of space on the vertical (population) axis. A more manageable curve is obtained (Figure 2.7b) by plotting the logarithm of the population versus time. Now one sees that the curve has become a straight line, which is to be expected since the increase in the culturable population follows the exponential term 2^n. Whenever a bacterial culture exhibits a linear growth curve as in Figure 2.7b, the culture is said to be in the logarithmic, exponential, or log phase of growth. Three other principal phases exhibited by bacterial cultures are lag, stationary, and death phases.

a. The Lag Phase

To initiate growth in a medium, a small number of viable cells (known as an inoculum) are transferred to it. When a medium is first inoculated, growth normally does not begin at once. This delay represents the lag phase, and its length depends in part on the nature of the medium from which the cells were transferred and the nature of the new medium. In spite of the lack of any visible growth, the cells are physiologically very active during the lag phase, shifting their metabolism to match the conditions of the new medium. When all necessary enzymes and cell structures have reached optimum levels, growth begins.

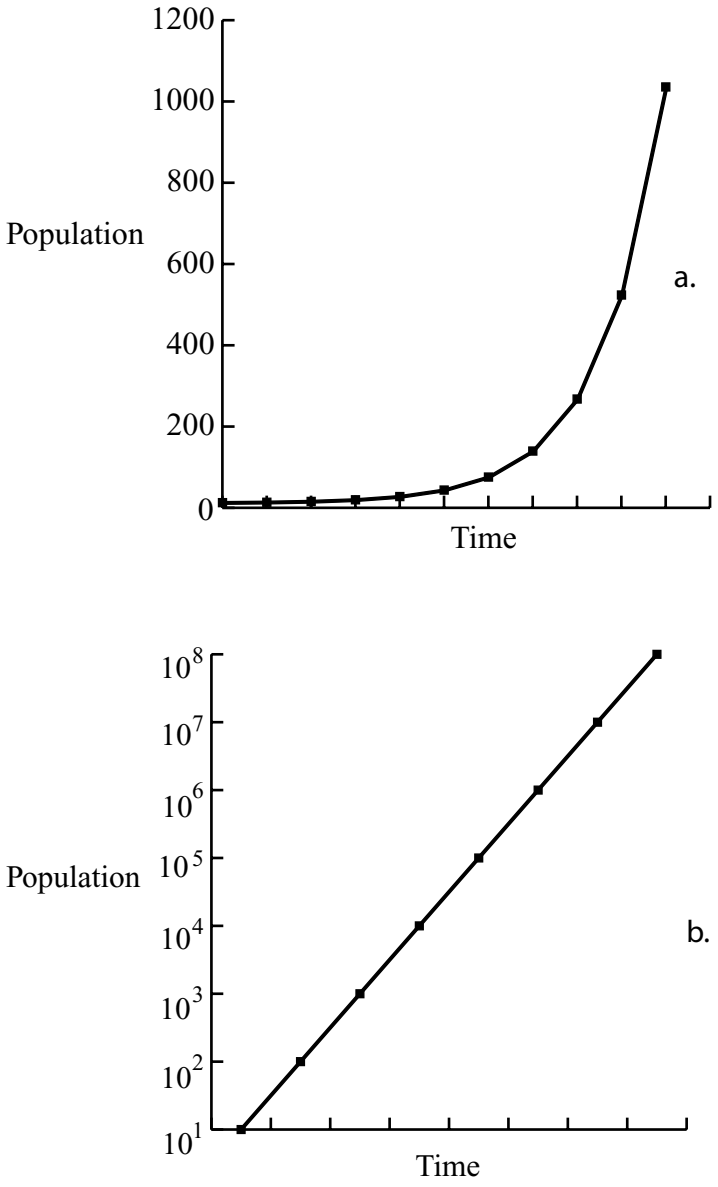

Figure 2.7 Bacterial growth curves. (a) When the population of bacteria growing in a broth culture is plotted on a linear scale versus time, the population increases so rapidly that the curve quickly runs off the chart. When plotting population on a logarithmic scale (b), the rapid population increase is easily accommodated on the chart.

b. The Log Phase

Soon most if not all the culturable cells in our new medium begin to divide at a fairly constant rate. Division is not synchronized, however, since the cells are dividing quite randomly throughout the culture. That is, cells are beginning division at different times, resulting in the smooth, straight line characteristic of the log phase. If division were synchronized (when every cell divides at exactly the same time), the growth curve would appear as stair steps but still following a straight line.

During the log phase, the cells achieve balanced growth, where every constituent or property of the cells, that is, protein, nucleic acid and cell wall concentrations, total mass, etc., is increasing at the same rate as every other constituent. That fact makes it possible to follow the growth of a bacterial culture by measuring any of the properties listed above. That aspect is covered in greater detail later in this chapter. The generation time, that is, the interval of time a given cell population needs to double in numbers, is also constant during the log phase. However, within a population of bacterial cells, generation times probably vary somewhat from cell to cell. The mean generation time of a culture can be calculated by rearranging Equation (2.2) and including t, the elapsed time between when P and P_0 were determined:

$$G = \frac{t}{n} = \frac{t}{3.3(\log P - \log P_0)} \tag{2.3}$$

where G is the generation time in minutes, P_0 is the starting population, and P is the population after t min (3.3 is 1/log 2). Typical generation times of some familiar bacteria are shown in Table 2.2. Generation times can span from 11 min for *Geobacillus stearothermophilus* to over 30 h for organisms such as *Treponema pallidum*. These generation times were determined

Table 2.2 Generation times of some bacteria

Species	*Generation Times (min)*
Geobacillus stearothermophilus	11
Escherichia coli	20
Streptococcus lactis	30
Lactobacillus acidophilus	75
Mycobacterium tuberculosis	360
Anabaena cylindrica	840
Treponema pallidum	2000

under optimal growth conditions. Generation times may vary depending on the medium and other growth conditions, such as temperature.

The log phase does not continue indefinitely. The depletion of necessary growth factors and the accumulation of toxic waste products eventually result in a relatively rapid leveling off of the growth curve. The culture is now entering the stationary phase.

c. The Stationary Phase

As the log phase progresses, the nature of the medium changes. Many essential nutrients become depleted and the pH has probably shifted out of the optimum range for the bacteria. The metabolism of the bacteria has become increasingly inhibited by toxic waste products, which include organic acids, alcohols, peroxides, and carbon dioxide. The growth curve soon becomes horizontal, a period known as the stationary phase. During the stationary phase, there is no apparent increase in the culturable population either because cell division has ceased entirely, or because equal numbers of cells are dividing and dying. Depending on the species of bacteria and the nature of the medium, the stationary phase may last from a few minutes to perhaps several hours.

d. The Death Phase

Eventually the culturable population of our bacterial culture begins to drop exponentially in a manner similar to the log phase, but probably with a different slope. The culture has entered the death or decline phase. Certain species exhibit a very steep death phase, so few if any culturable cells are recoverable after 48 h or so, while live cells of other species may be detected even after a year or more.

Combining all phases of a bacterial culture results in a typical growth curve as shown in Figure 2.8. The exact nature of each of the phases, that is, their duration and slope, is very dependent on the species of organism, the type of medium, and the culture conditions described above.

D. Continuous Growth

Even with strong buffering, the life of a bacterial culture is limited, as described above. For many bacteria the duration of the log phase is usually not more than a few hours. There is, however, an apparatus that makes it possible to maintain a bacterial culture in the log phase essentially indefinitely. The apparatus is called a chemostat, which consists of a growth vessel into which a slow but constant supply of fresh medium is added, and from which excess medium, waste products, and cells are

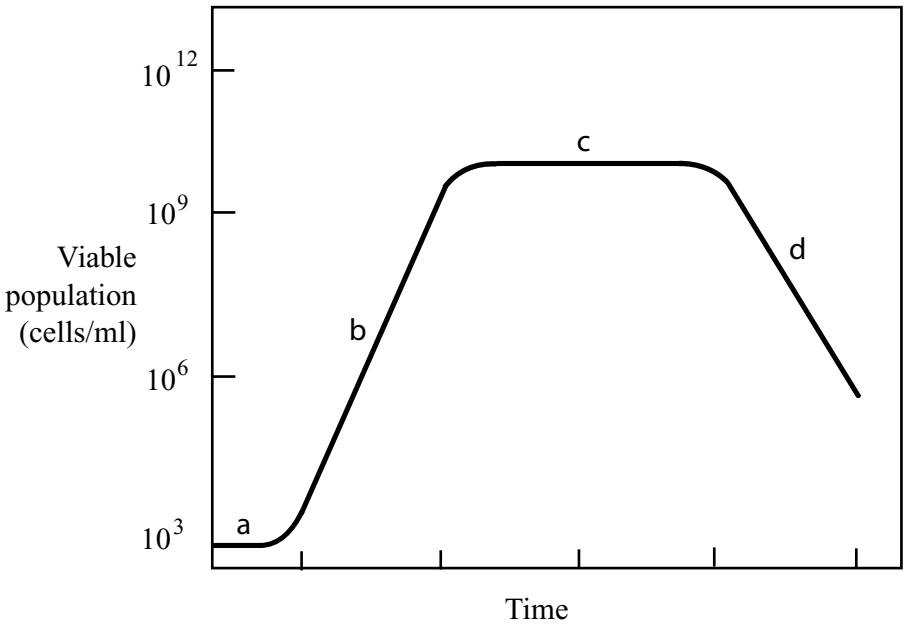

Figure 2.8 The complete bacterial growth curve. (a) Lag phase, (b) logarithmic or exponential phase, (c) stationary phase, (d) death or decline phase.

allowed to overflow. The fresh medium contains one limiting nutrient that acts as a throttle to control the growth rate of the bacteria. Growth rate can be regulated by merely adjusting the amount of fresh medium that is pumped into the vessel. The chemostat has a number of practical and research uses, such as having a ready supply of log-phase bacteria at any time ("bacteria on tap") or to study the accumulation of mutations over many generations.

E. Determining Bacterial Populations in Cultures

There are various reasons for a need to determine the population of a bacterial culture:

1. Constructing a growth curve
2. Determining the effects of antimicrobial chemicals on bacteria
3. Preparing a microbiological product such as a vaccine
4. Preparing an inoculum

There are a variety of methods for determining bacterial populations in laboratory cultures. The method chosen depends on whether one is

interested in just the culturable organisms or the total population (culturable plus viable but nonculturable). Also, methods for estimating populations in laboratory cultures as opposed to environmental samples (covered in Chapter 5) must deal with the extremely large numbers that are normally found in such cultures. A few methods for determining bacterial populations of laboratory cultures are described below.

1. The Viable Plate Count

To assess quantitatively the culturable population of a bacterial culture, a viable plate count is conducted. In this technique, carefully measured aliquots of the bacterial culture are removed, diluted an appropriate number of times, and placed onto a proper solid medium in order to produce isolated colonies. Following the incubation of the medium under suitable conditions, colonies are counted on each plate and the results are related back to the original cell suspension by this calculation:

$$\text{Colony forming units/ml} = \frac{\text{number of colonies}}{\text{plating volume} \times \text{dilution factor}} \quad (2.4)$$

Notice that the results of the culturable population determination are in terms of colony forming units per milliliter (CFU/ml). This awkward expression is necessary because each colony that appears on an assay plate may be the result of the growth of a single cell or it may have originated from an aggregate of two or more cells. Aggregates form when cells either fail to separate on division or clump at some later time. Since there is no way to distinguish between these alternatives, expressing the population in CFU/ml recognizes this uncertainty and avoids any implications as to the true origin of the colonies. Obviously viable (that is, culturable) counts are only estimates of the true population of a culture or environmental sample, but, if done carefully and consistently, they are highly reliable. Some microbiologists advocate the use of vigorous agitation or chemical wetting agents to break up cell aggregates, but, if not done consistently, the results can be misleading. The terms "plating volume" and "dilution factor" will be explained below.

a. Making Dilutions

As noted earlier, it is usually necessary to make suitable dilutions of the sample to be assayed because most bacterial suspensions, such as a typical 24-h-old broth culture, may contain upward of 10^9 cells/ml. As a general rule, the ideal plate count is considered one in which not more than 300 colonies appear on a standard 100-mm Petri plate. Assuming a broth culture

population of about 10^9 cells/ml and a plating volume of 0.1 ml, in order to achieve a colony count within the above acceptable range, the final 0.1-ml sample placed on a plate should have between 300 and 3000 cells/ml, requiring a million-fold (10^{-6}) dilution of the original suspension.

The reason for the 300 colony limit is as follows. As colony counts exceed about 300 per standard 100-mm Petri plate, the probability becomes significant that two or more colonies will overlap and be counted erroneously as one. In addition to estimating bacterial populations in cultures, overcrowded plates may also occur, for example, when assessing heavy microbial loads of raw materials. In this case, dilutions must also be made of the samples. Exceptions to the 300 colony rule are discussed below.

An acceptable assay plate also should not have fewer than 30 colonies. The lower limit rule is based on statistical considerations. The standard error associated with plates with fewer than about 30 colonies becomes a significant and unacceptable fraction of the count, nearly 20%. However, from a practical standpoint, very low colony counts are frequently seen in environmental samples taken in the more stringent cleanroom classes.

Dilutions of microbial suspensions must be carried out precisely and under strict aseptic techniques. Sterile pipettes, pipettor tips, dilution tubes, and diluent liquids should be used. Sterile saline, buffered saline, water, and broth are most commonly used as diluents, but they must be validated before use, as some diluents are injurious to certain species of bacteria. High-quality bacteriological pipettes or micropipettors should be used.

There are a variety of conventions for designating dilutions. For example, a cell suspension that is subjected to a ten-fold dilution is said to have undergone a 1:10, 1/10, or a 10^{-1} dilution. A hundred-fold dilution is variously described as 1:100, 1/100, or 10^{-2}. To prepare ten-fold dilutions, 4.5-ml diluent blanks are recommended, to which 0.5-ml aliquots are added. Hundred-fold dilutions are best handled by adding 0.1-ml aliquots to 9.9-ml dilution blanks. If using a micropipettor, smaller volumes are possible, but additional care must be practiced to assure each dilution step is thoroughly mixed before proceeding to the next step. Mixing 0.01 ml (10 μl) from a micropipettor into a 10-ml blank will result in a 10^{-3} dilution. (Technically, 0.01 ml should be added to 9.99 ml of diluent, but generally the error in preparing 9.99-ml blanks may be greater than any dilution error.) The individual dilution factors for each step are then multiplied together to arrive at the final dilution factor that is used in Equation (2.4) to calculate the original population.

A fresh pipette or pipettor tip should always be used for each dilution step since bacterial cells have a tendency to adsorb to the inner walls of pipettes and may be released two or more steps later, resulting in a large error in the assay results.

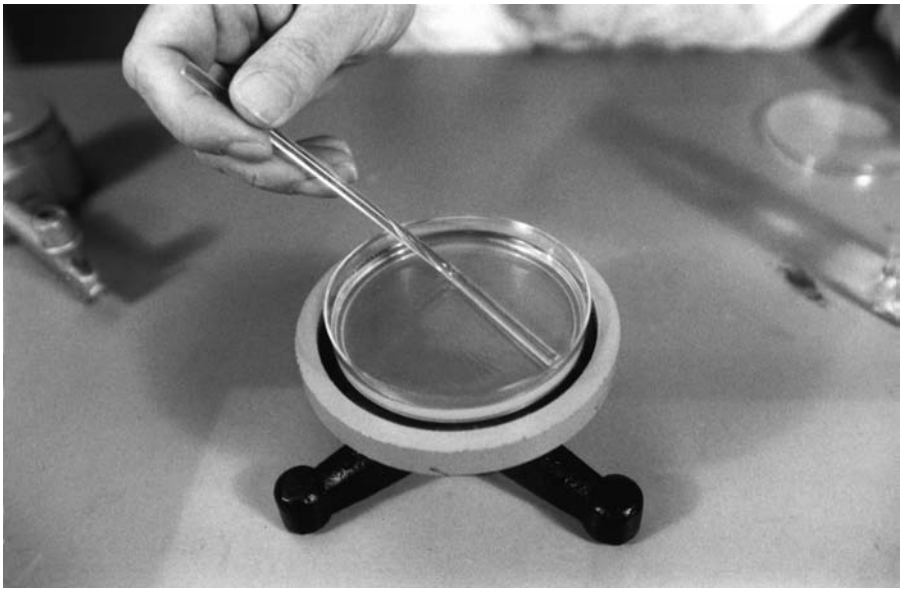

Figure 2.9 The viable assay. The spread plate method. A carefully diluted aliquot of a bacterial suspension is placed onto the surface of an agar medium. The cells are then spread evenly over the agar surface with a sterile glass rod. The plate is incubated, the resulting colonies are counted, and the original population is calculated.

b. Plating Methods

The plating volume is the volume that is transferred to the agar. Plating volumes are generally 0.1 to 0.5 ml. Volumes larger than 0.5 ml are usually not recommended because of the limited amount of fluid the hardened agar can absorb if using the so-called spread plate method. In this procedure, diluted aliquots are spread uniformly over the surface of the agar with a sterile glass rod or wire that is bent in the shape of a hockey stick (Figure 2.9). It is important that the aliquot be spread immediately after it is delivered to the agar surface, for the cells may in time become imbedded in the agar, which would prevent them from being spread. Batches of rods may be sterilized in advance and used once, or one rod may be used repeatedly and sterilized by dipping in alcohol and igniting the alcohol with a gas flame. The glass rod should not be overheated in the gas flame; it is only necessary to ignite the alcohol to sterilize the rod. Disposable, sterile plastic rods that do not require flaming are also available.

In some procedures an additional layer of melted agar (cooled to 45°C) is poured over the sample after the sample is spread. This is known as

a layer plate. Layer plates are often used when counting bacteria species that have a tendency to spread over the surface of the agar, making counting individual colonies almost impossible.

An alternate assay method, the pour plate, involves transferring the plating volume to a tube of melted agar (at a temperature not higher than 45°C), which is thoroughly mixed by spinning between the palms of the hands. The entire contents of the tube are then poured into a sterile Petri dish and allowed to solidify. This technique results in submerged as well as surface colonies, which are counted and the resulting population is then calculated in the same manner as for spread plates; see Equation (2.5). Alternatively, the plating volume may be delivered directly to an empty dish, followed by the molten agar. The dish is then gently swirled to disperse the cells in the agar.

In the layer plate method and the pour plate method, in which samples are added to the Petri dish prior to adding the agar, all of the colonies are submerged as they form and remain relatively small. This allows the formation of greater numbers of colonies without the problem of overcrowding. Therefore, the 300 colony limit discussed above does not apply to these techniques, but may be raised to as much as 1000 colonies.

For greater accuracy, viable assays are usually carried out in duplicate or triplicate. That is, two or three plates are inoculated from the same dilution tube.

c. Calculations

Colonies can be counted by hand or with the aid of a semiautomated counter, in which a register is activated when a grounded probe touches each colony or the bottom of the Petri dish. There are also fully automated counters that optically scan a Petri dish and electronically count colonies. These are obviously great labor-saving devices but may be subject to errors due to the presence of bubbles in the agar or marks on the Petri dish.

The calculation for a typical viable plate count in which the mean of three plates is 235 colonies, the plating volume is 0.5 ml, which was taken from the 1/100,000 (10^{-5}) dilution tube would look like this:

$$\frac{235 \text{ colonies}}{0.5 \times 10^{-5}} = 4.7 \times 10^{7} \text{ CFU/ml} \qquad (2.5)$$

Results of bacterial populations determinations are generally expressed in no more than two significant figures.

2. Filtration Methods

While most commonly used in environmental sampling, methods using filters can also be applied to determining bacterial populations in broth cultures. Filtration methods, described in more detail in Chapter 5, are especially useful when dealing with very low populations. A measured volume of liquid culture medium, diluted if necessary, is passed through a membrane filter having a porosity of 0.45 or 0.22 μm, which is sufficient to trap essentially all bacterial cells. The filter is then placed onto the surface of an agar medium or a paper pad saturated with sterile medium. The filter soaks up nutrients to support the formation of colonies, which then can be counted and related back to the volume of medium that was passed through the filter. When filtering a small volume, such as a few milliliters or less, the sample can be mixed with a larger volume of sterile diluent prior to filtration to distribute the sample uniformly over the entire surface of the filter. The volume of the diluent does not enter into the calculation.

While viable methods offer fairly accurate determinations of culturable bacterial populations, they exhibit one serious shortcoming. Due to the delay necessary for colony growth, usually at least 12 to 18 h, timely information is not possible. Some enumeration methods based on advanced technology have shortened the waiting time to a few hours or less, still short of instantaneous results. Some of these methods are described in Section 4 below.

3. Total Cell Counts

Total cell counts enumerate all cells, culturable, nonculturable, and nonviable. One rapid and simple technique for determining total bacterial populations involves the use of a microscope and a Petroff–Hausser counting chamber (Figure 2.10). The chamber consists of a microscope slide on which a pattern of squares is engraved. Each square is 0.05 mm on a side. Ridges 0.02 mm high support a cover slip above the grid. Thus each square as observed in the microscope can be thought of as a box $0.05 \times 0.05 \times 0.02$ mm, or 5×10^{-5} mm^3 (5×10^{-8} mL).

A small volume of bacterial suspension is placed between the cover slip and the grid. The exact volume of cell suspension is not critical. Counts are made with the aid of a microscope and the mean number of cells per square is calculated. This value when multiplied by the factor 2×10^7 results in the total number of cells per milliliter in the original suspension. In order to obtain statistically significant counts, the manufacturer's instructions should be consulted for guidelines as to how many squares to count.

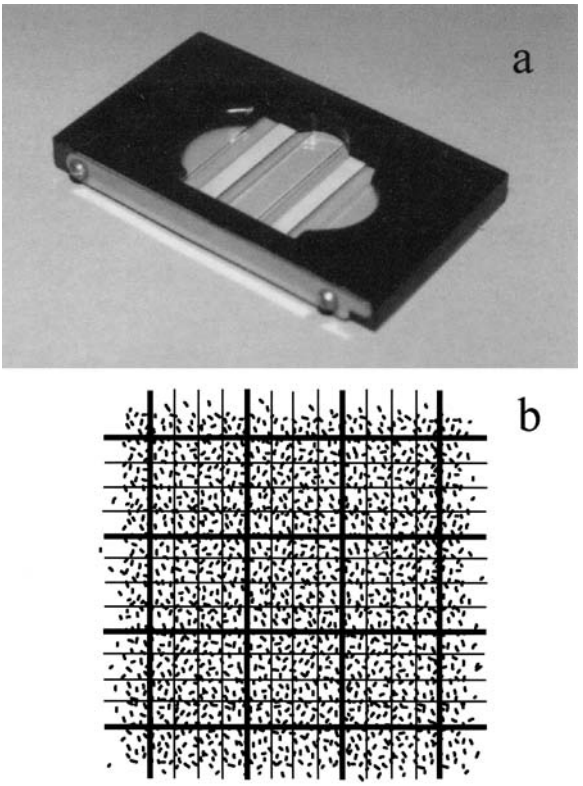

Figure 2.10 Total cell count with Petroff–Hausser counting chamber. Engraved slide in its holder (a) and the grid (b) as it appears in the microscope. Each of the smallest squares is 50 μm on a side.

Since it is not possible to distinguish live cells from dead cells in the Petroff–Hausser chamber, this method has obvious limitations, such as the inability to follow the stationary and death phases of the growth curve. These phases consist of mixtures of live and dead cells of unknown proportions. Also, the method is not applicable to determining population densities accurately much below about 10^7 cells/ml (less than one cell per square).

The advantage of conducting a total cell count such as just described is that an immediate result is obtained. If the Petroff–Hausser chamber is to be used on a routine basis with the same species, counts can be compared one time with viable counts and the proportion of viable cells determined. Then subsequent total counts can be corrected for culturable counts provided that standard culture conditions are followed.

4. Instrumental Methods

A number of semiautomatic and automatic instrumental methods are available for assessing microbial populations. They all are rapid and convenient, with many capable of returning results in a few minutes or less. Some of them, however, suffer from one or more shortcomings. They are not very sensitive, requiring microbial populations in liquids of at least 10^6 to 10^7 cells/ml. In addition, most of these methods are unable to distinguish live cells from dead cells and thus are not capable of determining culturable populations. Finally, the accuracy of some is limited. However, these methods still perform a number of very useful and often indispensable functions in the microbiology laboratory and continue to enjoy widespread use.

a. Turbidometry and Nephelometry

As pointed out earlier, bacterial cells are very nearly as small as the wavelengths of visible light, and they are colorless and transparent. Because of their small size and lack of color, bacteria scatter light in the visible wavelengths rather than absorbing it. Light scattering is defined as the redirection of a beam of light as it passes through a small, transparent object (Figure 2.11). The intensity of the light is not reduced; a portion of the light beam changes direction. Clouds are visible against a blue sky for the same reason: they are composed of very small, transparent water droplets that should be invisible, but because of light scattering they stand out quite noticeably.

A broth culture of bacteria of adequate population appears turbid to us because of the light scattering by the cells. The angle of scattering is seldom more than a few degrees but is sufficient to see without using

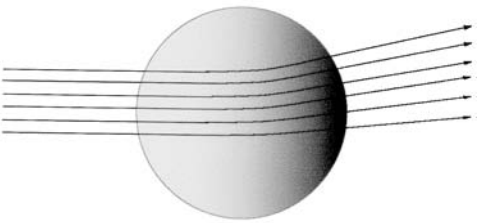

Figure 2.11 Light scattering by a bacterial cell. Because the cell is very nearly the size of the wavelengths of light that are used to observe the cell, the direction of the light path is slightly altered, but its intensity is the same. This phenomenon can be used to estimate bacterial populations by turbidometry or nephelometry.

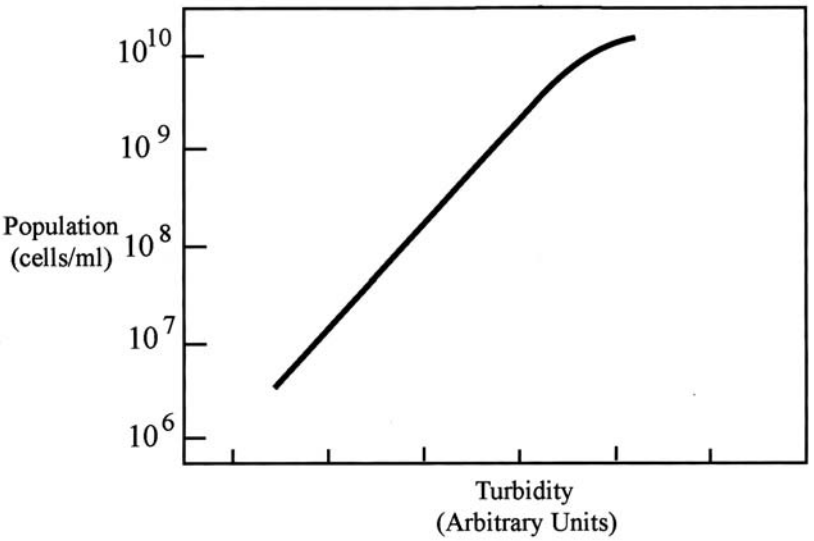

Figure 2.12 Turbidity standard curve. To determine bacterial populations by turbidometry, a standard curve such as this figure is prepared with known populations of cells. The population of an unknown bacterial suspension can be estimated by measuring its turbidity and referring to the standard curve.

appropriate instruments. Because the light beam is diverted from its normal path, its intensity (read on the absorbance scale) appears to be reduced when observed by a light-measuring device such as an ordinary spectrophotometer. This technique is known as turbidometry. By preparing a standard curve ahead of time in which absorbance readings are plotted versus known populations, an estimate of the bacterial suspension can be obtained (Figure 2.12).

The accuracy of methods based on light scattering is highly dependent on the care taken in preparing the standard curve. Light scattering of a particle is a function of its size and shape, which means the standard curve must be prepared with cells that are dimensionally the same as those that are to be measured. Preferably the same species and age should be used, if known. Wavelength is also an important factor: the smaller the wavelength, the more intense the scattering and the more sensitive the determination. The shortest wavelength that is practical is around 400 nm, provided the cells are suspended in buffer or saline. Broth contains materials that absorb strongly at these short wavelengths and therefore 530 to 600 nm is recommended for measuring turbidities of broth cultures.

When conducting turbidometry in a spectrophotometer, one is measuring the intensity of the undeviated light. One can measure the intensity

of the deviated light with the use of a special instrument called a nephelometer, where the light detector is placed slightly off-axis rather than on-axis, as in a spectrophotometer. This has the advantage of greater sensitivity, where populations down to about 10^6 cells/ml can be measured routinely.

The use of light scattering to estimate bacterial populations, particularly turbidometry, goes back nearly to the turn of the 20th century, and, in spite of its shortcomings, it continues to be one of the most popular methods for that purpose.

b. Flow Cytometry

In flow cytometry, cells suspended in a liquid are passed as a continuous stream through an intense beam of light, usually a laser. The signals from one or more sensitive light detectors that collect the light scattered from the cells are fed to a computer. Not only is it possible to count the number of cells in the liquid, size and shape can also be determined by the cells' light-scattering characteristics. In addition, various staining methods and advanced discrimination systems allow the determination of DNA and protein content or even the identification of specific species. In some instruments based on flow cytometry, individual cells are carried in separate droplets that are made to fall through the light beam, making it possible to sort cells according to a variety of measurements. As cells are analyzed, the instrument controls electrostatic charges that instantaneously divert the cells into various containers according to parameters such as DNA content, size, or staining reaction.

c. Methods Based on Electrical Resistance and Impedance

Microbial cells are relatively poor conductors of electricity and, consequently, if one or more cells are placed in the path of an electric current, the amount of attenuation of the current can be a measure of the population present. In instruments such as the Coulter counter, a cell suspension is drawn through a small orifice, through which an electrical current also passes. The magnitude of the current is measured continuously. Each cell is counted as it interrupts the current path. The population can be estimated with considerable sensitivity and accuracy, provided background counts due to dust and cell debris are not excessive.

Other methods are based on measuring the electrical impedance of an entire suspension of bacteria. Impedance is the attenuation of an alternating (AC) electrical current as it passes through a substance. Most bacteriological growth media contain undisassociated (uncharged) molecules such as carbohydrates and polypeptides, most of which are not very good conductors of an electric current. As bacteria metabolize, they release

by-products, such as organic acids, that disassociate easily, resulting in a drop in the impedance of the medium. A threshold occurs when the population reaches about 10^6 cells/ml. The population is estimated by determining the time of detection; that is, the time from initial measurement to the time of detection. Standard curves can be constructed with known starting populations. This method has been applied to automated instruments in the clinical microbiology laboratory for the assessment of antimicrobial drug susceptibilities.

d. Epifluorescent Microscopy

Bacterial cells that have been collected on a membrane filter can be counted directly with a microscope. Normally this approach offers considerable difficulties, since much of the particulate matter trapped by the filter is nonmicrobial. The trouble lies in the difficulty in distinguishing microbial cells from the noncellular debris that is common in most liquid bacteriological media. There are, however, fluorescent dyes, such as acridine orange, that preferentially stain microbial cells. An epifluorescent microscope is used to examine filters prepared using this technique. An ultraviolet light source is directed down through the objective lens. Viewed in a darkened room, stained bacterial cells show up as fluorescent objects against a dark background, whereas noncellular debris is either dimly outlined or invisible. Other stains make it possible to distinguish live cells from dead cells. Alternatively, antibodies prepared against specific species can be labeled with fluorescent dyes. In mixtures, only those cells for which the antibodies were prepared will become labeled and show fluorescence.

e. Chemical Methods

Adenosine triphosphate (ATP) is an important constituent of all living cells. It plays a number of roles, including as a precursor in DNA synthesis and as an energy transport molecule. The bond that connects the third phosphorus to the adenosine is referred to as a high-energy bond, which means that when the bond is broken, a considerable amount of chemical energy is released to supply energy to other reactions in the cell. The amount of ATP in a viable cell is generally constant, so a measure of the ATP content of a bacterial culture or an environmental sample is a good measure of the number of viable cells present. When a cell dies, its ATP is depleted rather quickly; consequently, only viable (that is, culturable and viable but nonculturable) cells are detected in this method.

A highly sensitive method of ATP analysis has been developed based on the light-emitting reaction of luciferin, the molecule that is responsible

for the light emission in fireflies. ATP is extracted from the bacterial culture to be assayed. In the presence of the enzyme luciferase, the luciferin emits one photon for each molecule of ATP present. A sensitive photometer is used to detect the intensity of light emitted by the luciferin/luciferase reaction, which is proportional to the number of viable cells present in the sample. With the help of properly prepared standards, one can then get an estimate of the viable bacterial population. The lower limit of sensitivity for this method is around 10^3 to 10^4 cells/ml. In an alternative method, cells are collected on a membrane filter, which is then treated with the bioluminescent reagents. The filter is then scanned with a highly sensitive light detection system. A sensitivity of 1 CFU is claimed in this method.

By measuring principal chemical components of the cells, like DNA, ATP, protein, total nitrogen, and dry weight, or metabolic by-products such as carbon dioxide, a good estimate of the population size can be made. Again, attention must be paid to the sensitivity limitations of the method. For example, the popular Lowry reagent can measure protein quantities down to about 50 μg/ml. This is equivalent to about 5×10^8 cells. Recognition must again be made of the fact that, except for methods such as ATP and carbon dioxide measurements, chemical determinations measure total cell populations without regard to viability.

It is quite clear that no one technique to assess bacterial populations is ideal. Counts based on turbidity, microscopic counts, or electronic counts are nearly instantaneous but, in general, are incapable of distinguishing live cells from dead cells, and most lack sufficient sensitivity to detect low numbers of organisms. Chemical methods are time consuming and frequently lack sensitivity. The viable plate count produces accurate and sensitive culturable population estimations, but requires a delay of at least 8 to 16 h to allow for colony growth. Many laboratories use a combination of two or more of these methods, and, when possible, use an instantaneous procedure as a guide and the viable plate count data for final calculations.

F. Microorganisms as Tools

Because microorganisms respond to media compositions in a predicable way, they can be used as a tool for some very sensitive assays. Suppose one needs to determine the concentration of the vitamin niacin in a sample. There are particular species of bacteria that require specific nutrients, such as niacin. A series of culture tubes containing a medium that lacks niacin is prepared and then various trace amounts of the vitamin are added back. For example, five tubes may contain 0.000, 0.001, 0.005, 0.01, and 0.05 μg of the vitamin. The tubes are inoculated with the organism that requires niacin and then incubated. The degree of growth

is measured by any of the methods mentioned above, such as turbidometrically. A standard curve is constructed by plotting vitamin concentration versus turbidity. If the correct range of niacin concentrations is used, there should be a relatively good linear relationship between turbidity and vitamin concentration. Samples containing unknown concentrations of niacin can then be assayed in a similar fashion. Provisions may have to be made to produce levels of the nutrient within the range covered by the standard curve.

Assays of antibiotics and other antimicrobial substances can also be conducted in this manner. In these procedures, the graded amount of substances produces various levels of growth inhibition rather than growth stimulation, but the principle is the same: comparing the amount of growth versus concentrations of agent.

III. GROWING OTHER MICROORGANISMS: FUNGI, PROTOZOA, ALGAE, AND VIRUSES

A. Fungi

While most fungi will grow well on ordinary bacteriological media such as TSA under the same culture conditions for bacteria, fungi generally prefer slightly different living conditions. For growing fungi in the laboratory, media specially intended for these organisms are recommended. One of the most popular media for growing the more common fungi is Sabouraud dextrose agar (SAB). Mycological agar and malt extract agar are also used for culturing fungi. These media are prepared with a lower pH and higher carbohydrate content, conditions that are more in line with these organisms' preferences. More about culturing fungi from environmental samples will be found in Chapter 5.

As noted in Chapter 1, the fungi consist of two types of organisms: yeasts and molds. Yeasts are single celled and thus population growth can usually be followed by methods such as microscopic counts and viable plate counts that are used for bacteria. Molds, on the other hand, form filamentous mycelia, which produce enormous numbers of spores, making it difficult to assess growth accurately by these methods. Instead, indirect methods are utilized that measure some by-product of fungal growth. The rationale for this approach is that for certain cell constituents, their concentration will be proportional to biomass. For example, the sterol ergosterol is commonly found in most fungi. The ergosterol can be extracted from samples of fungal growth and its concentration determined by chromatography. Alternatively, the organisms can be fed radioactive acetate, which is a precursor of ergosterol biosynthesis. The sterol is extracted from the fungal cells and its radioactivity is measured. When

proper standards are prepared, both methods offer reliable means of following mold growth in the laboratory.

B. Protozoa and Algae

Like most bacteria, protozoa require media with certain preformed nutrients, which are derived from animal and plant sources. While some protozoa can grow axenically (free of other organisms), many require the presence of bacteria, algae, or other microorganisms to feed on. The medium must therefore support both the protozoa and their live prey. All of the other conditions such as temperature and pH are also critical, and, for those protozoa that must feed on algae or contain symbiotic algae, a suitable light/dark cycle must be provided.

Algae do well in liquid media as well as on agar, where they will form visible colonies. The use of the layer plating method will prevent spreading of motile species. Since algae are generally considered autotrophic, their media normally consist entirely of mineral salts, although the addition of a few vitamins and simple organic sources of carbon and nitrogen are known to stimulate their growth. Since algae are photosynthetic, they require light as an energy source. A typical light/dark cycle for a culture might be 16 h on and 8 h off.

C. Viruses

Viruses grow by synthesis and assembly within living host cells. To culture viruses in the laboratory, appropriate host cells must be found and procedures for their growth and maintenance must be developed. The development of the techniques of cell culture, the ability to grow cells of higher plants and animals in laboratory glassware, has made it possible to cultivate many types of viruses. Human cells, for example, can be grown in bottles and infected with any of a number of human or other animal viruses. Primate cell cultures such as monkey kidney cells also support the growth of many human viruses. As one might expect, the nutritional and atmospheric requirements necessary to grow animal cells are considerably more complex and the skills involved are more demanding than those necessary in the cultivation of most other microorganisms.

Not all viruses can be grown in cell cultures, and microbiologists sometimes have no choice but to use a whole animal or plant host in order to obtain viruses to study. Alternatively, fertile poultry eggs have proven to be susceptible to a number of animal viruses and can act as hosts for virus cultivation. For instance, some human viruses for commercial vaccine production, such as influenza and yellow fever, are produced in chicken or duck eggs. Growing plant viruses in whole plants

is often necessary, making a plant virus laboratory look more like a nursery greenhouse.

1. Counting Viruses

Once a method for growing a particular virus in the laboratory has been developed, the next step is to confirm that virus multiplication has actually occurred by carrying out an assay of the virions that have been produced by the host cells. This number is frequently referred to as the titer. Viruses do not form colonies, but for many viruses an assay method somewhat akin to a colony count may be applied. Called a plaque assay, the method takes advantage of the effect some viruses have on their host cells. Viruses may produce a cytopathological effect, visible changes in the appearance of their cultured host cells as a result of viral activity. The most severe effect is the death and lysis of the host. A suspension of viruses may be made to infect a thin layer of host cells that have been growing on a solid surface such as agar or glass, called a "lawn" if the hosts are bacteria and a "monolayer" if the hosts are animal or plant cells. The localized damage or destruction of host cells will produce circular, visible areas called plaques, in the layer of cells (Figure 2.13).

A plaque begins when a single virus infects a host cell. At the end of the infectious cycle, the infected host cell releases newly minted virus particles into the surrounding medium. These viruses infect cells immediately adjacent to the initially infected cell. These infected cells in turn release more viruses. The viruses spread out from the initial host cell and eventually the area of infected cells is sufficiently large to form a visible plaque. Knowing what volume of the original virus suspension was placed in the host cell culture and assuming that each plaque began with a single virion infecting a host cell, the titer of the virus suspension can be determined.

Viruses are generally not a problem in most cleanrooms concerned with the manufacture of pharmaceuticals and medical devices. A significant exception involves those products that are derived from mammalian cell cultures, such as vaccines and active proteins. Genetically engineered animal cells and tissues and organs prepared for transplantation can also be a source of viruses, as well as being susceptible to virus contamination. For facilities involved in these activities, microbiological monitoring of raw materials and the environment must include an assessment of adventitious viruses.

IV. ASEPTIC TECHNIQUE

It is clear that when one is involved in growing a culture of microorganisms for any reason, it is imperative that no unwanted microorganisms are

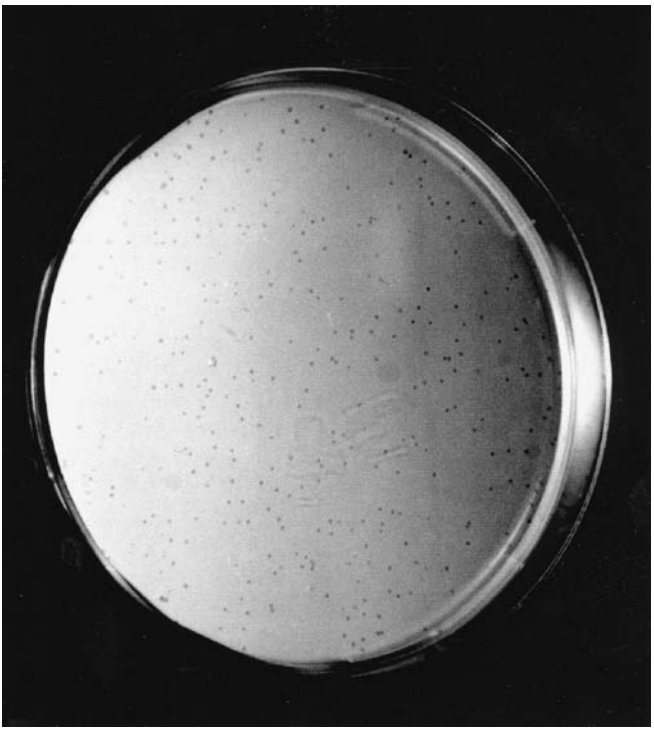

Figure 2.13 Determining virus populations by plaque assay. Dark spots or plaques in a uniform "lawn" of confluent bacterial growth are the result of localized destruction of cells due to bacteriophage infections. The number of plaques can be counted and the titer of the original bacteriophage suspension can be calculated.

allowed to enter the culture during the entire process, from the preparation of the sterile medium and its initial inoculation to the end of an experiment or production process. The precautions taken by the microbiologist and other facilities personnel to assure that the operations are not contaminated are generally referred to as aseptic techniques. Some basic aseptic techniques that are familiar to every microbiology student are illustrated in Figure 2.14. Aseptic technique must become second nature, for a contaminated culture is useless and, in some situations, dangerous. All media, glassware, and any other items that come into contact with a culture must be sterilized prior to use. The mouth of any vessel that is to be inoculated should be flame sterilized before and after inoculation, as should the inoculating loop or needle. In addition, all used materials and glassware that have come in contact with viable microorganisms must be sterilized prior to reuse or disposal.

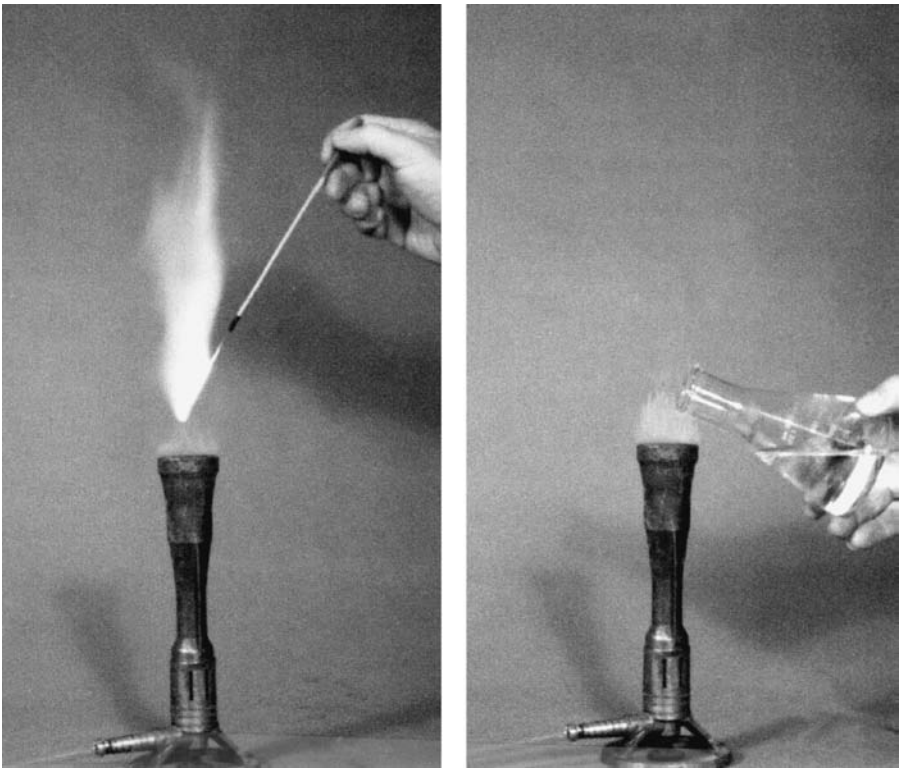

Figure 2.14 Aseptic techniques. Flaming an inoculating loop and the mouth of a culture vessel is a routine application of aseptic technique used in a microbiology laboratory.

V. SAFETY ISSUES

Growing microorganisms in a laboratory usually involves the production of bacteria, fungi, and viruses in numbers considerably larger than normally found in nature. While most of the microorganisms dealt with in the cleanroom laboratory may be relatively harmless for normal, healthy individuals, those personnel with weakened immune systems due to chemotherapy, infections, hereditary or other conditions may be susceptible to opportunistic infections by these normally benign microbes. In addition, cultures derived from environmental samples can contain more aggressive microorganisms such as *Staphylococcus* and *Streptococcus*. The rule should be established in every microbiology laboratory that every culture should be treated as though it contains pathogens. Adequate training of all personnel, including janitorial, is a must. Emergency standard operating procedures (SOPs) must be in place to deal with spills, and

strict controls over access to cultures should be maintained. Government regulations consider viable microbiological cultures to be hazardous medical waste that must be sterilized by appropriate means such as autoclaving or incineration prior to discarding. Live cultures are never poured down the sink. Sterilization of unwanted cultures should preferably be done on-site to avoid the risks of transporting hazardous material to another location. A prudent rule is that all laboratory cultures are sterilized by autoclaving for not less than 60 min prior to discarding. Contaminated glass- and plasticware and other equipment must also be sterilized before washing and reuse or before it is discarded.

All viable cultures, culture collections, and other concentrations of live microorganisms should be stored in a manner that prevents their release in the event of an earthquake, fire, storm, or other major disaster. Incubators and storage cabinets containing live cultures should be clearly labeled to alert firefighters and other emergency workers.

VI. SUMMARY

Many microorganisms can be cultured in the laboratory on various media. In the case of viruses and other more fastidious microorganisms, living cells or whole plants or animals must be used as hosts. In either case, care must be taken to supply all the chemical and physical conditions (temperature, atmosphere, etc.) necessary to support the desired organisms and, when appropriate, their hosts, as well.

There are many techniques at the disposal of the microbiologist for measuring microbial populations; some determine only culturable populations, others measure total (culturable, viable but nonculturable, and nonviable) cell numbers. There is no one ideal method, and frequently one must rely on two or more different techniques to satisfy a particular need.

Due to the large populations normally involved, cultivating microorganisms may expose laboratory and maintenance personnel and the general public to certain hazards. Special precautions should be practiced whenever microbial cultures are handled and discarded.

3

CONTROLLING GROWTH AND ACTIVITIES OF MICROORGANISMS

I. INTRODUCTION

It is well known that the activities of microorganisms can cause

1. Spoilage of food and pharmaceutical products
2. Deterioration of materials
3. Infectious diseases in plants and animals
4. Disruption of electronic microcircuitry and nanotechnology devices

These occurrences can be avoided by excluding microorganisms entirely from processes and products or by controlling the growth and activities of existent microorganisms. Cleanroom and other contamination control techniques have made significant progress in reducing microorganisms from sensitive products such as pharmaceuticals and medical devices. Heat, cold, radiation, and filtration are examples of physical methods of controlling microorganisms, while the use of disinfectants, antiseptics, preservatives, and sterilants represents chemical means. In this chapter we will explore these methods, but first we must deal with a few key definitions.

II. DEFINITIONS

"Decontamination" refers to the reduction of the microbial population in or on an object to some lower value, but not necessarily to zero. "Disinfection" refers to the reduction in numbers of the vegetative forms of

pathogenic microorganisms present in or on an inanimate object. A disinfectant is a chemical that is used to carry out disinfection. "Pathogenic" means able to produce disease. Sanitization, when applied to food handling operations, is the reduction of microbial populations to levels considered "safe" by public health standards. It is carried out with sanitizers. When applied to cleanrooms, sanitization more generally refers to reduction of microbial contamination. Preservation involves the use of antimicrobial chemical preservatives to prevent the multiplication of microorganisms in pharmaceuticals, foods, and other products. "Sterilization" means the removal or destruction of all living organisms. Sterility is an absolute condition — an object is either sterile or it is not — whereas an object that has undergone disinfection, decontamination, or sanitization may still be contaminated. Sterilization usually requires exposing materials or items to much harsher conditions than does decontamination. It is therefore often useful to recognize instances when sterilization may not be necessary and decontamination would suffice.

Culturable means the ability of an organism to reproduce when placed in a suitable environment. Conversely, nonculturable is defined as the lack of a cell's ability to reproduce when it is placed in an environment that would normally support its growth. Cells that do not form colonies may be physiologically dead or viable but nonculturable, as described in Chapter 2. Microorganisms may appear nonculturable under one set of conditions and culturable under another set. It is therefore important when reporting results of the testing of a disinfectant or a sterilization process, or when conducting environmental sampling, to follow standard conditions faithfully and consistently as to type of medium, incubation temperature, and other culture conditions.

The bioburden is the total recoverable viable microbial population in or on objects just prior to sterilization. The bioburden for a particular item may vary depending on the specific process used for collecting the organisms that adhere to the item, which underscores the need for consistent and reproducible sampling methods.

III. PHYSICAL METHODS OF CONTROL

A. The Kinetics of Microbial Death

Under ideal circumstances, a population of microorganisms exposed to lethal doses of heat or radiation dies off in an exponential fashion. That means that a graph plotting the logarithm of the surviving population versus time of exposure results in a straight line. If heat is involved, the slope of the resulting thermal resistance curve (Figure 3.1) reflects the rate of death of that particular organism; that is, the measure of the

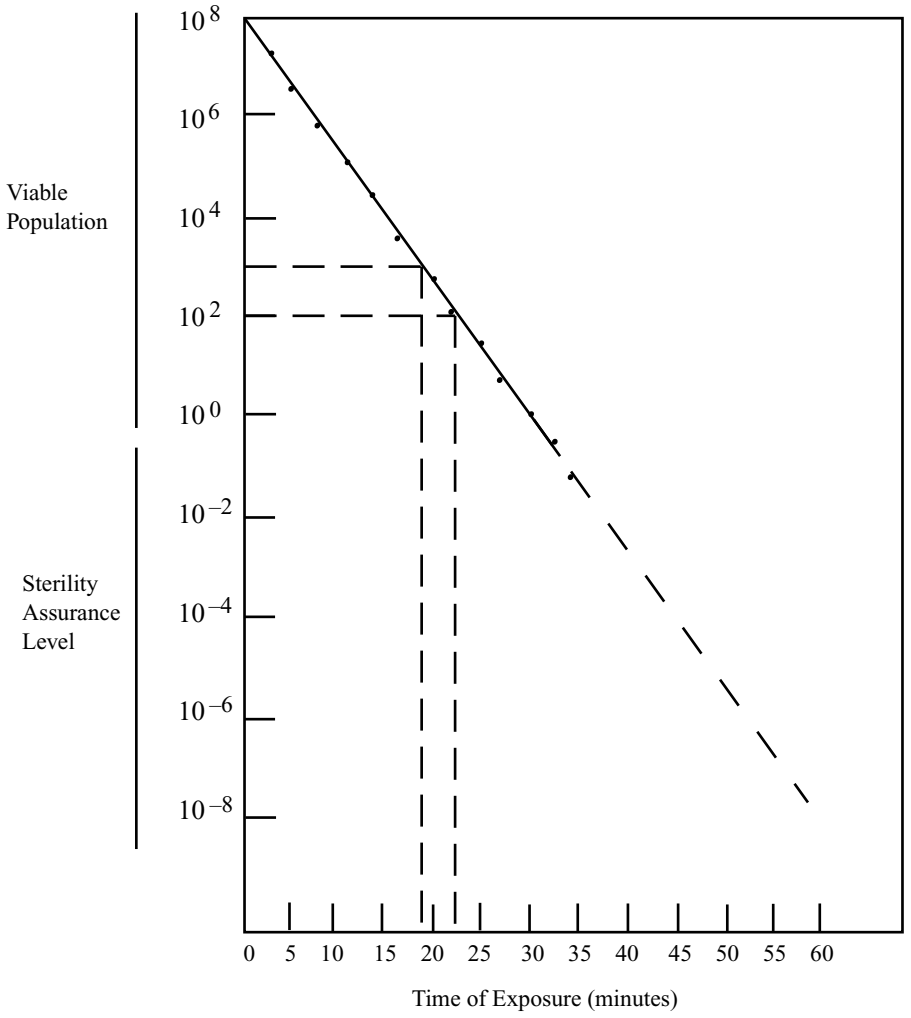

Figure 3.1 Thermal resistance curve. A suspension of 10^8 viable bacterial cells was exposed to 160°C. During that time, frequent samples were taken and subjected to a viable count. By about 30 min, viable cells were no longer detected in the suspension. By determining the time necessary to reduce the viable population by 90% or one log (from 10^3 to 10^2 in the example here), one arrives at the D-value, about 4 min. By extrapolating the plot beyond 30 min, the time necessary to achieve a given SAL can be determined.

Table 3.1 Some factors that influence the germicidal effects of heat

Age of cells
Form of cells (vegetative or endospores)
Presence of organic matter (blood, grease)
Presence of moisture
Nature of atmosphere (oxidizing or reducing)
pH

organism's relative resistance to heat. Thermal death is a complex phenomenon that is dependent on a number of factors, some of which are listed in Table 3.1. Inactivation curves like Figure 3.1 illustrate an important lesson: when microorganisms are exposed to heat or radiation, they do not die instantaneously. At typical intensity levels of lethal agents, it takes a certain amount of time for the agent to kill the target population, and of course it follows that the larger the population, the longer it takes.

For a given thermal resistance curve as depicted in Figure 3.1, we can determine the decimal reduction time, or D-value, which is the time, at the stated temperature and under other standard conditions, that is necessary to reduce the microbial population by 90%, tenfold, or one logarithm; e.g., from 10,000 to 1,000. It must be pointed out that D-values for the same organism may vary depending on a number of factors, such as the nature of the material on which the bacteria were attached and the moisture content and age of the cells. When sterilization protocols based on D-values are being designed, the conditions under which the D-values were determined must be defined.

By extrapolating the resistance curve to a surviving population of less than one cell (there is no zero on a log scale), we can determine the time necessary to sterilize the specific population in question at that temperature and under the same conditions. The extrapolation below one cell that the sterilization plot is extended is considered the measure of the sterilization assurance level (SAL), or overkill. The SAL can be looked upon as a probability that one or more items that have been exposed to a sterilization treatment remain unsterile. If a load of 10,000 syringes is sterilized to an SAL of 10^{-3}, one may expect $10{,}000 \times 10^{-3}$, or 10 syringes, to remain unsterile. A typical SAL for critical medical products is usually set at 10^{-6}.

The usefulness of the D-value is in estimating how much time would be necessary to achieve a particular SAL at a particular temperature under standard conditions. For instance, with a bioburden of 10^8 bacteria and an SAL of 10^{-6}, 14 D-values (8 + 6) would be necessary. In our example in Figure 3.1, the D-value appears to be about 3.75 min. Therefore, 14 ×

3.75 or 52.5 min would be needed to sterilize that load for a SAL of 10^6 at 180°C.

The thermal destruction curve (Figure 3.2) is an extension of the thermal resistance curve. To obtain a thermal destruction curve, thermal resistance curves are plotted for various temperatures for the same species and a D-value is determined for each temperature. A plot of the log of the D-values vs. temperature also results in a straight line. The use of D-values when calculating the cumulative lethal effects of the prolonged heating and cooling of large numbers of items will be discussed in the section on sterilizing large loads.

B. Heat

Heat remains the most reliable method for controlling microorganisms. Two forms of heat are used in decontamination and sterilization: wet or moist heat, and dry heat. Moist heat refers to situations where an object is heated in the presence of saturated steam; that is, at 100% relative humidity (RH). Dry heat means anything less than 100% RH, but normally refers to ambient RH. The two kinds of heat are discussed separately because one is considerably more efficient at sterilization than the other. Moderate numbers of bacterial spores are reliably killed in 15 to 20 min by saturated steam under 15 psi pressure at 121°C, whereas exposure to dry heat would require over 6 h at the same temperature to kill an identical number of spores. While the principal mechanism is the same for both types of heat, the thermal denaturation of critical cellular macromolecules, the dramatic difference in killing efficiency is primarily due to the greater ability of steam to deliver lethal thermal energy to the cells. The heat transfer coefficient of saturated steam is some two or three orders of magnitude greater than that of air at the same temperature.

1. Moist Heat

Moist heat means heat that is accompanied by 100% relative humidity. For sterilization of laboratory items, aqueous solutions, bacteriological media, and small to medium production runs, the use of moist heat is the most popular and reliable method and is normally applied in the form of saturated steam under pressure in an autoclave (Figure 3.3). Two types of steam autoclaves are used for sterilization: gravity displacement and prevacuum. In the gravity displacement autoclave, the lighter steam displaces or forces out the denser air from the autoclave chamber through a vent at the bottom of the chamber. A thermostatic valve closes the vent when the steam reaches the bottom of the chamber. Steam pressure can then build up to the required level. With prevacuum autoclaves, most of

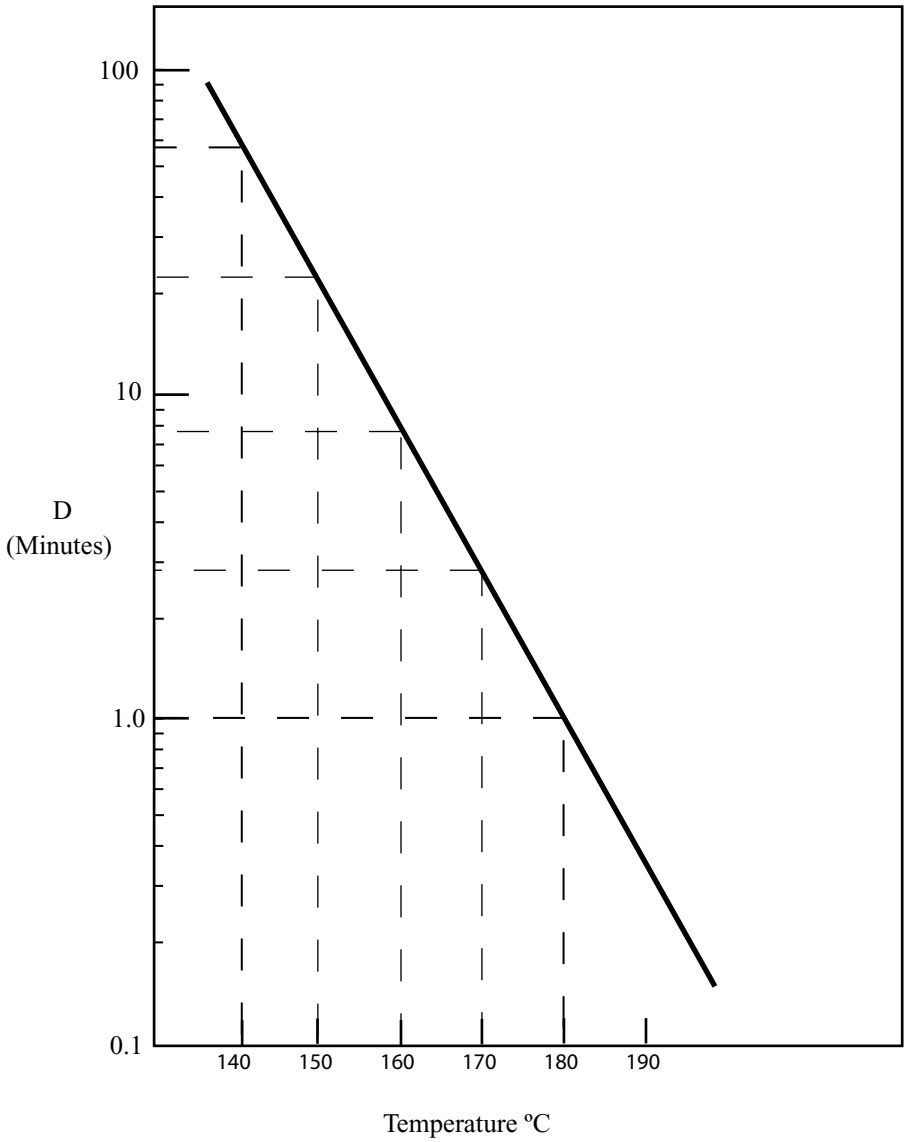

Figure 3.2 Thermal destruction curve. Using data from thermal resistance curves at several temperatures, D-values for a specific organism are plotted against temperature. The straight line that results is called a thermal destruction curve. It allows one to determine a D-value for any lethal temperature for that organism.

Figure 3.3 A typical steam autoclave for sterilizing small laboratory and surgical items, microbiological media, and other materials that can withstand the effects of 20 to 30 min in saturated steam at 121°C.

the air in the chamber is removed from the chamber by a vacuum pump before the steam is introduced. One hundred percent humidity cannot be achieved if appreciable amounts of air remain in the chamber.

We must remember that it is not the heat in the autoclave alone that kills microorganisms, but the combination of heat and saturated moisture. Consequently, the nature of items to be sterilized in the autoclave must allow the steam access to all their internal spaces and surfaces. Empty

glassware must have loose-fitting caps or cotton plugs to allow steam to enter them. Items such as filtration setups, syringes, surgical instruments, tubing, or mechanical parts from pumps and similar items must be completely disassembled and have all surfaces exposed to the steam. These items must be wrapped in Kraft paper, muslin, or other material that is permeable to moisture but not penetrable by microorganisms when the items are subsequently removed from the autoclave and handled. Frequently, to assure a high level of protection from contamination following sterilization, items for sterilization are double or triple wrapped. Then, when the items are to be used, outer wrappings are removed in a clean environment but their final wrapping is removed and the items are assembled under aseptic conditions such as in a biosafety cabinet or an isolator.

Dense items, such as towels, surgical drapes, uniforms, pouches, and bandages must be loosely stacked in the autoclave to allow complete steam penetration. Generally, items that have aqueous liquids sealed in them, such as ampoules or serum bottles, may be adequately sterilized in the autoclave, but certain areas inaccessible to the steam, such as between the layers of overcaps or double seals on bottles may escape sterilization and remain contaminated. In addition, if such items containing aqueous product have large headspaces, trapped air may prevent achievement of 100% humidity. Large, empty vessels such as flasks and carboys, even though they may be loosely capped, also may escape sterilization internally if steam is prevented from fully entering due to the presence of (heavier) air in the vessel. Such containers should be placed on their sides to allow the steam to displace the air. This situation is less of a problem with the prevacuum autoclave. Contaminated waste sealed in plastic biohazard bags will not be sterilized reliably unless there is adequate water present in the bags. The bags should either be left open during autoclaving, or at least 250 ml of water should be added before sealing.

Dense powders and oily substances (sand, talcum powder, paraffin wax, or petroleum jelly) cannot be sterilized in the autoclave because the steam cannot penetrate these materials. They must be sterilized by dry heat. Also, many types of plastics cannot be autoclaved without significant distortion, although some, such as Tygon and polyethylene, hold up reasonably well. Always check the manufacturer's literature before attempting to autoclave any items or their packaging that are wholly or partially composed of plastic. Most items of rubber survive autoclaving, such as latex tubing, gloves, and pipetting bulbs, but they begin to show signs of deterioration following repeated sterilizations.

Fifteen to 20 min exposure in the gravity displacement autoclave at 121°C (15 psi) is generally adequate to sterilize a load of small to medium size items, such as surgical instruments in moisture-permeable wrappings and heat-stable solutions or bacteriological media in volumes of up to

about one liter. If sufficient steam pressure is available and the objects can withstand the higher temperature, shorter processing times for the same load are possible by operating the autoclave, for example, at 136°C (46 psi). This is known as flash sterilization and only 10 min would normally be required at that temperature. The manufacturer's recommendations should be consulted.

Autoclave manufacturers' recommendations should also be consulted when sterilizing bulky items and large volumes of liquids, since these require additional time to reach sterilization temperature; viscous liquids especially require additional time, because heat transfer within the liquid is less efficient due to slower mixing by convection. As a safety margin, additional time (at least two- to three-fold over normal) should be applied to heavily contaminated material such as discarded laboratory cultures or environmental samples.

All modern autoclaves have provisions to operate under "slow exhaust" or "liquids" cycles for sterilizing unsealed aqueous liquids such as saline solutions and bacteriological media. When using this setting, the steam pressure within the chamber is released gradually at the end of the sterilizing cycle. This prevents liquids from boiling over when the chamber pressure is suddenly reduced after the liquids have been heated to over their normal boiling point. However, extreme care must be taken when removing liquids from the autoclave, since they still may be above their boiling temperature and can erupt superheated liquid if shaken. Nonliquid items such as empty glassware and surgical instruments may be subjected to a "fast exhaust" and perhaps an additional drying step, where pressure is released rapidly and a vacuum is applied to the chamber to draw off excess moisture. The newest models of autoclaves are preprogrammed to accommodate a variety of loads and conditions.

Pasteurization is an example of the use of heat to decontaminate rather than sterilize. When milk is pasteurized, it is subjected to 72°C for 15 s, or alternatively to 63°C for 30 min, a treatment that assures the destruction of all pathogenic organisms commonly known to occur in raw milk. As a bonus, 90 to 99% of the spoilage organisms in the milk are also destroyed, thereby extending shelf life. Certain fruit juices that have been known to contain pathogens are also pasteurized. Beer and other fluid food products that do not normally contain pathogens are frequently pasteurized to reduce spoilage organisms.

2. Dry Heat

Dry heat sterilization involves the application of heat at ambient humidities of less than 100%. Because it is less efficient than moist heat, dry heat must be applied at much higher temperatures and for longer times. The

Table 3.2 Examples of recommended conditions for dry heat sterilization

Items	*Conditions*
Hypodermic needles	2 h at 160ºC in tubes with cotton plugs; no stylets (inner wire)
Glass syringes	2 h at 160ºC; plungers and barrels separate; wrapped in muslin
Surgical instruments	1 h at 160ºC; must be clean and on a metal tray
Talcum powder	2 h at 160ºC; or 1 h at 170ºC; in not more than 1 cm layers

use of dry heat sterilization is limited to items that can withstand the effects of temperatures of 160 to 180°C for 2 h or more. Clean, empty glassware, some metal objects, powders, and oily materials are most commonly sterilized by dry heat; items consisting of volatile liquids, plastic, cloth, or rubber usually are not. The volumes of oils and powders must be kept to a minimum to allow adequate heat penetration. Layers not over 1 cm thick are recommended. Cotton plugs generally survive without discoloration if not heated above 160°C. Table 3.2 lists some common conditions and limitations for dry heat sterilization of selected medical items. Dry heat sterilization can be carried out in any gas or electric oven-like device that can be held at 160 to 180°C. Such a device must have good interior air circulation, which is critical for even heat distribution. This can be accomplished with fans or blowers.

Dry heat is often used to destroy pyrogens on containers and closures destined for parenteral solutions or on equipment that comes in contact with sterile products. Temperatures from 220 to 350ºC are usually used.

3. Sterilizing Large Loads

In large-scale industrial sterilization operations utilizing either moist or dry heat, calculating the time necessary to sterilize very large volumes of fermentation media, bulky pieces of equipment, or large numbers of packaged products can become quite complicated. Figure 3.4 shows a typical situation, the sterilization of a very large load of empty glass bottles. It may take several hours for the innermost items in the load to reach the sterilization temperature and then additional hours for the items to cool to room temperature. In other words, the load does not instantaneously reach the required sterilization temperature nor does it drop immediately to room temperature at the end of the heat cycle. During those times of heating and cooling, some bioburden killing occurs, which can be factored into the total time required to sterilize the load. This determination can

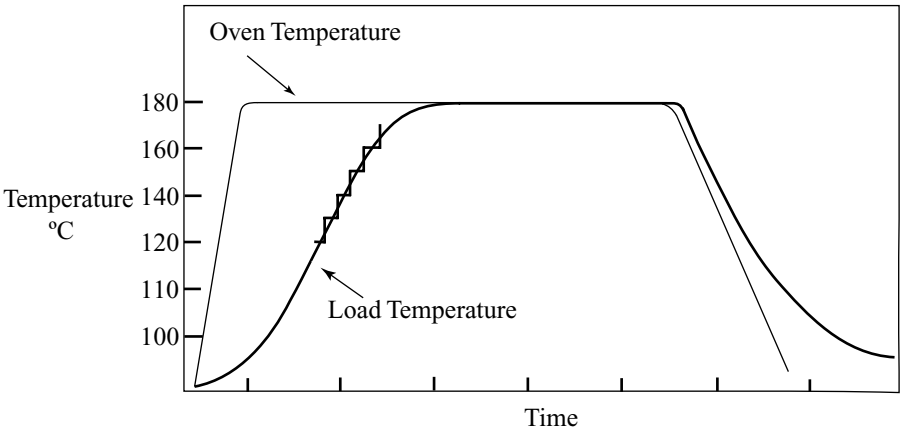

Figure 3.4 Sterilizing a large load. Time–temperature profile of a large load of product undergoing heat sterilization. While the oven heats up quickly, the innermost parts of the load take much longer to reach proper temperature and longer to cool to room temperature. However, some killing will occur as the load is heating and cooling, which can be factored into the total exposure required to sterilize the load. By assuming heating and cooling occur in discrete steps at specific temperatures, the accumulated lethal effects of the heat can be determined. See text for additional explanation.

minimize the overall time the bottles actually need to be exposed to the maximum temperature, saving both time and energy.

Suppose Figure 3.4 was based on heat treatments of an assumed bioburden in our load of bottles. At 180°C, the D-value is 1 min. The time that is necessary to kill the bioburden on the bottles (plus that required for overkill) is then calculated for the sterilizing temperature, 180°C. For example, suppose the bioburden for the entire load is estimated at 10^9 CFU. For an overkill of 10^{-6}, 9 + 6, or 15 D-values, would be needed, which calculates to 15 × 1 or 15 min at 180°C. But, as noted above, the load experiences some killing as it is being heated and while it is cooling down. All temperatures are measured at the innermost point of the load, the slowest heating zone (see Section IV.C. below).

A general example of how to determine the time necessary to sterilize a large load can be presented by assuming the temperature rises in steps during the heating phase as shown in Figure 3.4. For instance, suppose we visualize steps that are 10 min wide, and then estimate the average temperature at each step. From a thermal destruction curve prepared previously (Figure 3.2) we can see that at 140°C, for example, D = about 58 min. That is, the amount of lethality that takes 1 min at 180°C will take 58 min at 140°C or 1/58 of a D-value. Since we are assuming the

load was experiencing an average temperature of 140°C for 10 min, that would be equivalent to 1/58 × 10 or 10/58 of a D-value, or 0.175 D-values. D = about 22 min at 150°C, or 0.45 D-values for the 10 min the load is exposed to that temperature, and so on. D-values are additive, so from 140 to 170°C the accumulated lethality that the bottles have experienced amounts to a total of 5.36 D-values or the equivalent of being exposed to 180°C for 5.36 min. For this exercise let us assume the load experiences the same number of D-values as it is cooling for an overall total of 10.72 D-values. The load has accumulated 10.72 D-values as it undergoes heating and cooling. That leaves 15 − 10.72, or 4.28 D-values, of heat exposure required at the maximum temperature, or 4.28 min. The 15 min of exposure at 180°C have been reduced to 4.28 min, saving over 10 min of throughput time and associated energy.

The exercise just described is accomplished by the use of D-values, which, it should be remembered, are determined under specific conditions that may not represent exactly the conditions within a load of product. Dependence on these calculations must be backed up by validation of the sterilization process. Needless to say, these calculations are now handled by computer. In doing so, the width of the steps can be set close to zero, allowing for an almost infinite number of steps and a much more accurate determination of the effects of the heat on the bioburden.

C. Low Temperatures

Storage of perishable items like foods and heat-sensitive biochemicals at low temperatures is an excellent and widely used method of inhibiting microbial activity. Refrigerators operate at about 5°C and freezers from –20 to –80°C. Generally, mesophilic microorganisms exhibit little or no growth at typical refrigerator temperatures. Psychrophiles and psychrotrophs can grow at these low temperatures, although slowly, which is why refrigerated foods and other perishables still eventually spoil. Household freezers operate around –20°C, while freezers designed for laboratory and industrial use can reach –80°C or lower. Microbial growth is not possible at these low temperatures. Although some viability may be reduced at freezer temperatures, exposure to low temperatures is not a reliable method of decontamination. On the contrary, microbial suspensions are frequently preserved for later use by freezing. Once thawed, cultures of microorganisms that had been frozen will usually resume growth.

D. Radiation

Radiation is electromagnetic energy. It comes in a variety of forms that range from relatively weak radio and television signals to light to enor-

mously energetic gamma and cosmic rays. Certain types of radiation can be harnessed and used to control microorganisms.

1. Ionizing Radiation

Ionizing radiation is radiation of sufficient energy to cause the ionization of atoms and molecules. Alpha-, beta-, gamma-, and X-radiation, and electron beams (E-beams) are all examples of types of ionizing radiation. Because of the relative ease of producing them, gamma radiation and E-beams are the most common types of radiation used for the control of microorganisms. The principal application of ionizing radiation is in the sterilization of drugs, disposable plastic medical devices, bacteriological laboratory ware, and other healthcare products; the decontamination of foods; and the reduction of sprouting in potatoes.

When matter is exposed to ionizing radiation, it absorbs some of the energy of the radiation. The absorbed energy is often expressed in SI units called grays (Gy), or, more often, kilograys (kGy), for doses necessary for sterilization. One gray is equivalent to the absorption of one joule of energy per kilogram. The rad (Rd) is also used in connection with radiation. One rad equals 0.01 gray.

"Cold sterilization" and "picowaving" are terms that are sometimes applied to the use of ionizing radiation for sterilization or decontamination. Typical doses required to sterilize medical devices with a total bioburden of, for example, 10^3 CFU are generally of the order of 25 kGy or 2.5 megarads (mRd), which can be delivered in less than a minute by an E-beam source. (As a comparison, a typical chest x-ray delivers less than 0.001 Gy to the body. The median lethal dose for a human is estimated to be about 4 Gy.) Electron accelerators, giant versions of the electron gun in a television picture tube, provide the E-beams. Sterilization by gamma ray sources, usually cobalt 60 (^{60}Co), requires considerably longer exposures than E-beams because, while gamma radiation is able to penetrate matter to a much greater depth, the resulting energy that is transferred to the target is spread out over a longer pathway. Penetration of matter by E-beams is less, but the energy the target receives is more concentrated, requiring less time to effect sterilization.

Radiation kills cells by destroying vital molecules, principally DNA. When a gamma photon or E-beam electron collides with an electron in a cell, the energy transferred to the electron knocks it out of its orbit and sends the electron on a path where it soon collides with other electrons, resulting in a trail of broken chemical bonds and inactivated molecules. Death of the cell occurs when sufficient critical molecules are destroyed. In addition, toxic free radicals that are formed as a result of ionization

events also contribute to the death of the cell by their reactions with critical molecules.

Because facilities for sterilizing products by radiation are enormously expensive to build and operate, few manufacturers have their own radiation facility. Radiation sterilization most often is contracted out. Radiation sources are installed in heavily shielded rooms to protect the operators from radiation. Products to be sterilized are moved by automation through the radiation beam on a moving belt, on carts, or hanging from a monorail. Contrary to popular opinion, objects sterilized in this manner do not become radioactive.

Because of its great power of penetration, ionizing radiation can often be applied to sterilize items in their final bulk or case packaging. The density of the items is a critical factor in determining whether the radiation will penetrate the innermost spaces of the product and effect sterilization of the product's bioburden. In order to ensure that every item in a load will receive adequate levels of radiation to achieve the prescribed SAL, dose-mapping is conducted. Dose-mapping is a process by which a three-dimensional diagram of radiation levels is determined for the particular arrangement of product that will be sterilized by radiation. Devices known as dosimeters are placed in various locations within the load, and the load is subjected to sterilizing levels of radiation. Measurements of radiation doses are recorded by the dosimeters and are subsequently retrieved for analysis.

Just as we see in the case of heat sterilization, materials used in products that are to be sterilized by radiation must be chosen carefully. The physical characteristics of some materials may be affected by the radiation. Products and their packaging should be tested by exposing them to doses of radiation at least as high as those that they would be subjected to during routine sterilization processing. The products should then undergo thorough testing to determine whether they have retained their intended function. Some effects of the radiation may be unexpected. For example, certain materials may become brittle, and others may produce heat when irradiated, which may cause damage to the product or its packaging.

2. Nonionizing Radiation

Electromagnetic energy of a wavelength in the range of 240 to 280 nm with a peak at 260 nm is highly lethal to microorganisms. This form of radiation is in the ultraviolet light region of the electromagnetic spectrum and is of the nonionizing type, for it is not sufficiently energetic to cause ionization of molecules. This type of radiation is still lethal to living cells. The most convenient source of this type of radiation is low-pressure mercury vapor, or "germicidal" lamps commonly seen in microbiological laboratories.

These lamps emit a large proportion of their energy at 254 nm, which is very close to the absorption maximum of nucleic acids (around 260 nm), the principal target of UV radiation. Germicidal lamps should not be confused with black lights or Woods lamps, which emit ultraviolet light principally in the 320 to 420 nm range. These lamps are intended to activate fluorescence in mineral specimens, chromatography preparations, fluorescence microscopy, and other applications and do not produce significant radiation below 300 nm. The different types of UV lamps can be distinguished by the appearance of the lamp tube or bulb: black lamps have a dark tube, whereas the germicidal lamps have a clear tube.

Except under carefully controlled conditions, ultraviolet light is not a reliable sterilizing agent, and generally can only be used for disinfection. This is because of two characteristics of UV light: it follows the inverse square law, and it is not very penetrating. The inverse square law refers to the fact that as a target moves away from the source of radiation, the intensity of the radiation decreases in a manner inversely proportional to the square of the distance from the object to the lamp. That is to say, if a bacterium should be moved from one inch to two inches from a UV lamp, the intensity of radiation the cell feels would have been reduced to 1/4 of what the cell felt at one inch, and at about three inches, the radiation intensity would drop to almost 1/10 of its power. What that means on a practical basis is that the target organisms must be relatively close to the UV lamp to receive significant levels of radiation.

A second characteristic of germicidal UV light that limits its usefulness is that it does not penetrate dirt, dust, glass, most plastics, most bacteriological media, or more than a few micrometers of cytoplasm. UV light generally cannot be used to sterilize dirty or heavily contaminated objects, solutions in glass or plastic containers, or solutions that are cloudy or contain organic material or dense microbial populations. UV light is successfully used to sterilize clear water and air and the surfaces of clean objects. It can be used to internally sterilize empty bottles of certain types of low-density polyethylene provided that the radiation source is of sufficient intensity. Despite these shortcomings, UV light has some useful applications in the laboratory. One of its most common uses is in the disinfection of confined air spaces such as the interiors of biohazard and transfer hoods, isolators, and air ducts. UV is also used to control microbial activity in water systems, which is discussed later in this chapter.

Great care must be practiced in the presence of operating germicidal lamps, as their radiation is highly damaging to the eyes and skin. In a few seconds a high intensity UV lamp is capable of delivering a painful sunburn that would take several hours to obtain at the beach. Gloves, long sleeves, and a full-face shield should be worn whenever work near operating UV lamps is necessary.

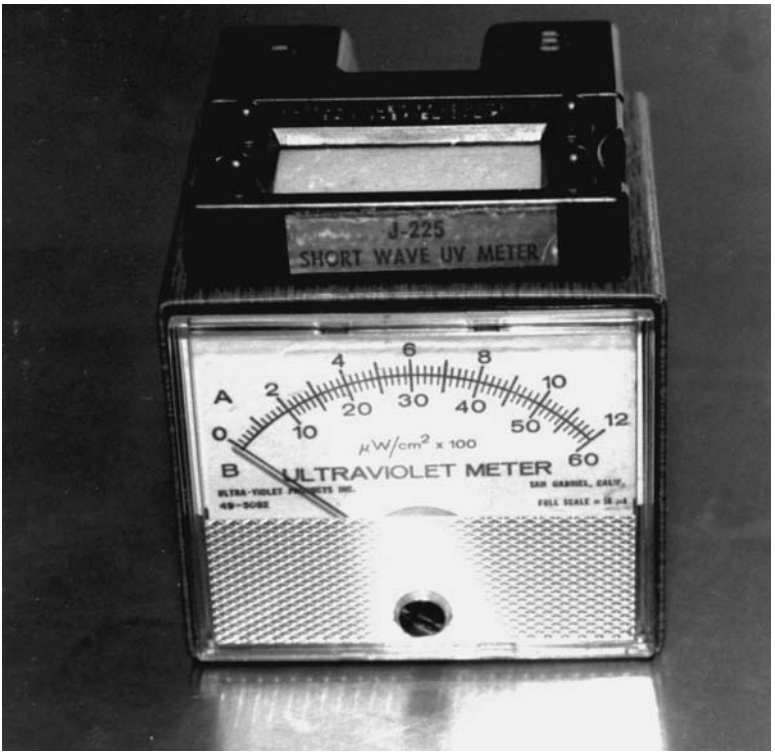

Figure 3.5 An ultraviolet intensity meter.

The output of an ultraviolet lamp should be monitored on a regular basis, since such lamps have a tendency to deteriorate over time. In addition to normal aging, the accumulation of dirt and grease on the outer surface of the lamp can severely reduce delivery of the lethal wavelengths. These lamps must be cleaned periodically. UV light meters are available for monitoring germicidal lamps (Figure 3.5).

Microorganisms subjected to radiation generally follow exponential kinetics similar to what has been described for killing by heat (Figure 3.1), allowing one to predict the effects of a particular dose of irradiation on a given population of microorganisms. Parameters such as D-values can be obtained for the lethal effects of irradiation on microorganisms.

E. Filtration

Filtration often is employed to remove particulate matter, including microorganisms, from ventilation air for laboratory and manufacturing facilities. Filtration is also used to sterilize fluid products that contain such materials

as enzymes, vitamins, and other components that cannot be sterilized by other methods due to heat or radiation sensitivities. The recent dramatic growth of protein-based pharmaceuticals and other heat labile products has caused a considerable increase in interest in sterilization by filtration. There are two basic types of filters: depth and membrane.

1. Depth Filters

Depth filters are composed of thick layers of materials such as glass and polypropylene fibers. Depth filters have the advantages of a high particulate capacity, relatively low operating back pressure, and high throughput, but their pore size rating is usually considered nominal, meaning typically they only are able to retain between 60 and 98% of particles at or above the size for which the filters are rated. Some special types of depth filters that are used to remove particulate contamination from cleanroom air are rated at over 99%. These are described in further detail in Chapter 4. Since depth filters do not exhibit 100% retention, they are only used for decontamination, not sterilization. Depth filters frequently are used as prefilters to capture large particulate matter in solutions prior to membrane filtration.

2. Membrane Filters

Membrane filters enjoy an absolute pore size rating, which means they are able to capture 100% of particles at or above the size for which they are rated. Membrane filters consist of thin sheets of organic polymers such as cellulose nitrate, cellulose acetate, polysulfone, or fluorocarbonate, in which precise, ultrafine pores can be introduced during their manufacture (Figure 3.6). Typically, membrane filters come as sheets, discs, and pleated sheets in cartridges in a variety of sizes and ratings. Membrane filters with a rated porosity of 0.20 or 0.22 μm typically are used for the sterilization of most liquids. Membrane filters qualified to sterilize pharmaceutical products must be able to retain 100% of a specified population of a species of bacterium known for its small size, *Brevundimonas* (*Pseudomonas*) *diminuta*.

Because of their size, most viruses are not retained by the filters rated at 0.20 or 0.22 μm, nor are mycoplasmas. If these microorganisms must be eliminated, filters of smaller porosity ratings or other methods of sterilization must be used.

3. Mechanisms of Filtration

Besides their physical and chemical differences, depth and membrane filters also differ in the principal mechanisms by which they capture

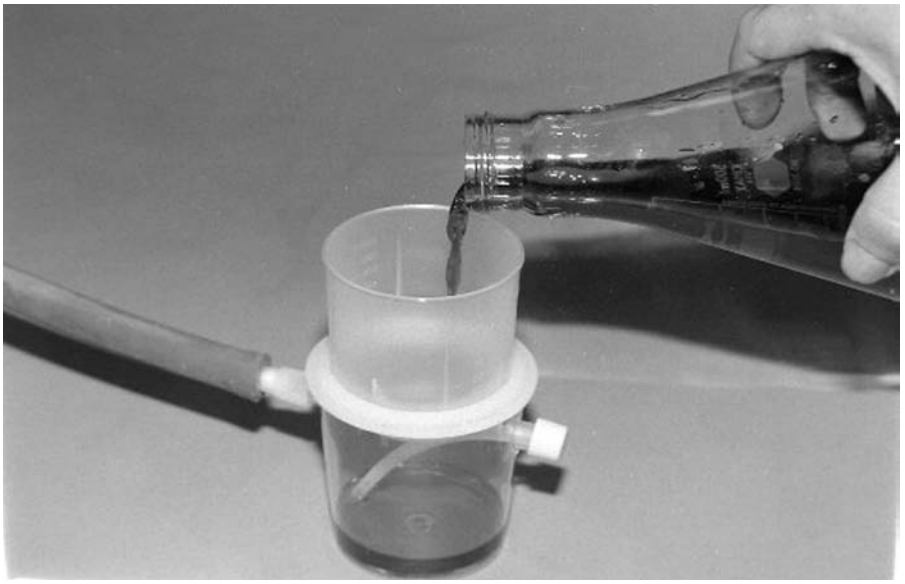

Figure 3.6 Sterilization by filtration. A solution can be sterilized by passing it through a membrane filter with a porosity of 0.22 μm.

particles. There are multiple mechanisms that operate in depth filters. Particles holding an electrostatic charge opposite that of the filter medium will be attracted to and held by the filter. Filter media can be manufactured with a permanent electrostatic charge or it can be applied to an in-place filter by an external electrical source. Particles small enough to be affected by Brownian movement may be trapped by diffusional collection. Due to their heightened diffusion rate, these particles may experience a high rate of collisions with the filter medium during their travel through the filter and are eventually captured. Larger particles are retained through inertial impaction. As they follow a circuitous path through the fibrous matrix of the filter medium, the inertia of large particles often will cause them to collide with the filter medium as the air or fluid stream they were riding is deflected sharply by the medium. Large particles also may be trapped physically by the medium by sieving; that is, the particles are just too big to pass through the spaces between fibers of the medium and are retained. The relative participation of each of these mechanisms depends on such things as qualities of the particles, the nature of the filter medium, and properties of the liquid or gas and its velocity as it passes through the filter.

Sieving appears to be the principal mechanism by which membrane filters operate. As mentioned above, the filters are manufactured with very narrow pore size ranges. Generally, particles larger than the pores cannot

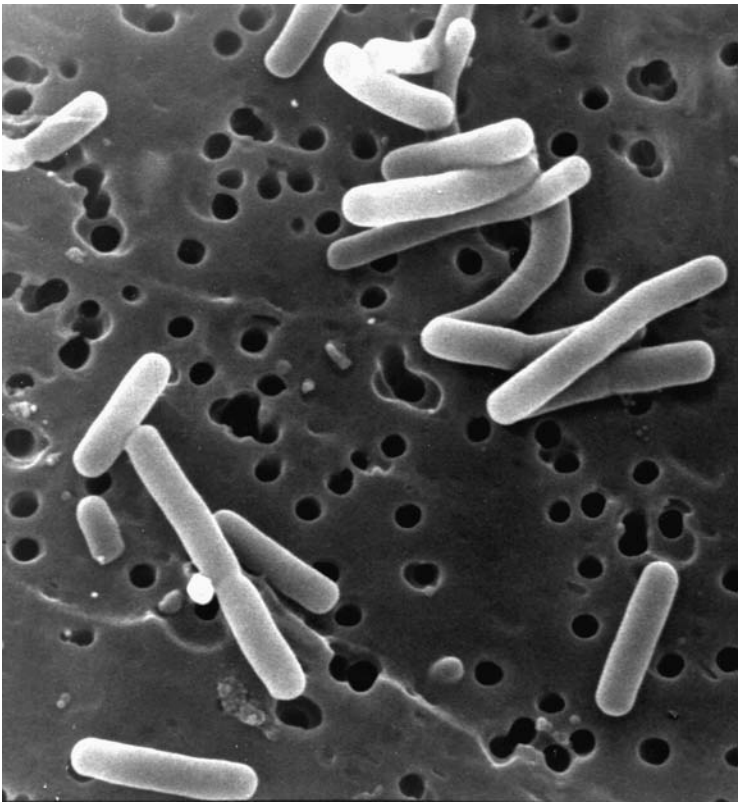

Figure 3.7 Bacteria collected on a cellulose acetate membrane filter rated at 0.22 μm.

pass through the filter and are retained (Figure 3.7). Other mechanisms listed above in connection with depth filters probably also play roles in membrane filters, such as adsorption and electrostatic attraction. For example, pyrogens may be retained through electrostatic attraction by some membrane filters even though pyrogens are considerably smaller than the rated porosity of the filters.

4. Validation of Filtration Sterilization

In validating filters for sterilization of liquids, the test liquid normally used is the actual product with added challenge bacteria. The use of the product as the suspending medium is recommended because the size, shape, and other features of the test organisms and their capture characteristics may be altered depending on the fluid in which they are suspended. Furthermore, some of the secondary mechanisms of retention

of the filter medium such as electrostatic attraction may also be affected by the nature of the product.

Compatibility is an important consideration when choosing a filter material that will come into contact with raw materials or product. Filter media are not totally inert and may adsorb constituents from the product or otherwise alter the product. Membrane filters may release chemical contaminants (known as extractables) into the filtrate, including chemical remnants from their manufacture, wetting agents, plasticizers, and endotoxins. Thorough testing must be carried out before adopting any specific filter medium.

IV. ENSURING STERILITY

It usually is not possible to prove that a batch of bacteriological media or a load of manufactured items is sterile without testing each item and, by doing so, compromising its sterility. Most standard sampling protocols call for a certain fraction of the items to be tested and the results extrapolated to characterize the entire lot. This approach is discussed in Chapter 5. Another technique is to validate the process of sterilization to affirm sterility of the items. In the manufacture of healthcare products, for example, there are two basic approaches that can be applied to validating the terminal sterilization of a product. These approaches are known as the overkill method and the bioburden method.

A. Overkill Method

In applying the overkill method, the product is subjected to sterilization conditions (heat or radiation) that would yield a specified SAL or overkill, such as 10^{-6}. The D-value used would be based on the assumption that the product is contaminated with a bioburden of a certain number of the most resistant bacterial spores. The particular time and temperature to be used are determined by the use of a biological indicator that would simulate the bioburden. Biological indicators (BIs) consist of known numbers of bacterial spores of known D-value. The spores have been applied to strips of filter paper or plastic and are enclosed in glassine envelopes or are sealed in vials. Prior to sterilization the BIs are inserted at various locations within the load, including the slowest heating zone. At the completion of the sterilization cycle, the BIs are retrieved to assess whether sterilization conditions were in fact met in the entire load. The spores in the BI are derived from bacterial species that are known to be exceptionally resistant to the specific type of sterilization to be tested: moist or dry heat, sterilizing gases, or radiation (Table 3.3). Since the actual bioburden of the product is not likely to consist entirely of large

Table 3.3 Sterilization biological indicators

Sterilization Method	*Biological Indicator (Spores)*
Moist heat (autoclave)	*Geobacillus stearothermophilus*[a]
Dry heat	*Bacillus atrophaeus*[b]
Ethylene oxide	*Bacillus atrophaeus*
Gamma radiation	*Bacillus pumilus*

[a] Formerly named *Bacillus stearothermophilus*. [b]Formerly named *Bacillus subtilus* var. *niger*.

numbers of extremely heat-resistant spores, such an overkill treatment is considered sufficient to sterilize the product well beyond the desired overkill of 10^{-6}; that is, the method represents a worst-case scenario. An overkill cycle can only be applied to products that can withstand the conditions (heat or radiation) necessary to achieve the target SAL. Should one have to deal with a more labile product, the bioburden method may be an alternative choice.

B. Bioburden Method

In the bioburden method, one must determine the actual bioburden of the product set for sterilization. Both numbers and relative resistance (that is, D-values) of the most refractory species in the bioburden population would have to be determined. The sterilization cycle would then be calculated based on that information together with the target SAL. Because of the need for continual laboratory testing of bioburdens and determinations of associated D-values for every batch of product to be sterilized, this method involves considerably more cost in terms of labor and materials than the simpler overkill method.

Methods of determining bioburdens are discussed in Chapter 5. Clearly, to sterilize objects with low bioburdens would require shorter exposure times to the lethal agent than would be needed for heavily contaminated objects. The ability to accurately match sterilization conditions to an actual bioburden provides many practical advantages. A major advantage of this approach is avoiding subjecting a product to excessive heat or radiation. On the other hand, if calculated sterilization conditions are off the mark and result in undertreated products, the products may suffer from shortened shelf lives due to the activities of contaminants that have survived treatment. For healthcare products, undertreated products create risks for consumers and liability for the manufacturer. Unnecessarily long exposures of products to sterilization processes raise costs both in terms of reduced throughput and increased energy consumption. Furthermore, many prod-

ucts cannot tolerate excessive exposures to heat or other sterilization agents. In spite of these points, the bioburden method does not appear to be applied as often as the overkill method in many industries involved in the manufacture of sterile products.

C. Validation of Sterilization Processes

The sterilization process must be monitored to determine that all of the equipment is operating properly and procedures associated with sterilization are followed carefully. Various monitoring methods can be used, such as placing electronic heat sensors (thermocouples, resistance temperature detectors, or similar devices) within a load of items to be sterilized to confirm that the items reach the proper temperature. The exact locations that are selected for monitoring should be representative of the load, but, most importantly, those that are the most difficult to heat, the slowest heating zones, should be monitored. The use of heat sensors is generally acceptable with dry heat sterilizers, but, as we have pointed out, moist heat sterilization depends on the combination of heat and moisture. Consequently, an item may have reached 121°C in an autoclave, but unless the correct amount of moisture is present, the item may not be sterilized.

In monitoring chemical sterilization, remote sensors can report concentration of the agent, humidity, and temperature as indicators that sterilization conditions were met. In addition, one may use paper or plastic strips that are impregnated with chemicals that change color when exposed to ethylene oxide, for example, confirming that the sterilant had reached the indicator. These are known as chemical indicators.

Biological indicators (BIs), described earlier, provide additional validation of a sterilization process. The indicators are placed in the innermost region of a load of items properly packaged for sterilization and retrieved following the sterilization procedure and tested for viability by transferring them to an appropriate growth medium. Signs of viability (nonsterility) can be turbidity in the test medium, or, for those BIs in which a medium is included, incubation indicates growth, which is detected by a color change in the medium. Since the indicators represent a worst-case situation in terms of numbers, levels of resistance, and location in the sterilizer, sterilization of the indicators generally assures that the entire load has been sterilized. The use of BIs is based on the assumption that the conditions (sterilant concentration or radiation intensity, temperature, humidity) experienced by the spores in the BIs were an accurate representation of those conditions actually felt by the bioburden of the objects or materials being sterilized.

The reliability of using commercial BIs in a specific situation may be questioned if it is shown that the conditions of the load or the nature of

the products are sufficiently different from those used to determine the resistance of the BI. For example, suppose the product is made of fiberglass or coconut fiber while the D-values of the BI were determined with spores dried on stainless steel. The relative susceptibility of microorganisms toward lethal agents such as heat and chemicals often depends in part on the surface on which they repose. Through validation tests for the examples cited above, it would have to be shown that the nature of the product would have no effect on the relative resistance of the organisms that make up the bioburden.

The objects or material to be sterilized may be contaminated with organisms that are more resistant to the sterilization conditions than those in the indicator. If this is the case, then the contaminating organism must be isolated and used as the biological indicator for sterilizing the objects. Along those same lines, if the numbers of contaminating organisms that make up the bioburden exceed those in the biological indicators, but have the same level of resistance as those in the BI, appropriate adjustments of sterilizing conditions must also be made.

Complete documentation that all processes are working properly is known as "validation." Maintaining complete validation records, preferably in electronic form for easy retrieval and tracking, is a critical part of the plant microbiologist's responsibilities.

D. Maintaining Sterility

The continued maintenance of the sterility of items for the period until they are used is an important extension of all sterilization procedures. For laboratory glassware to be used immediately, loose-fitting metal caps, screw caps, or cotton plugs are adequate, but for items that are to be stored for extended times, including commercial products, additional coverings of foil, plastic, or paper should be applied prior to sterilization to protect the items from accumulated dust. As a general rule, items should be used as soon as possible after they are sterilized. Packaging of sterile items must be compatible with the type of sterilization used. Paraffin film would obviously not be appropriate for heat sterilization, and foil must not be sealed too firmly on empty vessels or other items for steam sterilization so the steam is not prevented from entering them. For wrapped items, the reliability of the packaging must match or exceed that of the sterilization procedure. Material for wrapping must not be permeable to external microbial contamination, and it must be tough enough to avoid damage. Sterile packages must be carefully examined before the items are used, and any that show the slightest damage must be repackaged and resterilized or discarded. Packages must be designed so that when they are opened the objects inside are not inadvertently contaminated.

Double and triple wrapping may be appropriate in some cases. For certain types of packaging with limited shelf lives, expiration dates must be determined and shown on the items.

V. CHEMICAL METHODS OF CONTROL

The use of chemicals to control the activities of microorganisms goes back at least 5000 years, when humans discovered that salt would retard food spoilage. The first application of chemicals to prevent infectious diseases appears to have occurred early in the 19th century with the introduction of antiseptics and disinfectants in the practice of surgery. This predated the establishment of the germ theory of disease by nearly a half a century. Present-day uses of chemicals to control microorganisms are encountered in as diverse applications as food and beverage manufacturers, retailers, restaurants, manufacturers of healthcare products, healthcare providers, the construction industry, the military, and the consumer market. While biocleanrooms and contamination control technologies are encountered in many of these fields, generally cleanroom design has yet to achieve truly microorganism-free facilities that would assure completely microorganism-free environments or products. Consequently, the application of antimicrobial chemicals is a necessary supporting activity in every biocleanroom.

A. Definitions

Antimicrobial chemical agents for controlling environmental microbial activity are generally classified into four categories: disinfectants, antiseptics, sanitizers, and sterilants. Antibiotics, another category of antimicrobial chemical, are used in media for growing fungi. They serve to inhibit bacteria that may overgrow more slowly growing fungi. Antibiotics will be covered briefly later in this chapter. Antimicrobial chemicals are supplied as liquids, water-soluble powders, or gases. Disinfectants, sanitizers, and antiseptics are relatively effective in killing or inhibiting the vegetative forms of microorganisms, but have little effect on bacterial spores. Tuberculosis bacteria are also known to be highly resistant to these products.

The word “sanitizer” is occasionally used interchangeably with disinfectant. Although the same chemical often may be labeled as both a sanitizer and a disinfectant, the terms are used quite differently as reflected in the tests used to validate their efficacies. For example, challenge organisms and performance criteria for products labeled as sanitizers are very different from those sold as disinfectants. Preservatives are chemicals that are added to foods, pharmaceuticals, and other products to kill or prevent the multiplication of microorganisms that may be present and

cause impairment of the product. Sterilants are chemicals that are sufficiently powerful to sterilize objects; that is, to free them of all living organisms including bacterial spores. Chemical sterilants are usually highly reactive toward tissue and some materials and must be handled with care.

The names of classes of chemicals that kill microorganisms frequently end with "-cide," with the root of the word referring to the kind of organism affected. Agents that kill bacteria (vegetative forms only), fungi, or viruses may be labeled as bactericides, fungicides, or viricides, respectively. Considerable overlapping occurs. For instance, many bactericides exhibit fungicidal and viricidal activity as well. Only those chemical agents that are fully lethal toward bacterial spores within a relatively short time may be labeled sporicides or sterilants.

If an agent only prevents growth of the organisms, but does not kill them, the suffix "-stat" is used: bacteristat, fungistat, etc. (There cannot be viristats, since viruses are not capable of independent growth.) Some chemicals are -cides at high concentrations, but become -stats at lower concentrations. Since exposure to -stats does not kill microorganisms, growth normally resumes when the agents are removed and the organisms are placed in an appropriate growth medium.

B. Factors Influencing Antimicrobial Chemical Agents

Just as in heat sterilization (Table 3.1), there are many factors that alter the effectiveness of chemical disinfectants and sterilants. Time, temperature, and concentration are parameters that are under the control of the user. Time of exposure is critical because, just as we saw in the case of heat killing, microorganisms do not die instantaneously in the presence of antimicrobial chemicals, but succumb gradually over time. Sufficient time must be allowed to complete chemical disinfection or sterilization. The labeling of antimicrobial products should show the required contact time for a given formulation. As a general rule, elevated temperatures improve the effectiveness of chemical agents, provided the agents do not decompose or evaporate too rapidly. Increasing the concentration of an antimicrobial agent will not necessarily improve its effectiveness. "More is better" is not always the rule. For example, 70% ethanol is more effective as a disinfectant than 100%. It is imperative that manufacturers' instructions be followed carefully with regard to the proper concentration for specific disinfectants in specific applications.

Disinfectants are usually supplied as concentrates that are to be mixed with water or water–alcohol mixtures to produce working solutions. Since the water normally makes up a major proportion of working solutions, the quality of the water is of immense importance. For example, some disinfectants are inactivated by the mineral content of hard

tap water. Also, tap water has been known to contain bacteria that are capable of growing in many types of disinfectant solutions. Even distilled water, if allowed to stand for any extended time, may accumulate significant levels of bacteria. Consequently, most antimicrobial chemicals are best diluted with sterile, purified water (PW) or water for injection (WFI). Manufacturers' literature should be consulted for specific formulations. Care must be practiced when making up solutions with hot water. Some disinfectants such as hypochlorite and hydrogen peroxide become unstable at elevated temperatures and should be diluted with room-temperature water.

Excipients such as surfactants (wetting agents), buffers, acids, or bases and chelating agents are frequently added to antimicrobial formulations to increase efficacy. This is most common in so-called one-step disinfectants that combine the disinfecting agent with components that increase the cleaning ability of the products.

The age of a working solution is another critical consideration. Working solutions of many chemical antimicrobial agents have limited shelf lives, some as short as two days. All working solutions must have the preparation and expiration dates clearly marked on the container. Different chemical agents should never be mixed together unless approved by the product manufacturers. The effectiveness of an antimicrobial agent depends on its chemical activity, which can be destroyed if mixed with incompatible material. By the same token, soil on items to be treated may have a neutralizing effect on some antimicrobial agents. In this context, soil usually refers to concentrated organic matter such as bacteriological media, grease, blood, serum, and fecal matter. Depending on the agent, such soiled items may have to be precleaned before they are treated with the antimicrobial agent.

Other factors that severely impact the effectiveness of a disinfectant include the type and age of the microorganisms that are to be eliminated. A few generalizations follow: As has already been pointed out, bacterial spores can be orders of magnitude more resistant to chemical agents than vegetative cells. An object contaminated with bacterial spores will not be disinfected significantly by most chemical agents. The vegetative cells of Gram-negative bacteria tend to resist antimicrobial agents better than Gram-positive cells, while members of the genus *Mycobacterium* (including the species that causes tuberculosis) are eminently successful in resisting many disinfectants. Nonenveloped viruses tend to be more resistant to chemical agents than lipid-enveloped viruses. Fungi are more resistant toward disinfectants than are vegetative bacterial cells. Fungal spores, while somewhat more resistant than vegetative forms, do not approach the resistance shown by bacterial spores.

C. Disinfectants

1. Characteristics of Specific Agents

Quaternary ammonium compounds, phenolics, halogen compounds, dilute solutions of hydrogen peroxide, and alcohols are some of the more popular disinfectants encountered in biocleanrooms.

Quaternary ammonium compounds (quats) are extremely popular, broad-spectrum disinfectants and sanitizers. Examples are benzalkonium chloride and cetyl pyridinium chloride. They are frequently one of the active agents in one-step cleaners/disinfectants. Even alone, quats, which are detergents, owe some of their antimicrobial activity to the physical removal of cells, but they are also moderately effective bactericides, viricides, and fungicides in their own right. At concentrations as low as 0.0005%, quats can still be bacteristatic. Since they are cationic, the action of quats appears to involve the penetration of the negatively charged cell membrane, leading to its disruption. Most popular mouthwashes contain these agents, which attests to their low toxicity for humans. However, quats also show low toxicity toward certain types of bacteria, such as pseudomonads and tuberculosis bacilli; consequently, they cannot be relied upon to disinfect medical instruments or for similar critical applications where this type of contamination may occur. The quats are not effective against bacterial spores. Quats can be inactivated by phenolics, anionic detergents, and some surfactants (wetting agents), meaning that products containing quats must never be mixed with or alternated with other products containing these interfering materials. Quats must always be diluted in deionized or distilled water, since they are also inactivated by hard water.

Phenol (carbolic acid) is an effective disinfectant, but its toxicity and corrosiveness have severely limited its general use. Derivatives of phenol, known as phenolics, such as *o*-phenylphenol and *p*-tertiary amyl phenol, are considerably less toxic, more effective than phenol, and very popular for decontaminating hard surfaces of equipment, furniture, and floors. Phenolics show little effect on bacterial spores. Hexachlorophene, another derivative of phenol, is a good skin antiseptic, but its use must be carefully controlled. It is absorbed through damaged skin in adults as well as the normal skin of young babies and can be highly damaging to the central nervous system. The antimicrobial action of the phenolics appears to be related to their ability to precipitate proteins, but even at very low concentrations, when precipitation would normally not occur, the phenolics retain some antimicrobial activity. This appears to be related to their additional capacity to disrupt cell membrane function, preventing essential nutrients from entering the cell or causing leakage of cell contents. It has

been reported that the phenolics are not compatible with nonionic or cationic detergents.

Chlorine and iodine belong to a group of chemical elements known as halogens. Chlorine is a highly toxic gas at room temperature and is used as a disinfectant of water for domestic consumption. However, its use is being drastically reduced because of evidence that shows it may be responsible for the formation of carcinogens in the presence of high levels of organic matter, a condition becoming more common in natural water sources. Inorganic chlorine compounds, principally hypochlorite (ordinary laundry bleach), are also widely used as disinfectants and sanitizers. In the presence of water, hypochlorite ($HClO^{3-}$) is converted to hypochlorous acid (HClO), a powerful oxidizer that is highly microbicidal. The principal effect of hypochlorite seems to be the oxidation of sulfhydryl groups that are essential for the functioning of many cellular proteins. Hypochlorite also may form chlorinated carcinogens in the presence of organic matter.

Common applications of chlorine and its compounds as disinfectants other than for water supplies are as disinfectants and sanitizers in biocleanrooms and in operations involved in the production and handling of food and beverages, including restaurants and dairies. Chlorine compounds are frequently used as sanitizers in a final rinse for eating utensils and glassware. Diluted hypochlorite solutions tend to be unstable, resulting in short shelf lives. Working solutions should always be prepared fresh daily. Commercial bleach (5.25% hypochlorite, 52,500 ppm available chlorine) solutions diluted 1:100 are useful disinfectants on clean surfaces, but if significant amounts of soil are present, a 1:10 dilution is recommended. Heavily soiled objects are best cleaned before decontamination. Hypochlorite solutions show some limited sporicidal activity at elevated concentrations but cannot be relied upon to sterilize objects. Chlorine dioxide and organic chlorine compounds known as chloramines have replaced gaseous chlorine and hypochlorite in many applications where the formation of carcinogens is to be avoided. An advantage of chlorine dioxide is that it can be generated in appropriate amounts at the time of application, avoiding the need to store large volumes of hazardous chemicals. Chlorine dioxide is discussed further in the section on sterilants.

Iodine has been a familiar household antiseptic for generations, usually as an alcoholic solution known as a tincture. Iodophors, organic compounds of iodine, have replaced tincture of iodine for the treatment of wounds, since they are less irritating to open tissue, earning them the nickname “ouchless” iodine. Iodine and its compounds are good skin antiseptics since they are bactericidal and fungicidal, and they are widely used on patients’ skin before surgery. Other uses include sanitization of water and food-handling equipment.

The halogens and their compounds are strong oxidizers. Consequently, widespread industrial use of the halogens for the control of microorganisms has been limited due to their corrosiveness toward some metals.

Hydrogen peroxide has long been a popular antiseptic and disinfectant. Like chlorine and its compounds, it is a strong oxidizer. Used in concentrations of 3 to 6%, it is a reliable disinfectant on clean objects, but it is unstable in the presence of organic debris. At higher concentrations or as a vapor, hydrogen peroxide is an effective sterilant (see below). Solutions of hydrogen peroxide are unstable and are best kept refrigerated.

Alcohols, such as ethanol and isopropanol, as sterile, aqueous solutions are effective and widely used as decontamination agents. As in the case with cationic detergents, the effectiveness of alcohols appears to be due to a combination of cleansing action on alcohol-soluble soil and their antimicrobial activity. As organic solvents, their bactericidal effectiveness is probably partly due to their ability to extract lipids from the bacterial cell membrane and thereby destroy its function as well as denature cellular proteins. Water solutions of about 70 to 90% (by volume) of ethanol or isopropanol are bactericidal against vegetative cells, but have little effect on bacterial spores. The volatility of alcohols is both advantageous and disadvantageous. They leave no residue (except some types of denatured ethanol), but because they have a tendency to evaporate quickly, exposure time is often diminished unless they are applied in generous amounts and at room temperature.

Two additional potential downsides are related to the volatility of the alcohols. When used copiously, the alcohol vapors that are emitted from a facility add to the atmosphere's load of volatile organic compounds (VOCs) and may represent a significant contribution to air pollution. In addition, illnesses have been reported among workers exposed to elevated concentrations of alcohol vapors for extended periods of time, so use should be limited to well-ventilated areas.

2. Selection of Disinfectants

About 10,000 disinfectants are registered with the Food and Drug Administration, which closely monitors their labeling. However, what does not appear on a label is frequently more important than what does appear. Users should not assume capabilities of a disinfectant unless they are specifically and unequivocally expressed on the label or in the manufacturer's literature. A product that is labeled simply as a "germicide" or "disinfectant" without any further clarification may be effective only against relatively sensitive bacterial species but useless against more resistant species such as tuberculosis bacteria and *Pseudomonas*.

It must always be assumed that a given disinfectant is not sporicidal unless the manufacturer's literature specifically states that it is. Sporicides must be clearly labeled as such. Beware of products that claim to kill spore-forming bacteria, or words to that effect. This simply means that they kill the vegetative forms of the spore formers. Nor can one rely on products to kill viruses, fungi, or tubercle bacilli unless they are specifically labeled as such. In all cases, one should look for results of standard tests that back up the claims and then validate the products by using the same or similarly recognized tests.

3. Validation of Disinfectants

Some of the conditions that a manufacturer of a antimicrobial chemical agent must list when claiming the efficacy of a formulation are time of contact necessary to achieve the antimicrobial effect claimed and the condition of the organisms; that is, were they in an aqueous suspension or dried on a hard surface? Also included must be whether soil was present and how much, the diluent that is required (distilled or tap water or alcohol) to prepare working solutions, and the recommended temperature range during application.

The AOAC use dilution method is the most common test of the efficacy of a disinfectant. A major advantage of this test is that it uses the very concentration of formulation that actually would be used (the use concentration). Older tests (i.e., the phenol coefficient test) often used concentrations that had no relation to the use concentration. The AOAC test involves disinfectant exposure by test organisms that have been dried on a specified number of small, sterile, stainless steel or porcelain cylinders, simulating environmental surfaces common in cleanrooms, hospitals, and laboratories. Following exposure to the disinfectant for a specified time, the cylinders are placed into a growth medium that contains substances that neutralize the disinfectant. The disinfectant passes the test if no growth is observed after a prescribed period of incubation. Dried control samples must contain at least 10^4 CFUs.

Disinfectants that claim a broad spectrum or general efficacy are tested against *Staphylococcus aureus* and *Salmonella choleraesuis*, which represent typical Gram-positive and Gram-negative pathogens, respectively. In addition to these organisms, a successful test against *Pseudomonas aeruginosa* will gain the product's acceptance for use in medical settings. *Pseudomonas aeruginosa* is a common soil and water inhabitant and ubiquitous airborne contaminant. This organism is a cause of wound infections, particularly in burn patients, and is known for its resistance toward many common disinfectants and antibiotics. It is also prudent to use organisms as test

cultures that are isolated from the facility in which agents are to be used in the event that especially resistant species are present.

Variations of the AOAC test involve the use of glass carriers in place of stainless steel or porcelain, and the use of other test organisms such as fungi and *Mycobacterium bovis*, a stand-in for the human tuberculosis bacterium. When testing the activity of a disinfectant toward viruses, the organisms are dried onto the bottoms of glass Petri dishes, on glass slides, or on other hard-surfaced carriers and then exposed to the product. Again, the disinfectant must be tested at its use dilution and other conditions must simulate actual usage. Following exposure, the viruses are quantitatively scraped off the glass, diluted, and subjected to a plaque assay as described in Chapter 2 or using another appropriate method to determine extent of inactivation. If a product is labeled specifically as viricidal, 100% inactivation per this test protocol is required.

4. Rotation of Disinfectants

It generally has been assumed that if a facility were to use the same disinfectant for extended periods of time, the resident population of microorganisms would eventually develop resistance toward the agent in a manner similar to what is observed when organisms are exposed to sublethal doses of antibiotics over a long period of time. Resistance to any antimicrobial agent acquired by a microorganism is caused by genetic changes. Because of the rarity of such changes, it generally requires large numbers of organisms for these changes to occur. Since large populations of microorganisms are not normally found in cleanrooms and other controlled facilities, it is not likely that the resident microorganisms would develop resistance to a particular disinfectant. Unless one has strong evidence that such resistant strains are appearing, switching to another disinfectant may cause more problems than staying with the same one. One reason for this is that many types of disinfectants are incompatible. Nearly all disinfectants leave a residue on surfaces on which they are used. If one were to switch to an incompatible agent on those surfaces, yesterday's residue might neutralize today's disinfectant, or at best the two agents may form an insoluble film that would make cleaning difficult. In addition, such films may protect populations of microorganisms from the effects of later applications of a disinfectant. It is therefore prudent to test resident microbial populations regularly for signs of resistance, and only if resistance is detected, switch to a compatible, substitute disinfectant. The new disinfectant must operate under a mechanism different from the old agent; otherwise, the organisms may be resistant to the new disinfectant as well. In summary, switching disinfectants should be avoided except under very compelling circumstances.

Rinsing of surfaces to remove residues left by disinfectants may be justified under certain circumstances, but the practice also may create problems. Unless done carefully, rinsing may recontaminate surfaces that have just been chemically decontaminated. This practice can be validated by microbiologically sampling the surfaces before decontamination and before and after rinsing. This procedure is covered in Chapter 5.

D. Sterilants

Sterilants are chemical agents that are sufficiently lethal toward microorganisms to sterilize objects treated with them. The ideal sterilant should have at least the following characteristics:

1. Demonstrates sufficient toxicity toward microorganisms, including bacterial spores, to kill all organisms in a relatively short period of time
2. Has little or no toxicity toward humans
3. Causes little or no damage to materials
4. Shows good penetrating power
5. Leaves little to no residue

The ideal chemical sterilant does not exist, and only a few chemicals come sufficiently close to fitting these criteria to be considered reliable enough for widespread use. Some of these are formaldehyde, ethylene oxide, glutaraldehyde, chlorine dioxide, hydrogen peroxide (vapor phase and solution), and peracetic acid, of which the last three are the most common ones encountered in biocleanroom applications.

1. Characteristics of Specific Agents

Formaldehyde is a colorless, highly toxic gas with an acrid odor. It is very soluble in water. A 37 to 40% (by weight) solution is known as formalin. Formaldehyde can be used either as a solution (usually 4 to 8%) or in the gaseous form. The gaseous form has been widely used to sterilize air spaces such as laboratories, hoods, biosafety cabinets, isolators, and air-handling ducts. The gas is most conveniently generated by heating the solid (flake) form of the chemical, paraformaldehyde, on an electric hot plate at 230°C in the space to be treated. The volume of the space is estimated and then completely sealed with tape and heavy plastic sheeting to prevent loss of the gas and to avoid danger to personnel in the surrounding facility.

The recommended amount of paraformaldehyde to use to sterilize a specific air space is 300 mg for each cubic foot of space to be treated.

Optimum conditions are a temperature of about 20 to 22°C and a relative humidity of 60 to 80%. The humidity can be raised by generating steam in the space just before the formaldehyde is released. This is accomplished by boiling a quantity of water on a second hot plate. Following treatment, the space must be thoroughly ventilated before it is used to allow outgassing of any residual formaldehyde, which can take as much as a week for some facilities. Generators for fogging air spaces with formaldehyde are available. They have the additional feature of releasing a neutralizer following the sterilization, thereby allowing an earlier occupation of the exposed space. The future use of formaldehyde remains in doubt since, in addition to its irritating odor and toxicity, evidence has accumulated that it is a possible carcinogen.

Glutaraldehyde is frequently used in dilute (around 2%) solutions to sterilize small medical devices, catheters, and the like. It must be activated with acid before use. Treated items must be rinsed with sterile water since, like formaldehyde, glutaraldehyde leaves a residue. To avoid exposure to glutaraldehyde residue by personnel, surfaces on which the agent was used also must be rinsed.

Ethylene oxide (ETO) is also a gas, but unlike formaldehyde, it has very limited solubility in water. It is frequently mixed with carbon dioxide, nitrogen, or a fluorocarbon to reduce the fire hazard, as it is highly flammable. It is an excellent general sterilant with an enormous ability to penetrate plastic or paper wrappings. Products such as bacteriological laboratory ware, surgical items, and medical devices may be sterilized by ETO in their final bulk packaging, but afterward they must be subjected to a lengthy quarantine period to allow for outgassing. ETO sterilization is carried out in airtight chambers similar to steam autoclaves. Commercial ETO chambers may exceed 4500 ft^3 in volume. Due to the time necessary for the gas to penetrate the innermost areas of a load of items, ETO sterilization can be slow, requiring 24 h or more for a large load. Humidity and temperature are also critical factors in using ETO. In spite of its shortcomings, about half of all medical supplies manufactured in the United States are sterilized with ETO.

ETO is used to sterilize many nonmedical items like spices, cosmetics, and museum artifacts. Growing evidence of the carcinogenicity of ETO has curtailed its use where repeated exposure to workers may occur. Use of ETO that is mixed with fluorocarbons has also been under criticism due to the damage fluorocarbons cause to the Earth's ozone layer.

Chlorine dioxide has recently been added to the short list of reliable chemical sterilants. It is a gas at room temperature, but almost impossible to use as such because of its toxicity and unpleasant odor, which has been described as a mixture of chlorine and fuming nitric acid. Aqueous solutions of chlorine dioxide, produced by mixing sodium chlorite with

an organic acid, are more often used, but with a shelf life of less than 48 h, they must be prepared fresh just before they are needed. Chlorine dioxide is known to damage aluminum and polycarbonate. Chlorine dioxide spray was used to decontaminate the postal facilities and other offices that had been contaminated by anthrax spores in the 2001 bioterrorism incident.

Hydrogen peroxide, both as an aqueous solution and in the vapor phase (VHP), is used to sterilize enclosed spaces such as biological safety cabinets and isolators (see Chapter 4). Humidity control is critical when VHP is applied, and a period of outgassing is necessary to allow adsorbed vapor to release from certain materials. Hydrogen peroxide is somewhat unstable, shortening its shelf life. An advantage of hydrogen peroxide over other sterilants is that any residue left behind breaks down to oxygen and water. It also is less corrosive toward metals and polymers than other oxidizers.

Peracetic acid has been used as an effective spray to sterilize enclosed spaces such as isolators and biohazard cabinets. Mixtures of hydrogen peroxide and peracetic acid have been used as sterilants, but they require long exposure times for full activity and they are somewhat corrosive toward soft metals. Due to the pungent odor of peracetic acid and its potential toxicity toward personnel, all formulations containing the agent must be used with caution.

E. Antibiotics

Antibiotics are discussed separately from disinfectants and other antimicrobial chemicals for at least two basic reasons, which are explained in the following definition. Antibiotics are chemicals that are produced by microorganisms that, in very small amounts, kill or inhibit other microorganisms. That is, antibiotics are natural compounds and are extremely effective in very low concentrations. Whereas working solutions of common disinfectants are relatively concentrated, antibiotics are effective at microgram levels. Antibiotics often act by inhibiting specific biochemical pathways in microorganisms such as DNA or protein synthesis. This ability can be a very valuable research tool, as described below.

We normally associate antibiotics with the treatment of infectious diseases, but most antibiotics manufactured today are actually used in a number of nonmedical applications. For example, they are used in microbiological research to control biochemical reactions. In certain types of microbiological studies it is sometimes necessary to selectively turn off protein or DNA synthesis in a culture. This is done easily by adding to the culture medium antibiotics that target the particular biochemical step. As mentioned earlier in this chapter, when processing environmental

samples for the presence of fungi, antibiotics are often included in the media to prevent the overgrowth of bacteria. Such selectivity is made possible because, as discussed in Chapter 1, fungi are eucaryotic and bacteria are procaryotic. Because of the differences in the two classes of cells, antibiotics can be made to target procaryotes but leave eucaryotes relatively unharmed. But the most significant consumers of antibiotics are farm animals. Over half of all antibiotics produced around the world are fed to poultry, hogs, milk cows, and beef cattle to stimulate growth. It is thought that this practice, along with the overprescribing of antibiotics by physicians, has led to the emergence of antibiotic-resistant microorganisms in our communities.

Antibiotics are produced by a surprisingly narrow collection of bacteria and molds consisting of just a few species from each group. For commercial antibiotic production, the organisms are grown in large quantities and the drugs are extracted from the media, purified, and packaged. Some years ago, chemists learned that natural antibiotics such as penicillin and streptomycin could be altered chemically to improve potency, stability, and other characteristics. These antibiotics are referred to as semisynthetic antibiotics. Methicillin and ampicillin are two examples of such antibiotics. There also are totally synthetic antimicrobial drugs that are known simply as antimicrobics.

VI. CONTROL OF MICROORGANISMS IN HIGH-PURITY WATER SYSTEMS

A. The Need for Pure Water

Many industrial processes require large volumes of pure water. Natural water is usually heavily laden with dissolved salts, gases, organic matter, inert particulates, and microorganisms, all of which must be removed to one degree or another depending on application. Typical water purification systems use one or more of the following steps: filtration, distillation, ion exchange, continuous electrodeionization, and reverse osmosis. Nearly every one of these processes, along with the water system infrastructure itself, can be potential sources of microbial proliferation that must be closely monitored and controlled. To assume that a pure water system will remain microbe free without intervention will only lead to disaster.

Industries involved in the manufacture of foods, pharmaceuticals, and semiconductors require water of varying purities. Generally, water that is designated as drinking or potable water is sufficiently pure for most applications in the food industry. Potable is defined as fit for human consumption; that is, free of harmful chemicals and microorganisms. Water for injection (WFI) must be sterile as well as essentially free of particles,

bacterial endotoxins (pyrogens), and other impurities. The purest water is required by the semiconductor and nanotechnology industries. A very large-scale integrated (VSLI) circuit, where thousands of electronic components are concentrated in less than a square centimeter, can be destroyed by the presence of a single 1-μm particle like a bacterial cell. During the manufacture of integrated circuits, the wafers (the substrates on which the circuits are assembled) are subjected to a number of chemical treatments, each of which is followed by a water rinse. Unless the rinse water is extremely pure, impurities can contaminate the circuit and render it inoperative.

B. Water Purification Methods

As noted previously, a variety of methods are used to purify water. Distillation, one of the oldest and most common approaches to producing pure water, involves heating feed water in a closed system until the water evaporates. The water vapor produced is essentially free of particles, dissolved mineral salts, microorganisms, and pyrogens, all of which are left behind. In most stills, the vapor rises through a series of baffles that ensures that nothing other than the pure water vapor reaches the condensers. The condensers are coils or plates that are cooled by circulating water or other fluid through them. On contact with the condenser, the water vapor is converted back to liquid water, which flows by gravity into a collection vessel. For ultra-pure water, the distilled water may be passed through a second distillation stage or through one of the other purifying methods mentioned above. Most commercial stills operate on a continuous basis.

Reverse osmosis (RO) is a common water purification process that removes inert particles, dissolved substances including salts, pyrogens, and other organic matter, as well as bacteria. RO involves forcing water under pressure through a membrane while leaving behind nearly everything else in the feed water. The porosity of the membrane permits only water molecules to pass through it. As the rejected material accumulates on the RO membrane, an environment eventually forms that is highly supportive of microbial growth. If not controlled, a biofilm eventually forms on the membrane surface accompanied by organic matter and a layer of mineral scalants. The organic matter provides sufficient nutrients to support bacterial growth and the scalants offer additional structural reinforcement for the biofilm. Biofilms are described in more detail below in Section C. As the biofilm builds (known as “fouling” or “biofouling”), backpressure increases and the efficiency of the RO membrane deteriorates. In time the bacteria may penetrate the membrane, causing a breakthrough into the purified water side of the membrane. Periodic chemical cleaning of the membranes together with hot (at least 80°C) water sani-

tization is necessary to maintain the RO operation. It has been shown that the older a biofilm becomes, the harder it is to remove. Hence, frequent cleaning of RO membranes is recommended. Cleaning and sanitizing chemicals for RO membranes should be chosen carefully, for some chemicals can damage certain types of membranes. For example, polyamide membranes have been known to be damaged by oxidizing disinfectants such as hydrogen peroxide or chlorine at normal use dilutions. Pretreating RO feed water to reduce organic matter, minerals, and bacteria is an effective way of slowing membrane fouling.

Organic contaminants in the RO feed water can be removed by pretreatment with activated carbon filters and ion exchange water softeners can remove scaling minerals. But both carbon filters and resin beds offer additional sites for bacterial proliferation. The dissolved organic matter that is adsorbed to the carbon filters provides sufficient nutrients to support significant populations of bacteria. Activated carbon beds must be cleaned periodically and sanitized with hot water. Organic matter that leaches from softener resins also provides nutrients for bacterial growth. While the resin bed can be sanitized with hot water, the brine that is used to regenerate the resin bed can be a significant source of bacteria. Bacteria known as halophiles (salt lovers) find near-saturated salt solutions to their liking. Stored brine can be made nearly microbe free by sanitizing it with hypochlorite.

Once water passes through its various purification stages, it comes in contact with the inner surfaces of piping, valves, heat exchangers, storage tanks, and other hard surface environments of the pure water system. These components all offer additional sites for the formation of biofilms. As noted in Chapter 1, so-called purified water can still contain sufficient organic nutrients to sustain populations of bacteria. Bacterial proliferation within such systems can be reduced by various precautions. For example, periodic or continuous cycling of the water at a temperature of at least 80°C has been shown to be effective in reducing microbial growth. Injection of purified steam also can be used to sanitize water systems, provided the systems are well designed. For instance, provisions must be made to bleed air from the system to allow full penetration of the steam, and drains must be placed at low points in the system to eliminate condensate. Such vents and drains must be protected from contamination. Recalcitrant biofilms that still form in the system can be killed by chemical means provided that sufficient concentrations and contact times are applied. Hydrogen peroxide/peracetic acid mixtures and ozone have been found to be effective in controlling biofilms. Obviously, the system must be thoroughly flushed of agents before being placed back into service.

Purely chemical treatments alone may not be sufficient to remove biofilms completely. Remnants of dead bacteria provide nutrients that may

offer an even more hospitable environment for the establishment of a new biofilm. Often, mechanical cleaning must be considered, such as brushing and high-pressure washing.

Poor designs of water systems invite the formation of biofilms and chronic contamination problems. Unused or seldom used "dead legs," sampling valves and pipe loops that may not be fully affected by cleaning and sanitization procedures, can be likely sources of bacterial contamination. Dead legs are dead-end sections of piping that are out of the main water circulation system. These features should be identified and removed whenever possible. The material that makes up the water system is also critical. For all inner surfaces of the system, highly polished stainless steel has been shown to reduce (but not eliminate entirely) the formation of biofilms. All welds and other potentially rough spots must also be polished, and cracks and crevices should be identified and repaired.

Even when internal sources of bacteria are under control in a pure water system, bacteria may gain entrance from the surrounding facility. Due to constant cycles of filling and emptying, storage tanks frequently experience positive and negative fluctuations in internal pressure relative to the local atmosphere. Negative pressure can draw microbial contamination into the tank through loose gaskets around sight glasses, access ports, sampling valves, and other openings. Changes in internal pressure can be relieved with vents, which should be equipped with filters to prevent contamination from entering the tanks. Filter media should be hydrophobic to prevent plugging by moisture and microbial grow-through.

C. Biofilms

It generally has been assumed that bacteria that are living in aquatic environments such as lakes and rivers or in industrial water systems are primarily planktonic; that is, suspended freely in the water as single cells or perhaps in short chains or small clumps. As described in Chapter 1, most bacteria in aqueous environments are found attached to surfaces as thick, tacky layers generally known as biofilms. Biofilms are found on a wide variety of natural and artificial surfaces that are continually wet, such as boat hulls, submerged rocks and plants, teeth, contact lenses, implanted medical devices like catheters and artificial joints, and, most relevant, the inner surfaces of pure water systems (RO membranes, pipes, heat exchangers, degasifiers, storage tanks, and other components of water purification units). It is thought that low nutrient levels in most of these environments are what trigger the formation of biofilms as a survival mechanism. In each of these instances, individual planktonic bacteria or small aggregates initially attach themselves to exposed surfaces with various adhesins, molecules that have adhesive properties. After a lag

phase, microbial reproduction occurs that results in the formation of a microcolony. Chemical signaling among members of the microcolony, known as quorum sensing, triggers the eventual establishment of a mature biofilm. The biofilm initially consists of scattered colonies, each containing millions of members that merge into a nearly continuous film of living organisms (see Figure 1.7). This film of cells provides a hospitable environment for additional planktonic cells to attach to the biofilm, adding additional layers to it.

Members of the biofilm release large amounts of sticky polysaccharides, which form a semirigid matrix known as a glycocalyx. The glycocalyx provides a number of advantages for the organisms in the biofilm. It stabilizes the biofilm to the surface, captures nutrients, and offers its members a degree of protection from disinfectants as much as two or three orders of magnitude over that exhibited by the organism's planktonic counterparts. Water channels between the colonies in the matrix function as conduits for nutrients and waste materials. In many natural settings, other bacteria, algae (if light is available), protozoa, worms, insects, and other higher forms may join the bacteria that initially established the biofilm. Such scenarios involving mixed populations occur frequently in natural settings but are not likely to occur in cleanroom pure water systems, where biofilms are more likely to consist entirely of one or a few species of bacteria. Nevertheless, a biofilm represents a breeding ground for bacteria. Once established and fed by traces of organic matter in so-called purified water, a biofilm can quickly become a springboard for large numbers of microbes that may be dislodged by turbulence, spreading to other sections of the pure water system. Of particular concern, biofilms can be a major source of pyrogens in pure water systems.

VII. SUMMARY

Many physical and chemical agents are available for the control of microorganisms. Some, such as heat or ethylene oxide, are sufficiently effective to be relied upon to sterilize objects, whereas most others are less effective and can only decontaminate. The choice of an appropriate method is not always obvious. Consideration must be paid to the compatibility of the method with the materials that make up the items to be treated. Many materials cannot tolerate the heat necessary to sterilize, while others are damaged by chemical disinfectants. Some situations may not require sterilization and decontamination may be sufficient. Raw materials, including water, must be handled carefully to avoid excessive bioburdens prior to sterilization of final product. Frequently, objects have to be precleaned to remove matter that would reduce the effectiveness of the disinfectant or sterilant. Purified water systems offer their own unique form of micro-

biological contamination problems. Maintaining a microbe-free purified water system has often been compared to shaving or mowing a lawn: It is an almost continuous effort, requiring constant monitoring and frequent remedial action.

4

CLEANROOM FACILITIES AND PERSONNEL CONTROLS

I. INTRODUCTION

Cleanrooms or controlled environments are manufacturing, medical, or research facilities that are designed to assure constant environmental conditions such as temperature and humidity and to reduce airborne particulate contamination. Cleanrooms may be designated as certified if they fully conform to recognized national or international cleanroom standards. Facilities that may lack full certification are more commonly referred to as controlled environments. Examples of manufacturing areas that require cleanrooms or controlled environments include microelectronic circuitry, nanotechnology, satellites, optical equipment, food, medical devices, and pharmaceuticals. Various forms of controlled environments are also frequently seen in pharmacies that dispense sterile formulations. Contamination control principles are practiced in the protection of hospital patients who are particularly susceptible to airborne infection. It has been demonstrated that surgical wound infections can be reduced when operations are carried out in controlled environments. Cleanroom principles have been successfully used to protect patients recovering from serious burns or suffering from severely compromised immune systems. The application of airborne contamination controls to isolate patients with highly contagious diseases such as tuberculosis can protect hospital personnel, visitors, and other patients. Certain areas of microbiological research that deal with highly infectious organisms also use many of the features and practices associated with the control of airborne contamination.

II. CERTIFIED CLEANROOMS

By design, certified cleanrooms can vary widely in acceptable levels of protection from contamination. These facilities are classified according to the measured number of airborne particles per unit volume of air of a specific size range. Airborne contamination levels usually are determined by automated particle counters. United States Federal Standard (FS) 209, issued in 1963, contained the first comprehensive guidelines for cleanroom classification. The standard was followed by four revisions that were issued over the next 29 years. Cleanroom classifications in FS209 were expressed in English or imperial units; that is, airborne particles per cubic foot within various particle size ranges. For example, a Class 100 cleanroom under FS209 meant it could have no more than 100 particles per cubic foot of air equal to or greater than 0.5 μm. A Class 10,000 cleanroom had no more than 10,000 particles per cubic foot 0.5 μm or greater, and so on. Levels in metric or SI units (particles per cubic meter) were added to the last revision (FS209E), released in 1992. FS209 was officially retired in 2001 and two international standards, ISO 14644-1 and 14644-2 were issued to replace it. These standards have added two additional, more stringent classes and one less stringent class that were not in FS209E. The new standards use particles per cubic meter as their unit of measure. Table 4.1 and Table 4.2 list some examples of particulate levels in the FS209 and the ISO cleanroom classification systems. Because the classification system used in FS209 has become so ingrained in cleanroom lore and literature for so long, it will probably continue to be referred to for some time.

Airborne particulate counts in a specific cleanroom will vary widely depending on its operational state when it was monitored. A completed cleanroom that is receiving clean air but is empty of equipment and personnel, known as "as built," should be the cleanest. An "at rest" room contains all of its normal complement of furniture and (nonoperating)

Table 4.1 Examples of cleanroom limits from FS209

Class	*Limits per Cubic Foot for Particles 0.5 μm or Larger*	*Class (SI)*	*Limits per Cubic Meter for Particles 0.5 μm or Larger*
1	1	M1	10
10	10	M2	100
100	100	M3	1,000
1,000	1,000	M4	10,000
10,000	10,000	M5	100,000
100,000	100,000	M6	1,000,000

Table 4.2 Examples of cleanroom limits from ISO 14644-1

Class	*Limits per Cubic Meter for Particles 0.5 μm or Larger*
2	4
3	35
4	352
5	3,520
6	35,200
7	352,000
8	3,520,000

equipment but is devoid of personnel. Such a facility will likely show some intermediate level of airborne cleanliness. An "operational" room is fully functioning with a normal array of equipment and personnel. It is expected to register the highest level of airborne particulate contamination. Thus, as operating equipment and personnel are added to a cleanroom, airborne particle counts increase, supporting the long-held view that these factors, particularly personnel, are significant sources of contamination in a cleanroom.

III. BIOCLEAN FACILITIES — VIABLE VS. NONVIABLE PARTICLES

Facilities where concern is also focused on the control of microbiological contamination are sometimes referred to as bioclean facilities or bioclean-rooms. In discussing the control of particulate contamination in bioclean facilities, we must distinguish between viable and nonviable particles. A viable particle is one that has one or more culturable microorganisms associated with it. If such a particle were to be placed in an appropriate microbiological growth medium, microbial growth would be observed. A nonviable particle has no detectable culturable organisms on it. Notice the word "detectable" in the definition of a nonviable particle. In essence, that recognizes that a particle may be culturable in one medium or under one set of incubation conditions, but nonculturable under others. That is why it is so important to use standard media and culture conditions when conducting any type of microbiological studies such as validation of disinfectants or environmental sampling. That point is discussed further in Chapter 5.

The presence of viable particles in parenteral and other products that come in contact with the bloodstream is an obvious hazard, but viable

particles are not the only particulate contaminants of concern in the manufacture of medical devices and pharmaceuticals. For example, nonviable particle contamination in products for parenteral use, that is, for injection into the body, can be equally hazardous for recipients. Such particles can trigger blood clots, irritate blood vessels, and induce other untoward reactions.

Whether a particle is viable or nonviable is generally a matter of how it was created. Airborne particles from soldering or metal grinding operations are usually nonviable, whereas dust particles from outdoor air, droplets emitted from a cough or sneeze, and sloughed skin particles are likely to be viable. Automatic particle counters that are routinely used to classify cleanrooms cannot distinguish viable from nonviable particles. Such instruments depend on the light-scattering properties of airborne particles, qualities that are not significantly affected by the presence of viable organisms. It should be noted that, in specific instances, while there may be rough correlations between viable particle counts as determined by biological assay and nonviable particle counts as measured by automatic particle counters, there is no general, industry-wide correlation between such counts; that is, there is no universal formula to convert total particle counts to viable counts. There can be facilities where total particle counts are very high, but viable counts are low, and in other locations viable counts may be very close to the total count. The same will be true for a particular cleanroom where the proportion of nonviable counts versus the viable counts could vary from day to day. To repeat, it depends on the source of the particles.

IV. CLEAN FACILITIES — GENERAL CONSIDERATIONS

By broad definition, a cleanroom is a room that is designed to remain clean while designated activities are being conducted in it. In this context, the definition of "clean" primarily means strict control of airborne particulate contamination within certain size ranges. The assumption attached to this statement is that such particulate contamination would be harmful to the activities within the cleanroom. In general, any room can become a cleanroom provided that it has certain features. The walls, floors, and ceiling must be constructed of smooth, seamless materials that do not shed particles and are easily cleaned. Wall, floor, and ceiling junctions must be coved for easy cleaning. Any furnishings, instruments, machinery, or incoming raw materials must not release significant quantities of particles. Airborne contamination from adjacent areas is prevented from entering the room by providing particle-tight doors and windows. Door and window frames should be flush with walls to avoid dust-collecting ledges. The room's ventilation air must be filtered and slightly pressurized

relative to surrounding rooms to prevent contaminated air from entering when a door is opened. The ambient pressure also prevents contamination from entering through small, undetected gaps in walls and ceilings. All known wall openings for utilities should be sealed. Personnel working in the room must be attired in nonshedding garments that block the release of particles from the body and underclothing.

A room's overall level of cleanliness is dependent primarily on both the quality and quantity of its ventilation air. Filtration is the most effective way of providing clean air. Particles that are released within the room can be flushed out by the movement of the air throughout the room. The cleansing effect of the ventilation air can be increased by raising the rate at which the air is replaced within the room. Two to three air changes per hour is the norm for conventional spaces such as offices and shops. By increasing that to 10 to 20 changes per hour, airborne particulate contamination that is released within the room would be diluted by the large volume of clean, filtered air that enters the room. Under these conditions, most airborne particles remain suspended in the air, where they can do no harm and are eventually carried out through one or more exit ducts. However, air movement in a room under conventional ventilation usually experiences turbulence. Turbulence creates dead spots — areas in the room where air velocity decreases significantly — causing airborne particles to settle onto the floor, work surfaces, or products.

V. UNIDIRECTIONAL AIR FLOW

While a room just described is based on conventional ventilation design in which air movement in the room can be rather turbulent, another concept minimizes turbulence by providing for the clean air to move through the room in a unidirectional manner. That is, supply air ducts may occupy an entire wall at one side of the room and exhaust ducts of equal size are placed at the opposite side. The air movement in unidirectional cleanrooms is often compared to a piston with an infinite number of faces, all moving in the same direction. The air movement within a cleanroom under unidirectional flow will approach laminar flow. True laminar flow where all air molecules are moving in parallel paths at the same velocity is not possible unless the room is relatively small and completely empty of furniture and other objects. For that reason, the term "unidirectional" is probably more accurate and is therefore preferred when describing air movement in this type of cleanroom.

Depending on specific needs, unidirectional cleanrooms may be designed for horizontal or for vertical air movement (Figure 4.1). In a cleanroom with horizontal air flow, clean, filtered air enters from supply ducts that may occupy an entire wall. Dirty air exits through the opposite

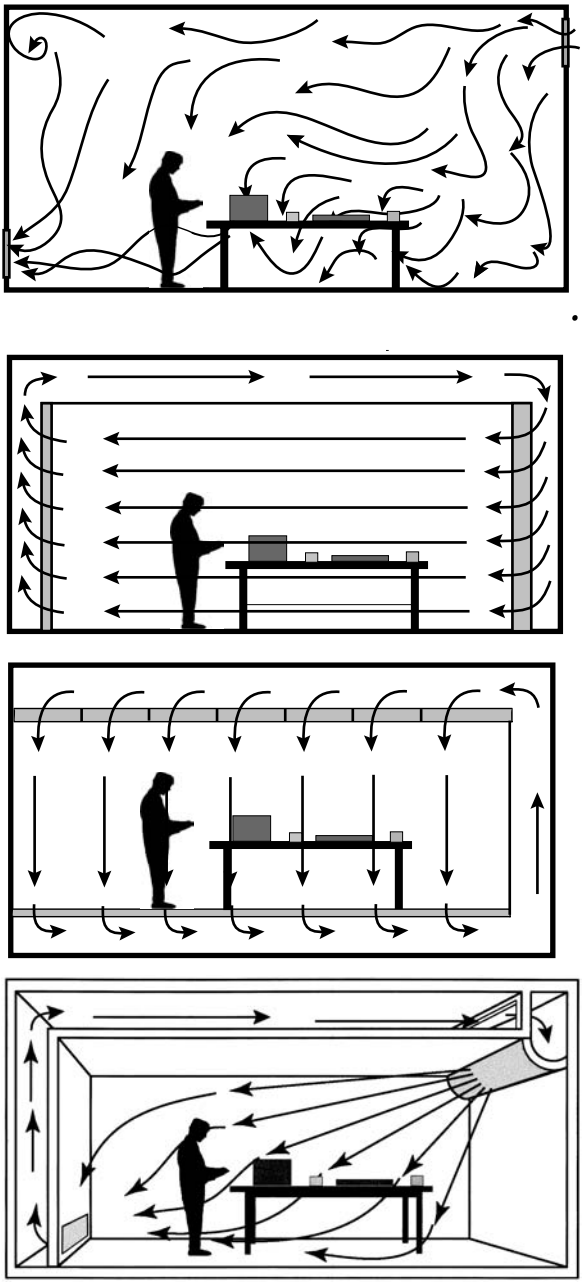

Figure 4.1 Comparison of cleanrooms. (a) Conventional ventilation with turbulent air movement, (b) horizontal unidirectional air flow, (c) vertical unidirectional air flow, and (d) vector unidirectional air flow. Blowers are not shown.

wall. In vertical flow cleanrooms, filtered air enters from perforations in the ceiling and exits through exhaust ducts that may be located in the floor or placed along the wall, adjacent to the floor. Supply ducts may occupy from 10 to nearly 100% of the ceiling area. In vector air flow, the supply grating may be installed along one ceiling/wall juncture and the return duct located at the opposite wall/floor juncture, creating a diagonal but essentially unidirectional air flow through the room.

The basic principle of unidirectional air movement to achieve a clean environment is to supply the room with air that is as clean as possible and that is moving nearly as a unified mass. By eliminating most of the turbulence that is characteristic of a conventionally ventilated room, air movement in a unidirectional cleanroom makes it less likely that airborne particles will settle onto critical areas, product, or processes. Unidirectional air flow has been shown to be two to three orders of magnitude more effective in controlling airborne contamination than conventional ventilation.

Levels of cleanliness higher than those encountered within a particular cleanroom may be achieved by installing workstations, clean benches, or enclosed devices known as isolators, all of which have their own integrated air filtration system. Such units are discussed in more detail later in this chapter.

Clean, incoming air is produced by passing it through depth filters that trap most suspended particles down to fractions of a micrometer in size. Particulate matter that is released in the room by personnel, equipment, or materials is swept away by the moving air. The cleanest area of a cleanroom is nearest the air supply ducts, and personnel, equipment, and other sources of contamination must be positioned downstream of items or areas to be protected from contamination. The velocity of the moving air at the filter face in a typical unidirectional cleanroom may be of the order of 50 to 100 ft/min (about 0.5 to 1 mph) depending on its classification. Care must be taken in the placement of furniture, machinery, and other bulky items in a cleanroom, particularly near the supply ducts. Such items may disrupt the movement of clean air through the cleanroom and create turbulence that would negate the very efficient cleansing effect of the unidirectional air stream. Blockage of exhaust ducts with furniture or equipment can also affect the normal air flow in a cleanroom and must be avoided.

The movement of personnel and materials in and out of a cleanroom must be limited to prevent additional contamination from entering the facility. Atmospheric pressure inside a cleanroom must be slightly higher than the pressure in adjacent, less clean areas so contamination is prevented from infiltrating through opened doors or cracks and openings in walls and ceilings. A pressure differential of about +15 Pa relative to surrounding rooms is usually sufficient. Entrance into a cleanroom should be through an air lock or vestibule with interlocking entrance and exit doors that

cannot be opened at the same time. Such entrances are useful in stabilizing air pressure in the cleanroom, but the air supply to the cleanroom must be sufficiently robust to maintain the pressure differential whenever a door is opened. Air curtains, that is, focused streams of filtered air, may also be used to prevent passage of airborne particles into clean areas. Observation windows and intercommunication systems should be installed to minimize foot traffic in and out of the cleanroom by personnel and visitors.

Dressing rooms in which personnel change into cleanroom garments must be immediately adjacent to the cleanrooms. Such rooms should be large enough to provide sufficient space for the personnel of a shift to change garments without interfering with one another. Various devices such as air showers and tacky floors or mats are usually placed at the entrance to cleanrooms to reduce the amount of tracking particulate matter that may adhere to workers' garments, carts, and other items into the cleanroom. Additional discussion regarding dressing room design and personnel attire will be found in the section on gowning.

VI. HEPA AND ULPA FILTERS

The passage of the supply air through HEPA (high-efficiency particulate air) or ULPA (ultra low–penetration air) filters is the secret to providing ultraclean air for a cleanroom. The HEPA filter (Figure 4.2) is a particular type of depth filter with an exceptional pore size rating for air. Their use in laboratory and cleanroom air supplies, and transfer and biosafety hoods considerably reduces airborne particulate contamination and provides significant protection for products and for laboratory personnel.

Typically, HEPA filters are made of continuous sheets of borosilicate glass microfibers combined with a water-repellent binder. The pleated design of the HEPA filters allows for large filters in a relatively small space. This provides air flows with greater than 99.97% filtration efficiency for particles 0.3 μm or larger with only 250 to 500 Pa pressure drop. Since most microorganisms are larger than 0.3 μm, HEPA filters are eminently successful in controlling airborne microbial contamination. For airborne microorganisms less than 0.3 μm, particularly viruses, it is doubtful that such organisms would survive for long. Without the protective effect of attachment to larger particles such as skin cells or saliva droplets, these organisms would likely succumb to the drying effects of air currents, toxic airborne pollutants, and other environmental stresses. These minute organisms do not appear to pose a significant source of viable contamination in rooms supplied by HEPA-filtered air. If control of such particles is necessary, ULPA filters exhibit efficiencies as high as 99.9999% for particles down to 0.12 μm. Since neither HEPA nor ULPA filters capture 100% of potentially viable particles, they cannot be designated as producing sterile

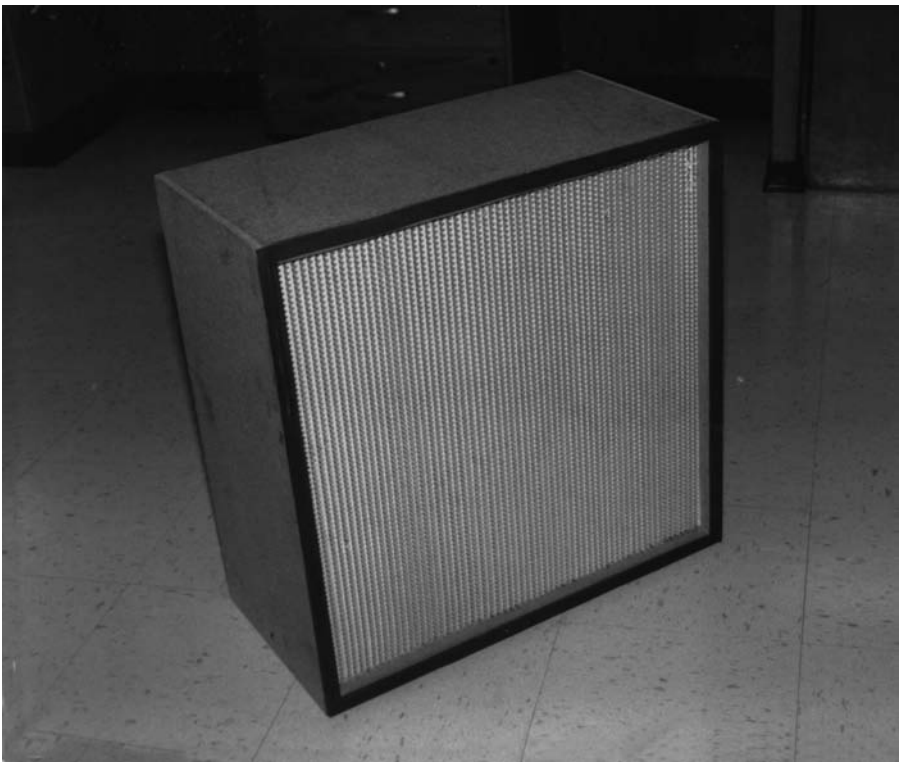

Figure 4.2 (a) A HEPA filter ready to be installed in cleanroom. (b) A curved minipleat HEPA filter for a vector air flow cleanroom showing inlet side. (c) A HEPA filter partially disassembled showing zigzag folding of filter medium around corrugated aluminum separators. Arrows show direction of air flow when filter is operating. ***Continued.***

air. The mechanisms that operate in HEPA filters were described in Chapter 3 in the section on depth filters.

HEPA filter units are manufactured in a variety of shapes and depths. To provide maximum filter area, the filter medium is pleated and the pleats are held apart with spacers of corrugated aluminum or with adhesives without spacers, known as minipleat construction (Figure 4.2b). A 2 × 2 ft and 7.5-in-deep minipleat HEPA filter actually consists of nearly 280 ft^2 of filter area. The entire assembly is sealed in a metal or composition frame and the completed unit is installed with gaskets on its edges to prevent air leaks around the filter box.

Once installed, the filters must be thoroughly tested for leaks, since filters may experience damage in shipping or installation. Testing is done by scanning the entire face of each filter and surrounding framework with

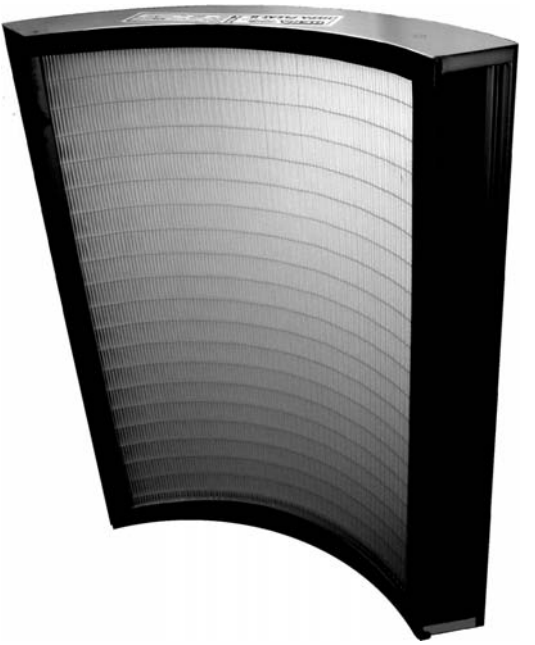

Figure 4.2 ***Continued.***

the probe of a particle counter while particles such as dioctylphthalate (DOP) smoke are generated in the supply duct. Particle counts on the exit side of HEPA filters should not exceed 0.01% of the counts on the supply side. Periodic testing to confirm filter system integrity, as part of a certification program, should be carried out every 6 to 12 months to ensure that the filters continue to function properly. Microbiological testing will be covered in Chapter 5.

VII. ASEPTIC FILL AREAS

The final preparation of sterile pharmaceuticals, medical supplies, and other products can be handled in one of two principal ways. In terminal sterilization, the product is placed into clean containers, sealed, and subjected to sterilization by heat or radiation. Note that product and container are not initially sterile, but are handled in a manner that assures a low bioburden. Aseptic filling involves the placing of sterile product into sterile containers and sealing with sterile closures of varying types. The filling operation may be automated, carried out by personnel fully suited in sterile garments, or by a combination of automated and manual operations. Aseptic filling can be conducted either in a conventional but highly stringent (ISO Class 5) cleanroom or within an enclosed facility known as an isolator, described below. In either case, in addition to requiring product, containers, and closures to be sterile, all equipment and materials that come into direct content with the product, including any gases, must be sterile and, when appropriate, pyrogen free.

Environmental controls for unidirectional cleanrooms where aseptic filling is carried out must be considerably more stringent than for manufacturing terminally sterilized products. Aseptic filling areas where sterile products and containers are exposed to the environment are called critical areas. According to FDA guidelines, critical areas must conform to the specifications of an ISO Class 5 (FS209 Class 100) cleanroom, plus exhibit a specified microbiological air quality level. This area is usually protected by a steady flow of filtered air aimed directly at the workspace. Human penetration into any designated critical areas is kept to an absolute minimum and is allowed only while wearing sterile garments and using sterile tools. Reaching into the critical area can produce turbulence, disrupting the protective effect of the unidirectional air flow.

Surrounding the critical area is the controlled area, a slightly less clean (ISO Class 6 or 7) space. Full barrier gowning is required for all personnel in this area, and all material and equipment must be either sterilized or thoroughly disinfected. Full barrier garments cover all exposed parts of the body, including the face, and are secured around the neck, wrists,

and ankles to minimize leakage of particle contamination from inner clothing and skin.

Blow-fill-seal (BFS) technology is an automated process in which containers are molded from hot, liquid plastic, aseptically filled with sterile liquid product, and sealed all in one continuous operation. For those products that lend themselves to BFS processing, the approach offers an alternate approach to protecting sterile products during the filling step.

VIII. BARRIERS, CLEAN ZONES, AND ISOLATORS

The unidirectional air movement in a cleanroom affords a process or product a certain degree of protection against airborne contamination. Additional protection can be achieved by installing barriers between the major source of contamination, cleanroom personnel, and the process or product. In addition to cleanroom garments, barriers can also include curtains, shields, or any other structure that acts as a physical separation between personnel and product. Clearly, a variety of barriers are in common use in all cleanrooms.

Clean zones or clean air devices are various units that are placed in a cleanroom to improve the level of contamination control within a localized area. Each of these units lends itself to one or more specific applications. Examples of such devices are biological safety cabinets, isolators, and biological transfer hoods. Generally these units have their own air filtration system, independent of the cleanroom air flow system. The workspaces within these devices can be one or more classifications cleaner than the surrounding cleanroom.

A. Biological Safety Cabinets

Biological safety cabinets (BSCs) are devices superficially similar to chemical hoods but that are primarily used to protect items in the work area from contamination from the surroundings, to protect the worker and the surrounding facility from contamination by hazardous microorganisms being handled within the cabinet, or both. BSCs should not take the place of chemical hoods for storing or handling large quantities of hazardous chemicals, gases, radionuclides, or volatile solvents. A serious risk of fire or explosion can occur if excessive amounts of flammable gases or liquids are used in a BSC. However, limited amounts of such materials can be handled in some types of BSCs, as discussed below.

Three classes of biological safety cabinets are presently manufactured and each class fulfills a particular purpose.

Figure 4.3 A Class I biological safety cabinet.

1. Class I BSCs

Class I biosafety cabinets (Figure 4.3) can be operated either with an open front or with a closed front and with or without arm-length gloves. Negative pressure maintained within the cabinet ensures an inward flow of air that protects the worker and the surrounding area from hazardous viable aerosols that might be generated within the cabinet. However, there is no protection for the work area from contamination originating from outside the cabinet when the front is open. When operated with an open face, velocity of entering air must be at least 75 ft/min. Exhaust air is filtered through HEPA or ULPA filters before being released into the atmosphere. Some models also subject the exhaust air to ultraviolet light or heat decontamination before release. Class I cabinets are often used to contain infectious aerosols generated by centrifuges, blenders, sonicators, cell homogenizers, and so forth.

2. Class II BSCs

In this cabinet, as in a Class I cabinet, the inward flow of air at the front opening protects the worker from exposure to hazardous organisms that

Figure 4.4 A Class II biological safety cabinet.

might be released within the cabinet. In addition, the work area is protected from outside contamination by a shower of HEPA or ULPA filtered air from above the work surface. Thus, Class II cabinets (Figure 4.4 and Figure 4.5) have the distinct advantage of protecting both operator and product and are widely found in numerous research and industrial applications. There are two major design variations within Class II, A and B, and several subclasses under B (B1, B2, and B3) (See Table 4.3). Type A cabinets recirculate the air within the cabinet and thus cannot be used to handle volatile, hazardous chemicals and radionuclides since these substances are not retained by the filters. The air in Type B cabinets is not recirculated but is exhausted to the outside, thus making it safe to handle small amounts of volatile chemicals in this type of BSC. Care must be taken, however, that levels of flammable chemicals do not create a fire or explosion hazard. This risk is especially high in the event of a spill. In those instances where chemicals released to the outside may

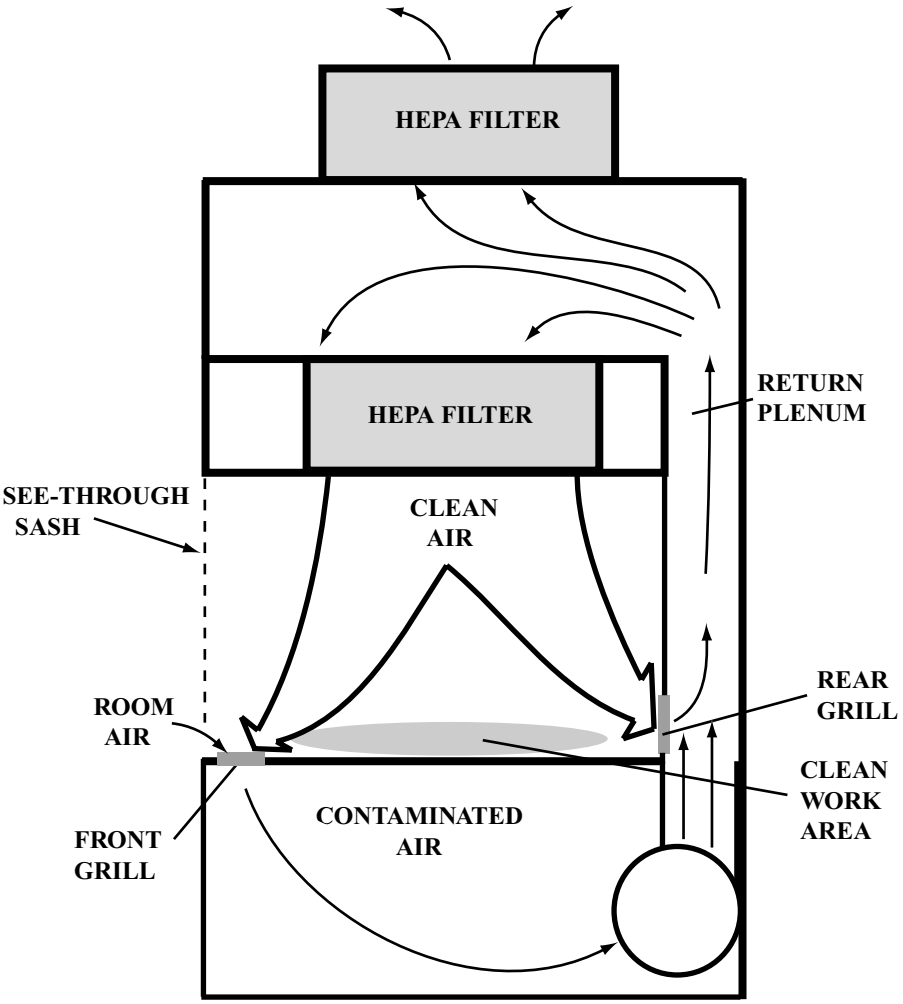

Figure 4.5 Cross section of a Class II biosafety cabinet. The lower HEPA filter delivers curtains of clean air over the work area. The front arm of the clean airstream together with the front grill prevents any contaminated air from the work area from exiting the cabinet. The front grill also intercepts room air and diverts it away from the work area. The room air that is drawn into the front grill creates excess air volume in the cabinet that is released through the upper HEPA filter where it can safely escape into the room or vent to the outside.

constitute an environmental hazard, the exhaust air may be passed through a charcoal filter prior to release. Neither Class I nor Class II cabinets are sufficiently effective to use with extremely hazardous organisms. For these, Class III cabinets must be used.

Table 4.3 Comparisons of Class II biosafety cabinets

		Permitted Applications	
Class	*Air Flow*	*Nonvolatile Toxic Chemicals and Radionuclides*	*Volatile Toxic Chemicals and Radionuclides*
IIA	70% recirculated to the cabinet work area through HEPA; 30% balance can be exhausted through HEPA back into the room or outside through thimble unit	Yes (minute amounts)	No
IIB1	Exhaust cabinet air must pass through a dedicated duct to the outside through a HEPA filter	Yes	Yes (minute amounts)[a]
IIB2	No recirculation; total exhaust to the outside through hard duct and a HEPA filter	Yes	Yes (small amounts)
IIB3	Same as IIA, but plenums are under negative pressure to room; exhaust air is thimble ducted to the outside through a HEPA filter	Yes	Yes (minute amounts)[a]

[a] In no instances should the chemical concentrations approach the lower explosion limits of the compound.

Source: From Centers for Disease Control and Prevention (CDC), in *Primary Containment for Biohazards. Selection, Installation and Use of Biological Safety Cabinets*, 2nd ed., CDC, Washington, DC, 2000, 37.

3. Class III BSCs

A Class III biosafety cabinet (Figure 4.6) offers maximum operator safety. It is airtight and the operator must manipulate objects in the cabinet through glove ports or by various remote control devices. These cabinets are usually reserved for handling organisms of the highest hazard level. All operations, such as inoculations, incubation, centrifugation, and staining are done inside the cabinet, or, occasionally, in several cabinets connected by airtight pass-throughs. When microscopic observations are necessary, only the ocular lenses protrude through airtight openings in the front panel. Air pressure in the cabinet must be maintained at least –125 Pa relative to the surrounding area, ensuring that no infectious material escapes in the event of a leak. Both supply and exhaust air are HEPA or ULPA filtered, and the exhaust air is double filtered for additional safety.

Figure 4.6 Class III biological safety cabinets. (Photo: CDC Public Health Image Library ID #2548.)

4. Proper BSC Operation

The protection of laboratory workers or products afforded by open Class I or Class II biological safety cabinets is only as good as the care and skill of the workers who use them. Partially blocked openings or quick movements by workers within or near the opening can disrupt the protective air barrier, allowing potentially contaminated air to cross the barrier either inward or outward. For example, since the inward air flow is usually somewhat under 100 ft/min, a person walking past the cabinet at a moderate pace of 200 ft/min would easily create a shockwave across the face of the cabinet that could disrupt the protective effects of the inward flow of air. Actually, exposure to cross drafts of as little as one half the inward flow velocity is considered sufficient to disrupt the normal air intake of a Class II safety cabinet. Open-face safety cabinets should be located away from foot traffic areas and at least 10 ft from sources of drafts such as doors or nearby heating and air-conditioning vents.

To take full advantage of the protective features of a Class II BSC, certain practices should be followed. A layer of plastic-backed absorbent paper should be placed over the work area to retain any splatter or minor spills. All items should be wiped down with a disinfectant before being

placed in a BSC, and then again when they are removed. The use of bulky equipment such as water baths and centrifuges within a Class II BSC should be kept to a minimum. These items can deflect the flow of air at the cabinet's opening and compromise the cabinet's functioning. Such items should be positioned as far back in the cabinet as possible without blocking the rear grill. In addition, they should be raised onto racks or stands to allow air to flow under them. Low profile items are preferred, such as trays rather than buckets for disposal of pipettes. All contaminated materials should be disinfected before being removed from the BSC, or placed in a biohazard bag while still in the BSC. All interior hard surfaces should be disinfected with 70% ethanol at the end of work.

The use of sterilizing burners in a BSC should be limited to those operations for which they are absolutely necessary. Microburners that can be turned on and off easily or electric furnaces are preferred over regular Bunsen burners, which create convection currents that disrupt the BSC's protective air flow pattern. In addition, the heat from a burner may damage the filter that is directly above the work area. Sterile, disposable inoculating loops and needles should be considered in place of the usual reusable metal variety. The unobstructed flow of air into the front grill is the key to preventing potentially contaminated air from exiting the BSC. Workers should not rest their arms or place notebooks or other items on the front grill.

Many BSCs come equipped with germicidal UV lamps that can decontaminate exposed walls and work surfaces, provided the surfaces are clean. Such lamps should not be operating while personnel are working in the cabinet. UV lamps should be cleaned periodically with 70% ethanol and their output should be tested with a UV meter. A BSC should not be used for long-term storage of items but should contain only those items of immediate use.

Personnel who work in safety cabinets should undergo thorough training on theory and operations of such equipment. Training should also include emergency protocols in the event of power failures and spills of hazardous materials.

5. BSC Certification

Biological safety cabinets should be certified when first installed and at least annually thereafter; whenever they are moved or any panels are removed for repairs or maintenance, their filters should be changed. Certification should be conducted by accredited personnel and include the following tests:

1. Filter leaks: using a particle counter as described for the testing of cleanroom filters, the entire filter faces plus all seals should be probed.

2. Air velocity: air coming out of the filter bank and air flowing into the front opening should have a velocity of 75 to 100 ft/min, depending on the type of BSC. To confirm uniformity of air flow, the entire filter face and cabinet opening should be tested by a handheld anemometer. BSC manufacturer's literature should be consulted for performance envelopes to determine proper balance of air flows for specific units.
3. Cabinet integrity: sheet metal fatigue may cause seams to open. Such defects can occur when a cabinet is initially installed, moved, or when panels are removed for maintenance. To check cabinet integrity, front and exit openings should be sealed and the cabinet body should be slightly pressurized with a tracer gas. Any leaks that are discovered must be sealed before the unit can be placed back into operation.
4. Air flow smoke patterns: air velocity measurements cannot detect slight defects in air movement around the front opening and within the work area. A small smoke generator can detect any such anomalies.

B. Isolators

Isolators are a type of clean-air device or enclosure that can provide essentially complete separation between product and its production equipment and the surrounding area and, most importantly, its attending personnel (Figure 4.7). Isolators may be employed for processes where a high level of product protection is required. For example, rather than operate aseptic filling machinery in an open ISO Class 5 cleanroom, an alternate approach is to enclose the entire operation in one or more isolators. Isolators have separate, built-in air filtration systems that can achieve extremely low particle counts. Because of their relatively small volume, their interiors can be sanitized readily with vapor or gaseous sterilants. To ensure asepsis while operating, light positive air pressure is maintained within isolators to prevent entrance of contamination from the surrounding environment via any openings. All interventions by workers are conducted through sealed glove ports. To protect workers when hazardous or pharmacologically active materials are being handled, isolators may also operate under negative pressure. In those instances where hazardous materials must also be contained under aseptic conditions, isolators are operated under positive pressure and attending personnel wear protective gear.

Isolators may be of an open or closed type. Closed isolators are totally sealed and no unfiltered or contaminated air can enter the unit while it is operating. Open isolators are used in aseptic processes in which product

Figure 4.7 An isolator enclosing an aseptic filler. Numerous glove ports provide sterile access to machinery when necessary. (Photo courtesy Bosch Packaging Technology/John L. Kirk.)

is continually exiting the unit through a port. The exit port ("mouse hole") is designed so that ambient air is prevented from entering the isolator, usually by maintaining outward flow of filtered air. Provisions must be made to seal the exit port when the interior is undergoing decontamination.

In a filling operation within an isolator, sterile product, containers, and closures are transferred into the isolator through a variety of openings such as air locks and rapid transfer ports. Rapid transfer ports can be quite elaborate and may include provisions for sterilizing incoming materials with dry or moist heat. Transfer isolators are portable enclosures that are used to transport sterile product or containers from one location to another without compromising sterility. For example, a transfer isolator is moved from a sterile prep area where it had been loaded with sterile product or containers. The transfer isolator is docked to the transfer port of the isolator that encloses the filling machinery. Sterile material is then moved into the filling machinery by remote control. Isolators can also be used for aseptic assembly of medical devices and for providing aseptic environments in which to carry out sterility testing (to be covered in Chapter 5).

Figure 4.8 Basic concept of a half suit isolator. Technician conducts sterility tests within a sterile isolator chamber while completely enclosed in an airtight suit. The suit is entered from below. Isolator normally would have several ports or air locks to transfer materials aseptically in and out of the chamber.

If a process that is enclosed in an isolator requires manual operations by personnel, access is afforded through glove ports or half suits (Figure 4.8). Even fully automated processes require occasional repairs, adjustments, and other interventions during a production run. Personnel contact through gloves must be minimized, however, as glove failures (rips, pinholes) represent the greatest threat to a sterile product.

The use of isolators may have advantages over aseptic filling that operates in conventional ISO Class 5 cleanrooms. Isolators can be placed in ISO Class 6 or 7 cleanrooms, avoiding the need to construct a Class 5 facility for the aseptic area. In general, isolators are easier to decontaminate, easier to monitor, and appear to provide higher sterility assurance than what can be obtained in a conventional cleanroom.

The physical constraints and the limited flexibility of isolators demand considerable advance planning. To avoid the need for frequent interventions, the enclosed machinery must have a high degree of reliability. When adjustments are necessary during operation, isolator ergonomics must allow for full and easy access through glove ports or half suits. When isolators are used in processes involving continuous manual operations, good design must provide for worker comfort.

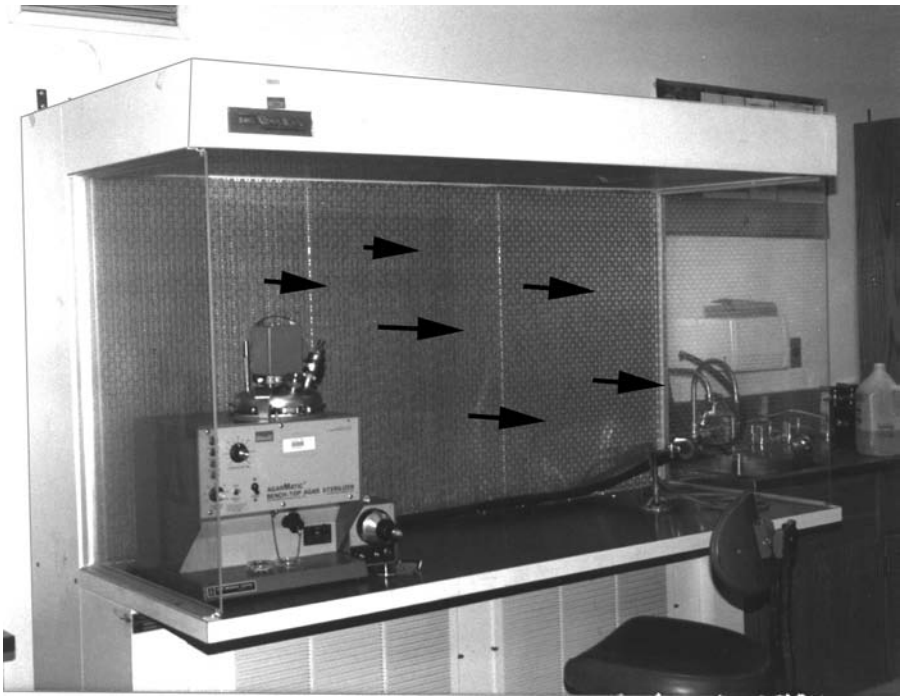

Figure 4.9 A unidirectional transfer bench or clean bench. These should be used only to protect the work area from contamination but should not be used to handle hazardous materials or organisms. Arrows show direction of HEPA filtered air.

C. Clean Benches

Workers must avoid confusing clean benches with biological safety cabinets (Figure 4.9). Variously known as transfer hoods, laminar flow hoods, or clean benches, these devices appear superficially like biosafety cabinets, but the principal function of clean benches is to protect media or other items or processes from contamination by an outward flow of HEPA-filtered air. Air flow may be either horizontal or vertical. Clean benches offer no protection for the worker and in fact are hazardous if used to handle harmful chemicals or pathogenic microorganisms, since the air moves from the interior of the hood into the face of the worker.

IX. BIOSAFETY LEVELS

In 1976, at the request of leading scientists, the National Institutes of Health (NIH) developed guidelines to be used in laboratories that engage

in research involving recombinant DNA (genetic engineering). Included in the guidelines were definitions of four classes of laboratory facility, ranked according to the relative level of protection afforded workers conducting experiments in the laboratories. Experiments that constitute little or no health risks to humans can be carried out in a P1 facility, which has a minimal level of safety features, whereas experiments of greatest risk require a P4 facility. Soon after the publication of the NIH guidelines, the Centers for Disease Control and Prevention (CDC) developed similar guidelines for handling pathogenic microorganisms, adopting the facility definitions of the NIH document, but renaming them BSL (for Biosafety Level) 1–4. Thus, organisms not known to be consistent hazards to human health may be dealt with in a BSL1 lab, while most known human pathogens are to be handled in either BSL2 or BSL3 labs. BSL4 facilities (Figure 4.10) are reserved for dealing with the most hazardous pathogens. Table 4.4 lists some of the principal features of the four biosafety levels.

Figure 4.10 A BSL4 laboratory. Technician wearing a positive pressure suit while working at a Class II BSC. Breathing air is supplied by coiled air hose behind worker's head. (Photo: CDC Public Health Image Library ID #1860.)

Table 4.4 Summary of recommended biosafety levels for infectious agents

BSL	*Agents*	*Practices*	*Safety Equipment (Primary Barriers)*	*Facilities (Secondary Barriers)*
1	Not known to consistently cause disease in healthy adults	Standard microbiological practices	None required	Open bench top sink required
2	Associated with human disease; hazard = percutaneous injury, ingestion, mucous membrane exposure	BSL-1 practice plus: Limited access Biohazard warning signs "Sharps" precautions Biosafety manual defining any needed waste decontamination or medical surveillance policies	Primary barriers: Class I or II BSCs or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials PPEs: laboratory coats; gloves; face protection as needed	BSL-1 plus: Autoclave available
3	Indigenous or exotic agents with potential for aerosol transmission; disease may have serious or lethal consequences	BSL-2 practice plus: Controlled access Decontamination of all waste Decontamination of lab clothing before laundering Baseline serum	Primary barriers Class I or II BCSs or other physical containment devices used for all open manipulations of agents PPEs: protective lab clothing; gloves; respiratory protection as needed	BSL-2 plus: Physical separation from access corridors Self-closing, double-door access Exhausted air not recirculated Negative air flow into laboratory
4	Dangerous/exotic agents that pose high risk of life-threatening disease, aerosol-transmitted lab infections; or related agents with unknown risk of transmission	BSL-3 practices plus: Clothing change before entering Shower on exit All material decontaminated on exit from facility	Primary barriers All procedures conducted in Class III BSCs or Class I or II BSCs Full-body, air-supplied, positive-pressure personnel suit	BSL-3 plus: Separate building or isolated zone Dedicated supply and exhaust, vacuum, and decon systems Other requirements outlined in the text (see reference below)

Source: From Centers for Disease Control and Prevention (CDC), in *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed., CDC, Washington, DC, 1999, 52.

A. BSL4 Facilities

Until recently, BSL4 facilities were a rarity in the United States, but with increased interest in emerging infectious diseases and bioterrorism, several BSL4 labs have been built or are planned. BSL4 facilities can follow two design models, referred to as cabinet laboratories and suit laboratories.

As the name suggests, cabinet laboratories depend on the use of Class III biosafety cabinets to protect workers from the highly infectious agents being handled. There are, however, some operations that cannot be conducted easily within a Class III cabinet, such as operating and maintaining large pieces of equipment. In those cases, workers operate in a specially built laboratory while wearing positive pressure suits.

B. Positive-Pressure Personnel Suits

As noted above, the use of Class III BSCs is necessary when dealing with extremely hazardous microorganisms or toxins. By wearing garb that is the equivalent of a Class III BSC in a suit laboratory, workers are able to acquire greater mobility while being afforded the protection of a Class III BSC. The suits consist of one-piece garments that cover the entire body with a heavy, impermeable material (Figure 4.11). Personnel suits operate under positive pressure; therefore, if any leaks do occur, air flow will be outward. Air is piped into the suits, which also carry self-contained back-up life support systems in the event of a failure of the main air supply. The BSL4 suit laboratory facility itself also is airtight and exhaust air from the laboratory is double HEPA filtered. The laboratory is essentially the equivalent of a Class III BSC. Workers who exit the suit laboratory must pass through a chemical shower that disinfects the outer surface of their pressure suits before they remove them.

X. CLEANROOM GARMENTS

The human body is a rich source of viable and nonviable particles and is, in fact, widely considered the principal source of contamination in a cleanroom. The skin of an adult human is an organ that grows from the inside out. Skin cells take about four weeks to move from the innermost dermis to the outermost epidermis where their life cycle ends. Dead skin cells are released from our bodies at the rate of about 2×10^7 cells per day, or the equivalent of an entire skin every four days or so. The released cells measure about 30 μm across and are easily broken into smaller particles. If a cleanroom worker were not wearing proper garments, depending on activity, he or she might release as many as 10 million dead skin cell fragments and other particles into the cleanroom air every minute, most of which would be carrying large numbers of viable micro-

Figure 4.11 A positive-pressure suit that is worn in BSL4 laboratories. (Photo: CDC.)

Table 4.5 Particles released during various activities

Activity	*Particles Released/min 0.3 μm and Larger*
Motionless: sitting or standing	100,000
Moving head, arm, neck, and leg	500,000
Moving head, arm, neck, leg, and foot	1,000,000
Walking about 2 MPH	5,000,000
Walking about 3.5 MPH	7,500,000
Walking about 5 MPH	10,000,000

Source: From Eudy, J., *A2C2*, April 2003, 7–11. With permission.

organisms (Table 4.5). One solution to this problem is to garb cleanroom workers in airtight suits that prevent all particles from escaping. This would work perfectly well if cleanroom personnel were reptiles, but that is not the case. In carrying out normal cleanroom operations, workers generate a considerable amount of body heat, which must be dissipated through perspiration. Some attempts have been made to use airtight, air-conditioned full bodysuits in conventional cleanrooms. As described above, this approach is used in BSL4 laboratories and may be satisfactory in certain industrial applications as long as the workers are not required to move about to any great extent. However, ease of movement and worker physical and psychological comfort is a paramount concern. Newer, more comfortable, nonshedding fabrics that "breathe" but prevent particulate penetration are now commonly used for cleanroom garments. However, leakage can still occur through openings at the neck, ankles, and wrists if they are not properly sealed. In addition, an uncovered face releases a considerable amount of particulate contamination from the sloughing of skin particles as well as from what is exhaled from the mouth and nose.

Full barrier cleanroom garments are frequently required where particulate release from exposed areas of the face must be prevented. These one-piece suits enclose the entire body and the face is covered with a mask and goggles (Figure 4.12). Some full suits use helmets to cover the head and face. The helmets may have various types of one-way breathing valves that allow air to enter the helmet, but close when the wearer exhales. Expired air is thus forced down the neck and into the bodysuit, where it filters through the suit material.

A. Correct Gowning Procedures

Cleanroom workers' garments provide the barrier between the recognized major source of contamination and the cleanroom environment. Examples

Figure 4.12 Cleanroom garment worn by personnel in aseptic filling facilities. (Photo courtesy Worklon/Pamela George.)

of some commonly accepted cleanroom practices and methods for proper gowning are as follows:

1. Access of personnel to cleanrooms should be through a series of rooms of increasing cleanliness. All excess personal property (coats, umbrellas, sweaters, and hats) should be stored outside of the gowning room, preferably in a separate locker room. Unnecessary personal items of value, such as jewelry, should be left at home; watches and wallets may be taken into the cleanroom provided

they are kept in inner pockets and not taken out. Rings with sharp corners that could puncture a glove should be removed. Other items such as keys, pens, and combs should be left in the locker room. If required by company policy, street clothing may be removed before entering the gowning room.

2. All makeup must be removed prior to entering the gowning room, or better, not used at all. If needed, moisturizing lotions may be applied to face and hands to reduce skin flaking.
3. All garment items, including jumpsuits, smocks, booties, gloves, hoods, masks, etc., should be left in their clean packaging or storage hooks until needed. Garments should be handled with gowning gloves or sterile gloves, as needed. Each item should be carefully inspected for frayed edges, open seams, tears, or missing or broken zippers or snap fasteners. Such defective items must not be used, but discarded or set aside for repairs. Workers should select garment pieces carefully, especially hoods and jumpsuits, for proper size. Items too small cause leakage and strains at openings; garments that are too large can create a bagpipe effect, forcing contaminated air to exit the garment when the worker moves.
4. The gowning room floor should be clearly divided and marked into a "clean" side and a "dirty" side. Under no circumstances should personnel walk in the clean area in street shoes. Most facilities require workers to remove street shoes or don shoe covers, head covers, and gloves prior to entering the gowning room. These items protect clean garments from becoming contaminated while being donned.
5. Ideally a low bench should be installed along the line dividing the clean side from the dirty side.
6. Gowning proceeds from the top down. If not already in place, a hair covering (bouffant) is put on first, or, if necessary, face mask and hood.
7. The coverall is donned by first holding the left and right legs at the cuff, ankle, and midsection with left and right hands, respectively. Stepping into each leg one at a time while taking care not to drag any part of the coverall on the floor or bench, the garment is pulled on. Leg and arm cuffs may be turned out to make it easier to don the jumpsuit. That also protects the outer parts of the cuffs from contamination in the event they come in contact with the floor or bench. The cuffs can then be turned down before booties and work gloves are put on.
8. Once the coverall is donned, the worker sits on the bench that separates the clean side of the room from the dirty side. One foot is moved to the clean side of the bench and without the foot

touching the clean floor, any shoe cover is removed and a bootie is put on. Covers are discarded on the dirty side of the bench. While keeping the foot with bootie on the clean side, the other foot is moved to the clean side and its bootie is donned. Leg cuffs should be tucked into the booties and secured with strapping.

9. Finally, gowning gloves are removed and work gloves are put on. Double gloving may be required by company policy. Gloves should overlap sleeves and, if necessary, be sealed with particle-free tape or strapping.

Gowned personnel entering a cleanroom should inspect each other for such things as exposed hair, skin, and open snaps. In addition, full length mirrors should be placed at all cleanroom entrances for self-inspection.

Normally, and depending on the classification of the cleanroom, regowning may be necessary whenever personnel leave the cleanroom to enter dirtier areas and return. Company policy will dictate whether personnel must change to fresh gowns when returning. If gowns are to be reused, they must be carefully hung up in a special location in the gowning area where they cannot become contaminated. Gowns must be laundered and packaged in clean facilities that are at the same level or better than that in which they are used.

B. Sterile Gowning

Certain types of products require special protection during their manufacture, such as those packaged by aseptic filling. In these and other circumstances the use of sterile gowns, masks, hoods, and gloves may be required. Generally they are donned in the same manner as other cleanroom garments, with the following exceptions: personnel scrub their hands and arms with antimicrobial soap and don sterile gloves prior to gowning. Workers should decontaminate their gloves with 70% isopropanol frequently during the gowning process and during the working shift.

XI. PERSONNEL PRACTICES AND TRAINING

While physical facilities and worker garments play critical roles in reducing contamination, good personnel practices become a third important factor in maintaining low contamination levels. These practices must be followed by everyone entering a cleanroom, including the normal cleanroom workers, inspectors, maintenance and cleaning personnel, visitors, even the company president.

Good personal cleanroom practices must be acquired through comprehensive and conscientious training. All employees who regularly enter cleanrooms, whether production workers or maintenance and supervisory personnel, must complete a training program that includes at a minimum:

1. Cleanroom terminology
2. Fundamentals of cleanroom design, operation, and monitoring
3. Standard operating procedures (SOPs)
4. Current good manufacturing practices (cGMPs)
5. Breaches of contamination control and their remediation
6. Sterility, aseptic techniques, and disinfection (if appropriate)
7. Fundamentals of biosafety cabinet design, operation, and monitoring (if appropriate)
8. Personal hygiene
9. Proper behavior in the cleanroom
10. Correct gowning procedures
11. Types and nature of products handled in the cleanroom
12. Record keeping
13. Safety, including emergency procedures for fire, earthquake, extreme weather conditions, spills of hazardous materials, power failures, occurrences of terrorism, and health-related incidents

Training sessions should include extensive video and hands-on demonstrations of gowning, aseptic techniques, and other appropriate operations. As part of qualification to operate in aseptic environments, trainees should undergo practical examinations in aseptic techniques, assembly and disassembly of equipment, and other practical requirements. If possible, at least some training should be conducted in actual cleanroom facilities. All personnel should attend periodic refresher sessions.

In addition, summaries of company policies regarding cleanroom operations, gowning, safety, and other important information should be conspicuously posted in all cleanroom dressing rooms. Eye-catching posters and frequent newsletters can be effective reminders of proper cleanroom behavior.

XII. PERSONAL HYGIENE

Proper cleanroom behavior begins at home. Scrupulous care of the skin and hair must be practiced on a regular basis. Frequent bathing and shampooing will ensure minimal shedding of skin fragments. Personnel with temporary skin conditions such as severe sunburn, or those with colds or allergies that cause abnormal nasal flow, coughing, or sneezing should be assigned to noncleanroom areas until the condition passes. Cosmetics

such as face powder, nail polish, hair sprays, and skin medications including aftershave lotions must never be worn in the cleanroom. Men should be clean shaven at all times or wear appropriate masks. Clothing worn under cleanroom garments should be clean, nonfrayed, and nonlinting.

XIII. GENERAL PERSONNEL PRACTICES

The following discussion covers numerous general practices that have been shown to improve the effectiveness of the clean air features of a cleanroom for controlling both viable and nonviable particulate contamination.

Avoid all rapid movements in the cleanroom. These not only disrupt the protective action of unidirectional air flow, especially around clean zones and biosafety cabinets, but they also cause increased leakage of particles at the neck and wrists by a bagpipe effect. Nervous movements like head scratching or chin rubbing may help us think, but they can contaminate the worker's glove and generate additional airborne particulate contamination. Such movements should be avoided. One should never remove goggles or face mask to wipe off fog or open or reach into his or her garment to retrieve an item while in a cleanroom. Talking also contributes to contamination levels and should be limited.

Personnel should not smoke immediately before entering a cleanroom as smokers have been shown to continue to exhale thousands of smoke particles long after their last puff.

Maintaining tools and materials in a neat and orderly fashion makes it easier to keep work areas clean and also reduces unnecessary movements. Tools and other objects that fall to the floor should never be used until they have been cleaned and, if necessary, sterilized. An object that falls to the floor should be picked up with a second tool and both items set aside for cleaning. Alternatively, the object can be picked up with a wipe cloth and set aside for cleaning; the wipe cloth should be discarded. Hands or gloves should not touch the floor or the dropped item. Gloves should be changed whenever they knowingly are contaminated.

Products that fall to the floor must not be returned to the process stream until they are inspected and cleaned, or discarded per company policy. It has been estimated that nearly 75% of pharmaceutical product failures due to contamination can be traced directly to the improper handling of products and containers by personnel.

Paper products such as books, notepads, or wrappers should never be brought into a cleanroom unless they are specially designated for cleanroom use. Only approved writing instruments should be used, never pencils. Ordinary paper and pencils are known to shed large numbers of particles.

Finally, eating and drinking, including gum chewing, are never permitted in a cleanroom. Not only are many food products and their wrappers considerable sources of particulate contamination, but movements of the mouth and face during eating will also contribute great numbers of particles to the surrounding air.

XIV. SUMMARY

A cleanroom is a facility that is designed to minimize particle contamination. Many important factors impact the quality of a cleanroom: the physical facilities, quality and quantity of ventilation air, incoming raw materials, and attire and behavior of operating personnel. Modern cleanroom design, thanks to over 40 years of evolution, has achieved a high level of effectiveness. Airborne contamination can be controlled through the use of unidirectional HEPA- or ULPA-filtered air. Strict manufacturing standards and a thorough testing program can prevent problems caused by dirty equipment and raw materials. That leaves cleanroom personnel as the major source of contamination. The contribution that personnel make can be minimized by proper training in cleanroom behavior, by the use of appropriate garments and tools, and careful personal practices both at work and at home.

5

DETECTION AND ENUMERATION OF MICROORGANISMS IN THE CLEANROOM

I. INTRODUCTION

In order to confirm that biocontamination controls are operating properly, it is necessary to assess the microbial levels of the air and surfaces of cleanrooms and controlled environments. In this context, biocontamination controls mean every action that is directed toward the detection, reduction, or elimination of microbiological contamination in cleanrooms and other controlled environments. That environment includes the air that ventilates the facility, the surfaces of the facility (walls, floors, ceilings, tabletops), the equipment, instruments and other hardware therein, and the personnel who occupy the facility. In-house cleaning materials and implements should not escape periodic testing. In addition, in a manufacturing setting, microbial levels should also be determined and controlled for raw materials, process water and the system that produces, stores and distributes it, gases, packaging, and final products.

II. OVERVIEW OF MICROBIOLOGICAL ENVIRONMENTAL MONITORING

A. Validation

Producers of foods, pharmaceuticals, medical supplies, and devices and other products that directly impact the health and safety of consumers

must be assured of the quality of their output. This goal is especially pertinent when dealing with sterile products. Each item cannot be tested to prove its sterility, and even the reliability of testing "representative samples" of a manufacturer's finished products is suspect. Instead, most attention is concentrated on the processes that create the sterile items. If it can be shown that the processes turn out sterile products with a high degree of assurance, the goal is met.

Microbiological attention also is focused on nonsterile products, which still may have microbiological limits. General limits may be for total bacterial or fungal counts or there may be limits on so-called objectionable microorganisms. These are organisms that may cause product deterioration or are deemed hazardous for consumers if present in a product. Objectionable microorganisms generally are species, product, and application specific. For example, nonsterile ophthalmic medications might have limits on organisms known to cause eye infections and feminine hygiene products might have limits on organisms that are known to cause vaginal infections. Assurances of high-quality products are established through a series of documented validations, or exercises that support "…by objective evidence that a process consistently produces a result or product meeting its predetermined specifications" (FDA Quality Systems Manual, 1996).

Validations are akin to scientific investigations in which hypotheses are formulated, experiments are designed and conducted, data are collected and analyzed, and conclusions are reached. And, like scientific discoveries, validations must be questioned and retested for the life of the process they cover.

Every aspect associated with microbiological monitoring must be validated. That includes samplers and implements, rinse fluids, diluents, media, incubators, automatic colony counters, the software that processes data — anything that would have an impact on the results of environmental sampling and the conclusions that are reached.

New facilities or processes undergo initial microbiological quality validation to demonstrate their capability to produce product that meets established standards. One or two months' data from daily microbiological monitoring may be collected from the outset to establish a baseline. As data accumulate, alert and action levels can be established. Monitoring should then continue throughout the life of the process in order to detect trends in contamination levels that may occur.

B. Monitoring Techniques

1. General Considerations

The results of microbiological environmental monitoring usually are expressed as total aerobic counts (TAC) or total bacterial counts (TBC)

Table 5.1 Indicator organisms

Organism	*Description*	*Typical Habitat/Pathogenicity*
Staphylococcus aureus	Gram-positive bacterium	Skin/human pathogen
Escherichia coli	Gram-negative bacterium	Intestine/usually nonpathogenic
Pseudomonas aeruginosa	Gram-negative bacterium	Water and soil/human pathogen
Salmonella spp.	Gram-negative bacterium	Intestine/human pathogen
Candida albicans	Yeast	Skin, mucous membranes/human pathogen
Aspergillus niger	Mold	Soil, air/human pathogen

and total yeast and mold counts (TYMC). The total viable count (TVC) is the sum of the TAC and the TYMC. Although sampling techniques are identical for determining the TAC and TYMC, the two counts are carried out differently with respect to media and incubation conditions, as described below. Monitoring for the presence of the indicator organisms (Table 5.1) also may be necessary. Indicator organisms are a specific selection of bacteria and fungi that are typical of pharmaceutical cleanroom contaminants and may present a hazard if allowed in a product. Indicator organisms should not be confused with Biological indicators (BIs), which are used to validate sterilization processes.

Assessing levels of airborne microorganisms generally is conducted by drawing room air through a sampling device that traps particulates either in a liquid or upon an agar surface. If collection is in a liquid, aliquots of the liquid can then be assayed for viable microorganisms by routine plating methods as described in Chapter 2. If particles are captured onto an agar medium, the agar only needs to be incubated under appropriate conditions to produce visible colonies. Various fallout methods detect the extent of settling by gravity of viable particles onto horizontal surfaces. These too are incubated to support colonial development.

Surface microbial contamination can be estimated by sampling a defined area with sterile swabs, contact plates, or other varieties of slides or strips. Workers' fingertips and other parts of the body that may come into contact with product or critical surfaces of machinery may be monitored in a similar manner. The collected viable particles can then be assayed by standard microbiological techniques.

Viable contamination in liquids and gases is conveniently assessed by passing them through membrane filters of porosities small enough to trap the microbes, after which the filters are cultured. The determination of

microbial contamination of solids and semi-solids such as foods or pharmaceutical products requires special procedures to free the microbes from the materials. The organisms are then cultured in the normal manner.

Microbiological population levels in properly maintained biocleanrooms tend to be low, requiring relatively sensitive detection methods. One of the great advantages of microbiological sampling is that in most instances it only takes one culturable cell to produce a visible colony. The detection of one bacterial cell in 100 ml of saline represents a sensitivity of one part in 10^{15}. However, the price for that sensitivity is time, for it takes a minimum of 8 to 12 h for a single cell to develop into a visible colony. Some newer methods have significantly reduced the time for a response. A few of these methods will be described later in this chapter.

The need for aseptic techniques while conducting environmental sampling cannot be overstated. Extreme care must be practiced when loading and unloading air samplers with collection plates or strips. The same precautions apply to contact plates and all other environmental sampling methods. Because microbial counts in cleanrooms tend to be low, any inadvertent contamination can be significant. Personnel conducting environmental sampling must undergo extensive, specialized training in general microbiological principles and specific techniques in sampling of viable microorganisms in addition to the normal training for cleanroom personnel.

There are three phases of microbiological analysis: detection, enumeration, and identification. These are defined in Table 5.2. An item that is labeled as sterile must be free of viable microorganisms, which is demonstrated by validated methods of detection. Mere evidence of growth in

Table 5.2 Phases of microbiological analysis

Phase	*Question to be Answered*[a]	*When Applied*
Detection	Are culturable microorganisms present?	Sterility testing
Enumeration	How many culturable microorganisms are present?	Environmental monitoring Bioburden monitoring Nonsterile product quality control
Identification	What culturable microorganisms are present?	All microbiological analyses

[a] By using traditional laboratory techniques. Newer techniques using DNA probes can detect and identify nonculturable organisms as well as culturable ones.

a medium is sufficient evidence to indicate nonsterility. The need to enumerate the viable microorganisms present in a sample comes into play when limits have been set for various materials or environments. For example, there are regulatory limits for viable microorganisms in drinking water, certain foods, raw materials for manufacturing, process water, nonsterile products, and the air around sterile filling processes. Identification means determining the identity of a microbial isolate down to genus and species. Identification of environmental isolates is often helpful as an aid to tracing the origin of contamination. Certain species are typically associated with the human body and others are common in water or soil. Identification of environmental samples is recommended during the validation phase of a new facility or system. If exceedances of alert or action levels occur at a later time, knowledge of what organisms are normal for the facility will be helpful in determining the cause of the exceedances.

Whether one is sampling the air of a cleanroom, the parts of a filling machine, or a load of product, one fact of life that is often overlooked is the distribution of contamination. Contamination is seldom distributed uniformly over the volume, area, or lot of product that is being sampled. Contamination may vary by many orders of magnitude within a few centimeters of a machine surface.

2. Sampling Sites

When conducting microbial sampling in a biocleanroom facility, every source of microbial contamination must be identified and examined. While personnel can be the major reservoir of microorganisms in a cleanroom, contamination can be transferred indirectly via the air or directly to sensitive products, surfaces, or processes within the facility. Thus, sampling not only must include personnel but the air and all exposed surfaces. In those facilities that involve the manufacture of a product in which microbiological contamination must be controlled, the environmental sampling horizon must be expanded to include raw materials, process water and the system that produces it, the finished product, packaging, and the machinery that comes into contact with the product.

Sampling sites should not be confined to central cleanroom locations, but might extend into auxiliary areas such as component and formulation preparation rooms and other support areas, vestibules, corridors, and dressing and gowning rooms. Finally, the laboratory in which environmental samples are processed must also be scrutinized. For consistency and reproducibility, sites that are chosen for sampling must be accurately defined and documented in the form of, for example, environmental monitoring maps.

3. Frequency of Sampling

Documented company policy should dictate where and how often sampling should take place and how many samples are to be taken. ISO and other advisory and regulatory documents have guidelines to aid one in these decisions. Sites may be prioritized according to their relative influence on the process or product that is to be protected from microbial contamination. As an extreme example, machinery parts or gases that come in direct contact with a sterile product should be sampled more often than dressing room walls. Sites that are difficult to sanitize such as spaces between machinery should also be given greater attention. Sampling should be conducted during every work shift and, if a manufacturing process is involved, samples should be taken at various times — at the beginning of a manufacturing run, in the middle, and at the end. Occasionally, times of sampling should be chosen that represent worst-case scenarios such as during overtime sessions and other stressful periods. Sampling intensity should be increased whenever major events occur in a facility such as replacement of room air filters, power failures, or significant changes in processes or personnel. Sampling must not, however, be so intrusive of sterile areas that a risk of inadvertent contamination becomes significant. It bears repeating that personnel conducting environmental sampling must undergo extensive, specialized training in general microbiological principles and specific techniques in sampling of viable microorganisms.

4. Transporting Microbiological Samples

Environmental samples for microbiological testing should be processed as soon as possible. If delays are necessary, samples should be protected from extremes of heat, humidity, and light. If processing is delayed by more than an hour, refrigeration is normally recommended to reduce multiplication of collected organisms. Freezing should be avoided since, as you recall, some mortality occurs when microorganisms are frozen. If the delay between collection and analysis is more than 8 h, survival of collected organisms must be validated. That can be conducted by inoculating sampling implements such as swabs with not more than 100 CFUs of each of the facility's specified indicator organisms. At the same time, control plates or filters are inoculated with identical numbers of the indicator organisms and incubated. The implements are held under the same conditions samples would experience if a delay in processing them would occur. Following the anticipated delay in processing, the stored samples are assayed. The two results (as CFUs) are then compared.

III. MONITORING OF AIR

Cleanroom air can act as a carrier of microbial contamination from cleanroom personnel and other sources to products and critical surfaces. Air sampling may be active or passive. Active sampling involves devices that dynamically collect particles from known volumes of air, allowing for quantitative determinations of air quality. Passive sampling devices are represented by a variety of qualitative methods to assess the fallout of viable airborne particles. They will be described in Section IV.

Two general types of active samplers are used for assaying airborne microorganisms: impactors and impingers. Impactors collect airborne particulate contamination onto a solid or semisolid surface such as agar; impingers collect the particles in a liquid. As defined earlier (Chapter 4), a viable particle is one that has one or more living microorganisms attached to it and, if captured in or on a nutrient medium, growth can result following suitable culture procedures.

Airborne viable particles, like specks of dust or skin flakes, nearly always have many — perhaps thousands or more — viable microbial cells attached to them. If captured on a solid medium the colonies that form do not necessarily reflect the true microbial population of the air that had been sampled. Due to air turbulence within the sampling device and the stress the particles experience on impact onto the sampling surface, the particles may break up randomly. A viable particle consisting of 1000 bacterial cells may result in the formation of anything from 1 to 1000 colonies. Consequently, airborne microbial populations are usually reported in terms of colony forming units (CFU) per unit volume of air, which avoids any implication regarding a precise population determination. As long as sampling procedures are consistent throughout and any remedial action is based on CFU trend analysis, such precise population determinations are not necessary.

A. Air Samplers

1. The Andersen Sampler

The Andersen sampler (Figure 5.1), developed in the 1950s by Dr. Ariel Andersen, remains one of the most widely used active viable air samplers. Its high efficiency coupled with its ability to fractionate collected particles into several size ranges are the major factors that contribute to its popularity. Various models of the Andersen sampler have eight, six, two, or one stage. The size ranges of collected particles for the six-stage model are shown in Table 5.3 on page 156.

a.

Figure 5.1 (a) Andersen six-stage sieve sampler. Cross section (b) shows typical pathway of air as it passes through successive perforated stages of decreasing hole size and increasing stream velocity. Captured particles are sorted according to size. See Table 5.1. ***Continued.***

Each stage consists of a perforated plate that is positioned above an open dish of agar medium. The holes in each stage are the same number and size, but become smaller in each successive stage that the air passes down through the sampler. The smaller the hole, the greater the velocity of the air directed onto the agar surface. Larger particles are impacted in the upper stages because of their greater inertia, but smaller particles pass through and are collected in succeeding stages. The sampler collects air at the rate of 28 l or 0.028 m^3/min. The resulting colonies that form are counted and reported in terms of CFU per liter or cubic meter of air according to the size of the collected particles.

When sampling air that contains moderate to high levels of viable particles, the probability becomes quite high that two or more viable particles may impact the agar at or near the same point to produce a single colony. The manufacturer of the sampler supplies a table to correct for this. Overcrowded plates are usually not a problem in cleanroom monitoring, however. More often than not, airborne microbial counts in cleanrooms may approach the lower limits of sensitivity of the Andersen sampler.

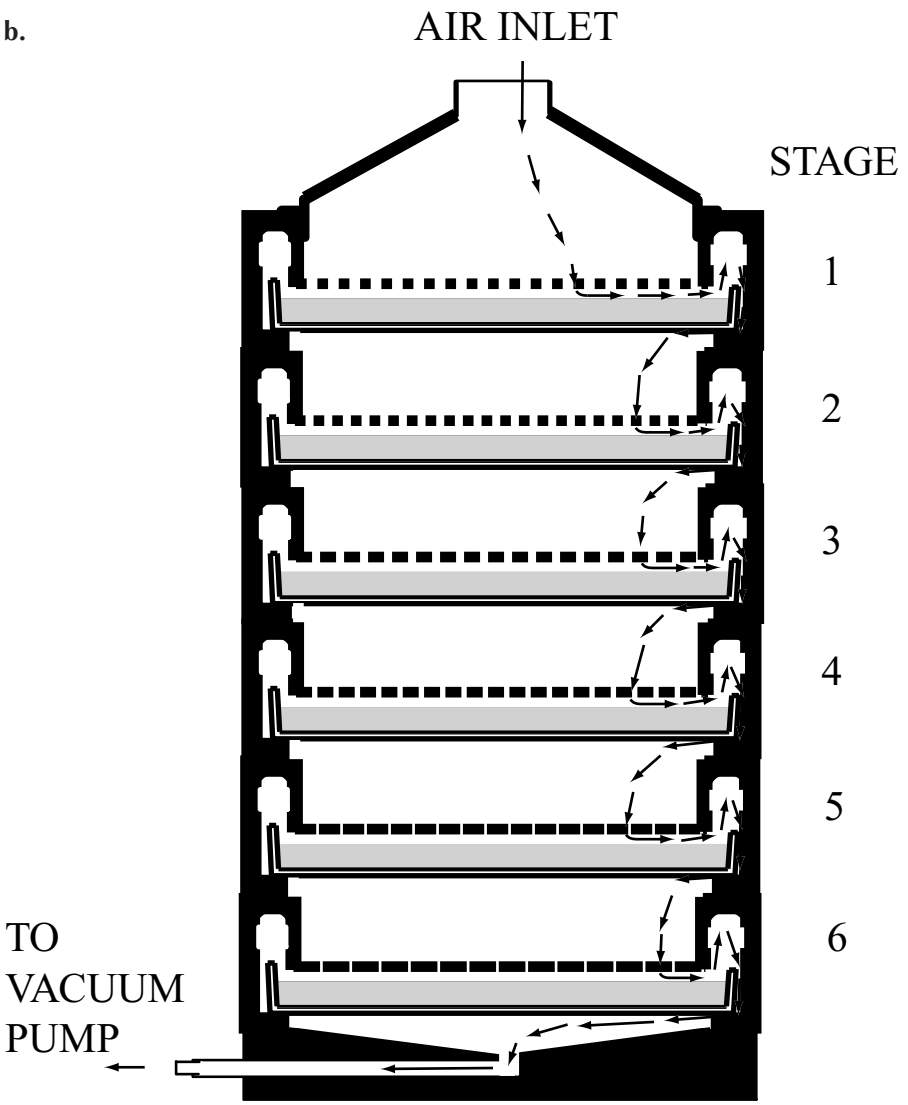

Figure 5.1 ***Continued.***

2. The Slit Sampler

The slit or slit-to-agar (STA) sampler is an impactor-type sampler in which the oversize agar plate rotates under the collection slit (Figure 5.2). Since the rotation of the plate is made constant by a clock mechanism, one rotation per hour, for instance, we obtain colony counts that can be related

Table 5.3 Particle size ranges of a six-stage Andersen air sampler

Stage	*Collection Size Range (μm)*
1	>7.0
2	4.7–7.0
3	3.3–4.7
4	2.1–3.3
5	1.1–2.1
6	–1.1[a]

[a] Lower limit of stage 6 is uncertain.

to the time of collection. This ability becomes very useful when one is trying to determine a source of contamination incidences. For example, if a careful record is made of all of the activities in a cleanroom during the period of sampling, any significant change in counts during the period can be related to specific activities, such as the switching off of anti-static devices, the opening of a door, or the centrifugation of a bacterial culture (Figure 5.2b).

Another positive feature of the slit sampler also has to do with the rotation of the sampling plate. Because the airstream is focused only on a relatively small area of the agar surface at any one time, desiccation of collected cells is less of a problem even after one hour of operation.

In order to maintain reproducible results from an impaction sampler, the distance from the perforated plate or slit to the agar surface must be kept constant. This is accomplished by assuring that all sampling plates contain precise amounts of agar; that is, prepared to a constant depth. That amount is usually listed in the sampler manufacturer's instructions. Some slit-to-agar samplers have a means of adjusting the distance from the slit opening to the surface of the agar. This feature avoids the need to fill the culture dish with precise amounts of agar.

3. Portable Samplers

Some microbiological air samplers are sufficiently light and compact to hand carry to sampling sites. Two such samplers will be described. Two models of SAS samplers (Figure 5.3) collect air samples at rates of 100 and 180 l/min. Particle collection is accomplished by impaction onto a single agar plate. The samplers are able to accommodate two sizes of culture plates: 55-mm contact plates and standard 90-mm Petri plates.

Although it is an impaction sampler, the Reuter centrifugal sampler (RCS) draws air by a different principle than we have seen in the other

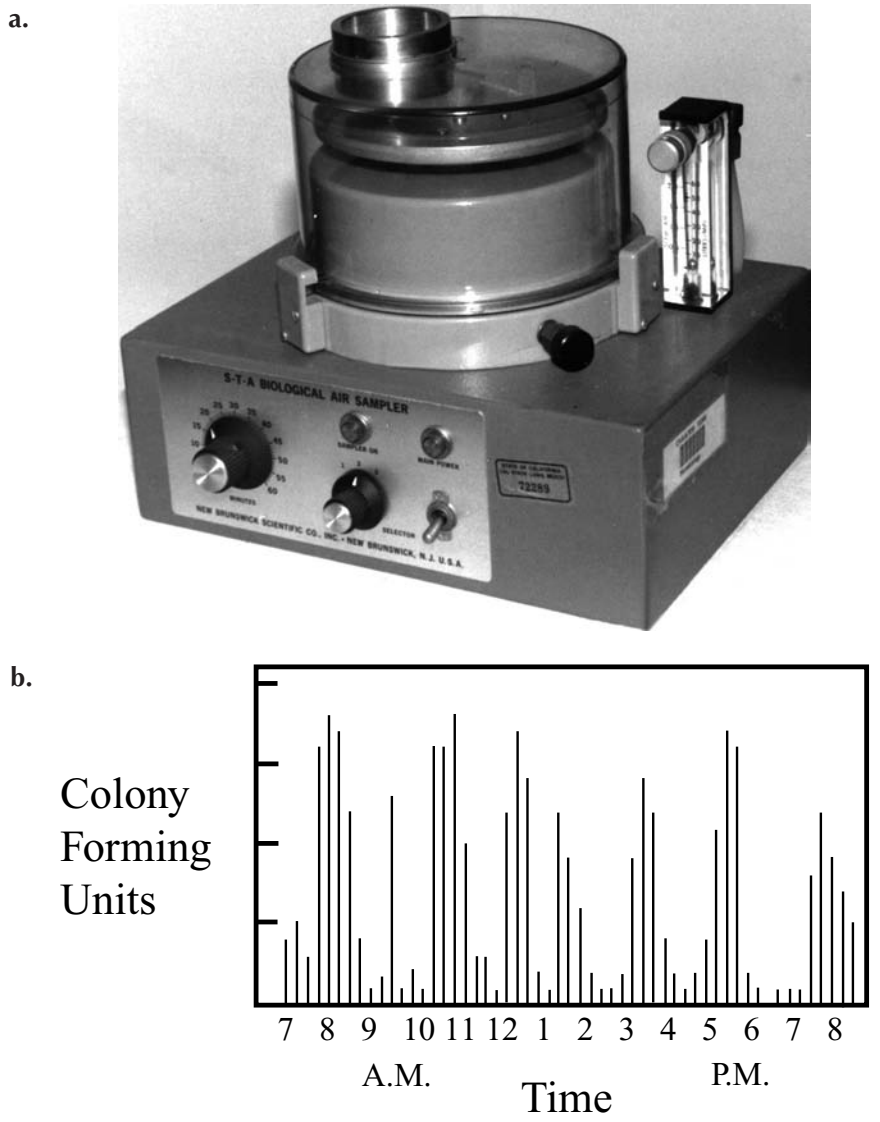

Figure 5.2 (a) Slit-to-agar sampler. A 150-mm agar plate rotates under the inlet slit, distributing viable particles over the agar surface. Colony counts can be made as a function of time. A typical plot (b) shows the fluctuations in airborne contamination that occur as a result of employee activity during a work shift in a manufacturing facility. Increased counts occurred at the start of the shift (8 A.M.), at the morning rest break (10:30 A.M.), the lunch break (noon), the afternoon break (2:45 P.M.), and at the end of the shift (5 P.M.). The cleaning crew began work around 7 P.M. Colony counts are for 15 min segments.

Figure 5.3 An SAS air sampler. (Photo courtesy Bioscience International, Inc./Marsha Pratt.)

impaction devices. Instead of drawing room air into the device by a vacuum pump, airborne particles, accelerated by the blades of a centrifugal air turbine, are impacted onto the collection surface (Figure 5.4). Depending on model, collection rates are listed as 40 to 100 l/min (.04 to 0.1 m^3/min). The collection surface consists of small segments of agar growth medium held in depressions in a narrow, flexible strip of plastic. The strip slides into the inner wall of the centrifugal turbine where it collects particles. After sampling is completed, the medium-containing strip is removed, its cover replaced, and incubated appropriately.

Figure 5.4 An RCS centrifugal air sampler, with three sample strips. Below, a sample strip following incubation. (Photo courtesy Biotest/Carol Julich.)

Samplers such as the RCS and SAS are quite portable, being only slightly larger and bulkier than a large flashlight. They are battery operated and contain built-in timers that allow a range of collection times. While very portable, it is recommended that these samplers be operated placed on a surface or mounted on a stand rather than handheld. That is because the microbial contamination from the operator may be collected by the sampler and create a biased estimate of airborne levels in the facility.

4. Liquid Impingers

While not widely used, impingers have an advantage not enjoyed by the impactors: collection liquids can be divided into several aliquots, each of which can be treated differently. The liquid impinger (Model AGI-30, Figure 5.5) is filled with 20 ml of collection fluid, into which a jet of air is directed as the result of a vacuum being drawn from the side arm. Airborne particles are impacted onto the bottom of the impinger and are immediately washed off by and suspended in the turbulent collection fluid. This very efficient sampler operates at 14 l/min. Following the collection period, the inner wall of the curved neck must be rinsed with sterile collection fluid, which is combined with the fluid in the sampler.

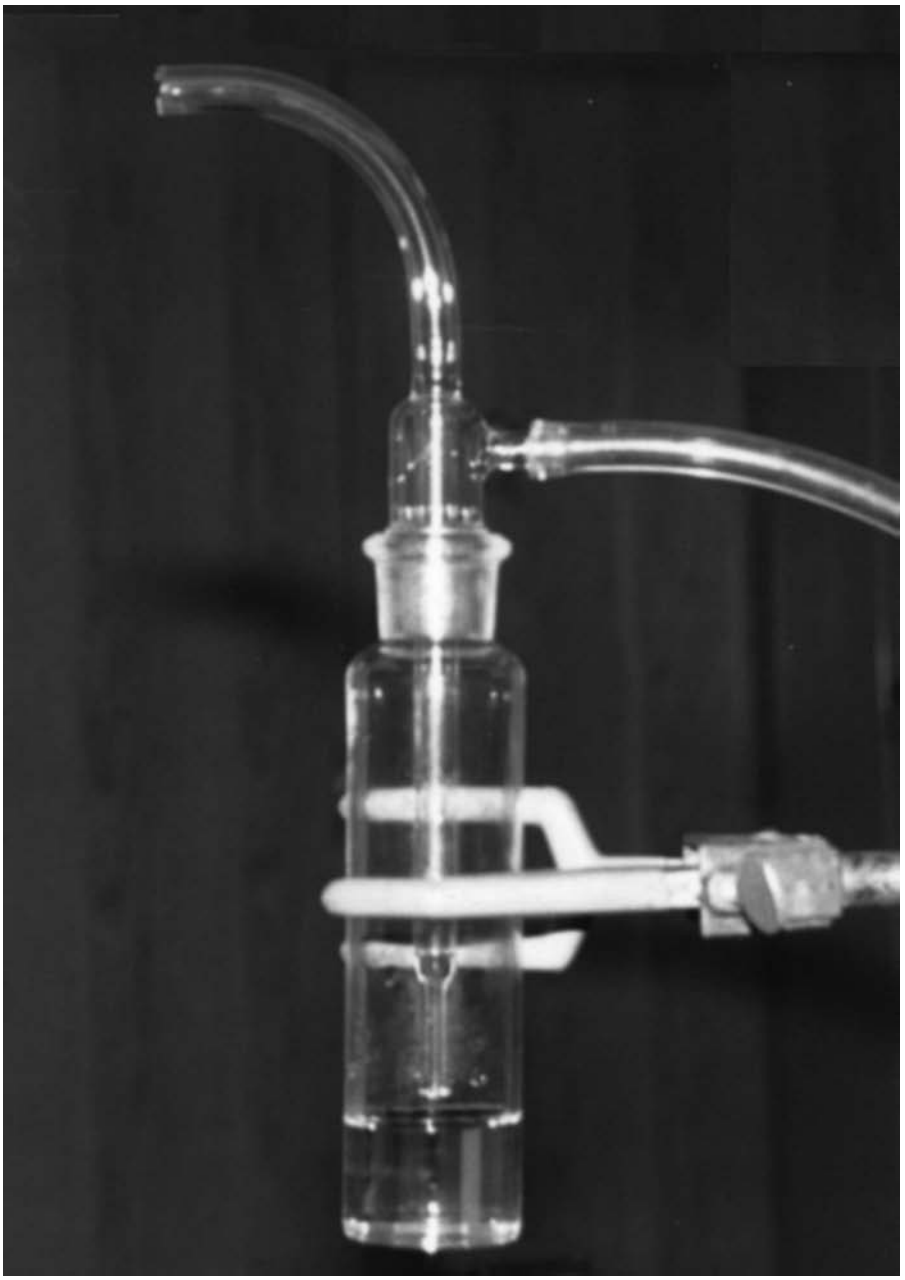

Figure 5.5 An AGI 30 impinger-type air sampler. The curved inlet tube is designed to simulate the human nasopharynx.

This is necessary because the neck was designed to trap particles larger than about 5 μm in order to simulate the human nasopharynx. The collection fluid can then be filtered through an appropriate membrane filter or plated on solid media. Various aliquots may be plated onto media specific for bacteria, fungi, coliforms, or heat shocked at 80°C for 10 min to detect bacterial spores.

5. Sampler Efficiencies

No air sampler is 100% efficient, and various types of samplers can vary enormously in sampling efficiency. Physical sampling efficiency is defined as the proportion of airborne particles within a sampler's theoretical size collection range that is actually trapped. It is meaningless, for example, to compare results from an Andersen sampler with those of an RCS sampler, unless the two samplers had been previously calibrated by running them side by side under very carefully controlled conditions.

Collection efficiencies of the same sampler can vary depending on its setup. For instance, the efficiency of the same sampler in a horizontal flow cleanroom will be different from that in a vertical flow room if the sampler inlet is not adjusted so that the room air flow direction and velocity relative to the inlet are the same in both instances. When the velocities of the room air and the air entering the sampler intake are exactly the same, the condition is known as isokinetic sampling. Isokinetic sampling is considered the ideal, but may not be achievable in all situations. In the absence of isokinetic sampling, it is imperative that all sampling be carried out consistently.

There also is the question of biological efficiency of an air sampler. Airborne microorganisms are exposed to considerable stress when they are collected and during the time they remain on the collection surface while the instrument is operating. As air passes over collected cells, dehydration can inflict serious injury on a microbial cell. As explained above, this is less of a problem with the slit sampler. The danger of loss of viability of collected cells is particularly present when microorganisms are collected on a dry surface such as a membrane filter. As one might predict, bacterial spores are fairly resistant to the forces of the collection process. However, vegetative cells may be injured when collected and fail to grow or show delayed growth. In either case, they may be overlooked, resulting in an underestimation of the microbial load of a sample. There is the temptation to sample for long periods of time in order to collect more statistically meaningful data. Sampler manufacturer's recommendations should not be exceeded. As long as all sampling procedures are fully documented and held constant and action is based for the most part

on trend analysis, the myriad errors that are apparently inherent in sampling of airborne viable particles can be essentially disregarded.

IV. FALLOUT METHODS

Fallout methods passively assess the level of contamination that may settle out of the air onto surfaces by gravity. Although fallout methods do not give an accurate assessment of airborne contamination levels and should never take the place of an active air sampling program, they can be a simple and relatively effortless measure of the general condition of a cleanroom. They are also useful in detecting specific areas of a cleanroom where air flow may fail to protect objects from biocontamination. There may be spots around equipment, for example, where the unidirectional air flow is sharply deflected, causing particulates to deposit onto a surface of the equipment, or in or on the product or container. Localized areas where air flow velocities are reduced because of turbulence may also experience particulate fallout. These, too, can be detected with fallout sampling. Fallout, settling, or settle plates are simply culture dishes containing appropriate growth medium that are left uncovered for varying lengths of time (usually an hour or more) to collect viable particles. There are automatic devices on the market that open a Petri dish to receive fallout and then close the dish after a predetermined time. Such devices avoid the possibility of operator contamination as they are being set up. The results of fallout plates are reported in terms of colony forming units per time period (minute or hour) per plate or per agar area (square inches or square centimeters). One of the advantages of settling plates is that the organisms that are captured are not subjected to the stresses that airborne organisms collected in impactors experience. Thus, assuming proper media are used, organisms growing on settling plates are probably more representative of the true range of microbial species in a cleanroom than what might be collected in an air sample.

Fallout, or witness strips, are small, sterile pieces of glass, metal, or plastic that are placed uncovered about the cleanroom. Glass microscope slides can be used for this purpose. After appropriate time intervals, the strips are aseptically collected with sterile forceps and placed into wide-mouth bottles of sterile 0.5% peptone water, which is then assayed on suitable growth media or subjected to membrane filtration. Some laboratories subject the bottles to agitation on a mechanical shaker (vortexing) or insonation in an ultrasonic bath before plating to increase release of particles from the strips. If sample bottles are subjected to insonation, the bath liquid should be at least as high as the rinse liquid within the bottles, and the time of treatment should be validated.

In a manner similar to that described for handling liquid impinger collection fluids, the rinse liquid from fallout strips may be split several ways, allowing one to test, for example, for aerobes, anaerobes, and fungi all from the same fallout strip. One aliquot may be heat shocked at 80°C for 10 min to assess the presence of bacterial spores. The strips can also be cultured directly by placing them into sterile Petri dishes and covering them with an appropriate molten agar growth medium. Following incubation, submerged colonies can be observed on the strips and counted.

V. SURFACE MONITORING

Environmental surfaces in a bioclean area should be monitored microbiologically on a regular basis to confirm that all contamination control precautions including cleaning operations are being conducted satisfactorily. Monitoring can be accomplished by directly sampling surfaces such as floors, walls, and equipment parts, as well as workers' garments, gloves, and fingertips, with swabs or contact plates.

Swabs are especially useful for sampling hard to reach or irregularly shaped surfaces to obtain a general estimate of surface contamination. Sterile cotton, rayon, or alginate swabs are slightly moistened with sterile water or transport medium and rubbed over a specified area of the object to be tested, say 25 cm^2. The area should be covered at least three times using the same swab, while reversing the direction of rubbing each time. The tip of the swab should be rotated during sampling. If the swab stick is handled by the worker the head of the swab should be broken off aseptically from the section that came in contact with the worker's glove and allowed to fall into a tube of a transport medium. Prior to assay, the fluid may be shaken, vortexed, or subjected to insonation. Commercially prepared swabs that are attached to the cap of the tube of transport medium (Figure 5.6) must be handled by the cap only. When sampling is completed the swab is reinserted into the tube. If contamination levels are expected to be high, dilution of the transport medium may be necessary before plating. If swabs are moistened with a nutrient transport medium, the areas that are sampled must be cleaned after sampling with alcohol or detergent wipes to remove residual organic matter, which could support growth of microorganisms.

Irregular surfaces on pipes, tanks, and filling equipment can also be sampled with contact samplers such as Biotest surface sampler (Figure 5.7). These are flexible plastic strips that are filled with appropriate agar medium that can be pressed gently against a curved surface. The sampler should not be rotated or rubbed over the surface. The strips are available with a variety of media.

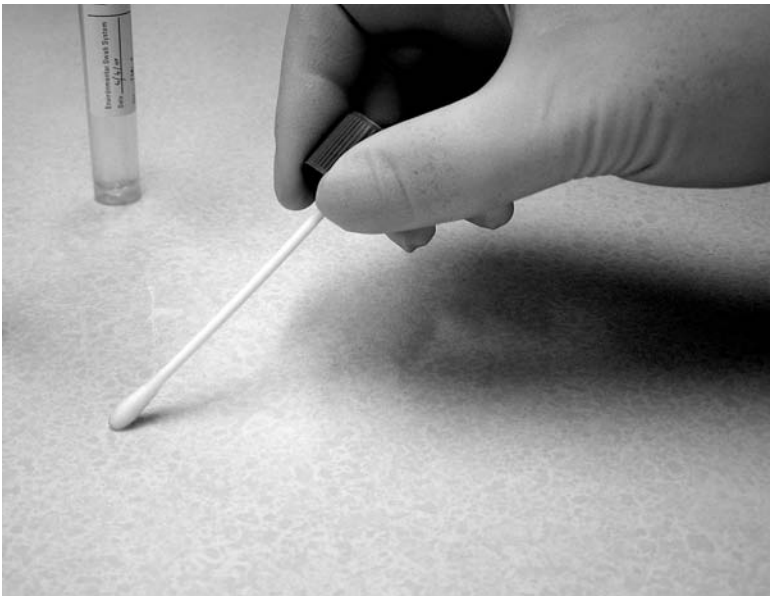

Figure 5.6 Sampling a surface with a swab.

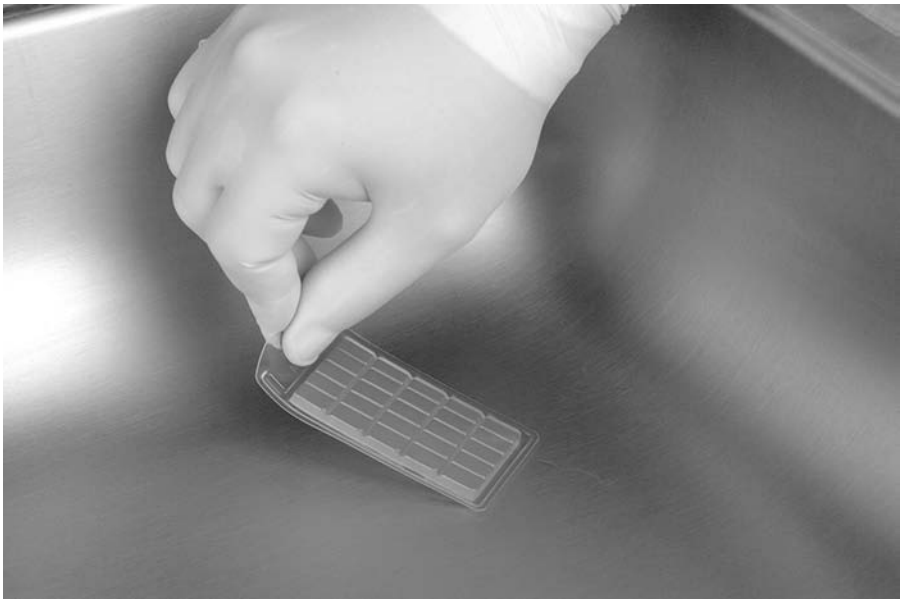

Figure 5.7 A Biotest contact strip sampling the curved surface of a sink. (Photo courtesy Biotest/Carol Julich.)

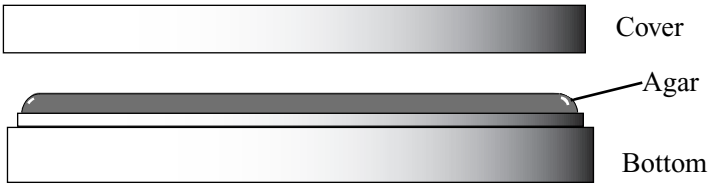

Figure 5.8 A RODAC plate with agar.

Contact plates, such as RODAC plates, are small plastic dishes that are overfilled with growth medium so that a meniscus of agar rises above the rim of the device (Figure 5.8 and Figure 5.9). To sample a flat surface such as a floor, tabletop, or wall, the agar surface is pressed against the surface, using gentle pressure to ensure complete contact. To standardize the pressure applied to contact plates when sampling horizontal surfaces, special weights that fit on the plates are available. Nonrigid surfaces such as gowns or packaging material can also be sampled in this manner if backed by a solid surface. The plates are then incubated under appropriate conditions and the resulting colonies are counted. Results can be reported either in terms of CFUs per plate, or CFUs per square inch or square centimeter by dividing the CFUs per plate by the area of the agar surface, which for RODAC plates is 25 cm^2.

Figure 5.9 Gentle pressure is applied to a RODAC plate when sampling a flat surface.

Nonpermeable surfaces that come in contact with media from the contact plates should be cleaned with an alcohol or detergent wipe to remove residual medium transferred to the surface. Fabrics and other permeable surfaces that have been sampled in this manner should be set aside for laundering or discarded, since residual medium cannot be removed easily and will support microbial growth. Prepoured contact plates and strips are commercially available with a variety of media for the recovery of bacteria and fungi. Empty, sterile RODAC plates are also available for facilities that need to prepare their own plates with specialized media.

If the surfaces to be sampled have residual disinfectant or cleaner on them, the medium used in the contact plates must contain appropriate neutralizers such as Tween 80 or lecithin. Each class of disinfectant requires a specific neutralizer. Since many neutralizers are heat sensitive, each may have to be sterilized separately and added to the medium aseptically just before preparing the plates. The need for neutralizers cannot be overstated. If traces of disinfectants are transferred to the medium, bacteriostasis may occur, limiting growth of the captured bacteria and leading to an underestimation of contamination levels. See Section X for more information about bacteriostasis and fungistasis due to disinfectant residues.

VI. TOUCH PLATES AND OTHER PERSONNEL MONITORING

Sterile gloves may become contaminated through inadvertent contact with nonsterile surfaces in the cleanroom such as skin or clothing. In addition, gloves may fail if punctured by jewelry, machinery, or tools, allowing skin bacteria to migrate to the outer surface of the glove. To detect the possible presence of contamination, the gloved fingertips of personnel who work in aseptic fill and other critical areas should be monitored at periodic intervals with touch plates. These are ordinary culture dishes containing sterile agar medium on which workers leave their gloved fingerprints. The plates are then incubated in the normal manner.

Figures for the numbers of microorganisms that are shed by humans during various activities were presented in Chapter 1. The figures cited are typical and do not reflect real situations. There are individuals who shed considerably more organisms than the estimated averages. These individuals, known variously as shedders or cloud workers, must be identified through personnel monitoring and transferred out of cleanrooms and other facilities in which microbiological controls are in place. Excessive shedding of microorganisms may be due to lax personal hygiene or improper gowning, which may be improved through counseling, but for

others it may be caused by inherent physiological conditions that may not be reversible.

All of the contact monitoring operations described above involve lifting microorganisms from hard surfaces, fabrics, or gloves. These operations are subject to significant sampling errors due to differences in the manner by which the samples are collected. The pressure one applies to the swab or contact plate, for example, and the number of scrubbings with the swab may have a great impact on the results of the test. Techniques must be standardized through full documentation and thorough personnel training.

VII. MICROBIOLOGICAL ASSESSMENT OF LIQUIDS

Water and liquids with viscosities near that of water can be passed through sterile membrane filters that have porosities of 0.22 μm, assuring that most bacteria and fungi will be trapped. Viruses generally will not be retained by these filters unless they are strongly attached to larger particles or are retained by mechanisms other than sieving. Viscous liquids such as syrups that are water soluble can be diluted with sterile buffer before filtration. Once a measured volume of sample is drawn through the filter, the filter is placed aseptically onto an appropriate solid medium or onto a paper pad that is saturated with liquid medium. In either case, nutrients soak into the filter membrane and support the formation of colonies that can then be counted and related back to the volume of liquid sampled.

The multitube or MPN (most probable number) method has been a valuable and labor-saving tool for estimating levels of coliform bacteria in drinking and recreational waters for decades. For that application a differential medium is used to detect the presence of the coliforms. The method has been adapted for estimating TAC by using a general-purpose liquid medium such as tryptic soy broth. Various dilutions of pure water system samples, bioburden rinse liquids, liquid impinger samples, and other aqueous samples are inoculated as various dilutions into multiple tubes of broth. That is, three tubes are inoculated with the undiluted sample, another three with a 1:10 dilution, and an additional three with a 1:100 dilution. Following incubation, the tubes are examined for signs of turbidity, indicating that at least one culturable cell ended up in a tube. The number of positive tubes in each respective dilution series is recorded and the results are analyzed with the aid of an MPN statistical table. For example, suppose three out of three tubes from the undiluted sample, two out of three from the 1:10 dilution, and two out of three from the 1:100 dilution all show turbidity. According to the MPN table, that represents an estimated TAC of 210 microorganisms/ml of original suspension.

VIII. MICROBIOLOGICAL ASSESSMENT OF SOLIDS AND SEMISOLIDS

If it is necessary to assess microbial populations that are embedded in solid or semisolid materials such as raw materials and finished food or pharmaceutical products, the organisms must be freed from the matrix before they can be cultured. If the solid is water soluble, such as sugar or an uncoated tablet, it may be dissolved in sterile buffer or broth medium (perhaps with the aid of a sterile mortar and pestle or blender under aseptic conditions). Certain types of soft foods or gels can be disintegrated in a stomacher, a device that consists of a heavy, flexible bag that is mechanically pummeled to break up the material. Gentle heating (not over 45°C) may be necessary. Extraction fluids that result are then analyzed as discussed above in the section on liquids. If the material has a moderate melting point (not over 45°C), such as, for example, gelatin, it may be possible to melt the sample, dilute with sterile buffer or medium if necessary, and filter or plate. If a material contains preservatives or other ingredients inhibitory to microbial growth, the cells can be collected on a membrane filter and thoroughly rinsed with sterile buffer or medium. If effective neutralizers are available, they may be added to the media. The filter may be plated directly or the cells resuspended and plated.

If the material is not water soluble, generally there may not be a solvent that would dissolve the solid and not be toxic to the microorganisms. Nonaqueous (organic) solvents, such as alcohol, toluene, ether, or hexane may cause death of cells and should be thoroughly tested before used. Alternatively, it may be possible to reduce the material mechanically to particles small enough to release some of the organisms. This can be done in a sterile ball mill or mechanical grinder or blender. However, it is virtually impossible to do this without significant cell damage and a resulting underestimate of the viable population. The extent of cell destruction can be determined by mixing known numbers of microbial cells into the solid formulation and then subjecting the formulation to the isolation procedure. For assaying some products such as creams, waxes, and ointments, the material may be soluble in isopropyl myristate, an organic solvent that is relatively inert toward most microorganisms. Alternatively, the product may be emulsified in sterile water or other aqueous diluent with the aid of an emulsifying agent. The resulting solutions then can be subjected to membrane filter assay. However, the procedures must always be validated with known populations of microorganisms.

If only the surface of a solid item is to be assayed and the item is small, it can be submerged in a sterile liquid such as 0.1% peptone water with 0.1% Tween 80. If possible, vortexing or insonation can be applied to increase release of particles from the object. The resulting rinse fluid is

then assayed as described above for liquids. For larger items, such as catheters, that are made of soft material, it may be possible to cut the items into smaller pieces using sterile tools before placing into the rinse liquid.

IX. MONITORING FOR SPECIFIC MICROORGANISMS

A. Bacteria

1. Bacterial Nutrition and Choice of Growth Media

The nutritional requirements for cultivating bacteria were reviewed in Chapter 2. As pointed out previously, bacteria as a group are noted for the immense range of substances that will satisfy their nutritional needs. Some bacteria found in nature can utilize the carbon in carbon dioxide (CO_2), the nitrogen from inorganic chemicals such as nitrates or ammonia, and derive their energy requirements from mineral sources or from light. These organisms, known as autotrophs, are commonly found in natural habitats but are not likely to be major contaminants in cleanrooms. Other species of bacteria obtain their carbon and energy from organic compounds, and these are referred to as heterotrophs. Most of the bacteria associated with the human body are heterotrophs, and thus the majority of bacteria that may be encountered in cleanrooms will be heterotrophs. They will probably also be mesophiles; that is, they will grow best at moderate temperatures. The most frequently recommended medium for assessing the levels of heterotrophs in cleanroom or controlled environments is variously referred to as soybean casein digest medium or tryptic soy agar (TSA). This medium is manufactured commercially by digesting the milk protein casein with pancreatic enzymes and soybean meal with papain, a proteolytic enzyme from papaya. The result is a medium that is ideal for growing most heterotrophs — rich in amino acids, vitamins, carbohydrates, and other nutrients. Other media such as plate count agar also are used.

A number of situations in cleanroom monitoring may call for special media. An alternative to the use of standard bacteriological media such as TSA might be in instances where one is trying to recover bacteria that have sustained sublethal injury due to exposure to heat or chemicals. A so-called resuscitation medium such as brain–heart infusion (BHI) medium may be employed. As the name implies, BHI medium contains water extracts of calf brain and beef heart tissue and is considerably richer in many nutrients than is TSA.

Another exception to the general use of TSA for the bacteriological monitoring of cleanroom facilities would be found in the culturing of bacteria that are common in purified water systems. As mentioned in Chapter 1, these organisms are capable of deriving their nutritional needs

from the traces of contamination that leach from plastic piping, dust, and other sources in the water system. Because these organisms, known as oligotrophs, are accustomed to very low levels of nutrients, the concentrations of nutrients in ordinary bacteriological media are frequently inhibitory to these organisms. For best results, standard media must be diluted considerably. For example, for growing the water contaminants, tryptic soy medium is prepared at a concentration of 0.3% (3 g/l) rather than the 3% that is recommended for most common heterotrophic bacteria. A medium known as R2A was devised especially for growing purified water contaminants and consists of very low levels of nutrients. Recovery of bacteria from pure water systems has been shown to be higher when using the R2A medium compared with standard concentrations of TSA.

Some types of media are formulated to fulfill specific purposes, such as selectively supporting the growth of specific groups of microorganisms. First described in Chapter 1, selective media contain specific ingredients that support the growth of organisms that are sought after but inhibit others. Anaerobes, as you recall, are bacteria that require the absence of gaseous oxygen. A certain fraction of biocleanroom monitoring should be aimed at the detection of anaerobic bacteria. When monitoring for strict anaerobes, thioglycolate broth may be used. Thioglycolate broth contains agents that reduce the oxidation/reduction potential of the medium to a level required by the anaerobic species. For best results, the broth must be boiled briefly and then cooled just before use. This procedure drives out remnants of oxygen from the medium. If anaerobic bacterial colonies are desired, the plates must be incubated in anaerobic jars or other devices as described in Chapter 2.

Differential media are especially useful in identifying microorganisms. These media contain components such as sugars, pH indicators, fluorescent dyes, and other additives that react in certain ways when specific organisms grow on the media. Differential media are especially useful in picking objectionable microorganisms out of the microbial milieu of an environmental or product sample. Besides staining characteristics, traditional methods of bacterial taxonomy depend almost entirely on the appearance of the organisms as they grow on differential media. The identification of bacteria is covered further in Section X of this chapter.

Commercially prepared media are available in the form of sterile, prepoured agar plates and tubes containing broth. Alternatively, media may be prepared in the facility where they are to be used. Such media are usually provided as dehydrated mixes that are dissolved in water, sterilized, and poured into sterile Petri plates or tubes. If media are prepared on-site, it must be conducted under aseptic conditions in a controlled facility. Strict adherence to media manufacturers' preparation instructions is critical. Media that are subjected to excessive heat steriliza-

tion may not support the growth of some bacteria due to the formation of toxic products. Understerilized media may contain viable microbes that will contaminate monitoring samples. All batches of plates and tubes of media should be marked with the date of preparation and used as soon as possible. Generally, when agar media are stored for long periods of time they can become desiccated and may not support the growth of some normally culturable bacteria.

Media that are to be used in environmental sampling should undergo media negative control tests to ensure that the media had not been contaminated prior to use. In these tests, a designated number of plates or tubes are incubated for an appropriate time and examined for signs of microbial growth.

The ultimate choice of media for cleanroom monitoring must be according to official guidelines. For example, the media that is used must be able to support the growth of indicator organisms that represent typical cleanroom contaminants (Table 5.1). In addition, the media must support the growth of designated objectionable organisms or representative organisms obtained from monitoring specific cleanroom facilities and associated personnel. A selected medium should undergo tests variously known as growth promotion tests or positive control tests. In these tests, various bacterial strains are inoculated into the proposed medium. Following appropriate incubation, growth is assessed to determine the medium's suitability. Any organisms that are isolated from positive sterility tests must also grow in the medium chosen for routine monitoring. In summary, any medium that is to be used in connection with microbiological monitoring must be fully validated.

B. Fungi

1. Fungal Nutrition and Choice of Growth Media

While fungi will grow on most common bacteriological media, there are several reasons to use media specifically devised for recovering fungi when sampling products or the environment for TYMC. Fungi generally grow more slowly than bacteria, frequently requiring several days to produce visible colonies, whereas bacterial colonies usually reach visible size within 24 to 48 h. In dealing with environmental samples, bacteria will almost always be present in the same sample with the fungi. When trying to grow fungi, it is therefore useful to avoid bacterial growth in order to prevent overgrowth and competition for nutrients. There are several media that encourage fungal growth while inhibiting the growth of most bacteria. Such media normally have higher levels of sugar and are adjusted to lower (more acid) pHs, both conditions being generally

inhibitory to bacteria. In some cases, antibiotics, which inhibit bacteria but which have little effect on the fungi, can be added to the medium. One of the most popular media for growing the more common fungi is Sabouraud dextrose agar (SAB). Its pH is adjusted to 5.6 and it contains 4% glucose, at least four times the concentration normally used in bacteriological media. Mycological agar, potato dextrose agar, and malt extract agar are also used for culturing fungi. Fungi are incubated for 5 to 7 days at 20 to 25°C. This temperature range is below the optimum temperature for many common mesophilic bacterial cleanroom contaminants.

X. RAPID METHODS OF IDENTIFICATION

Conventional methods of bacterial identification first require the isolation of the unknown species as a pure culture. The isolate is then subjected to Gram and other differential staining and to a series of inoculations into differential media. Under ideal circumstances, and if the isolate is a known species, the process may take 4 to 7 days. There are a few commercial products that have miniaturized the use of differential media. The devices contain up to 20 small compartments, each containing a differential medium. Examples are the Enterotube® (Becton, Dickinson, Franklin Lakes, New Jersey) and API® (bioMerieux, Durham, North Carolina). The compartments are inoculated with a pure culture of the unknown organism. Following 24 h incubation, the biochemical reaction in each compartment is recorded and the overall results for each unknown isolate are converted into a numerical code that is compared with keys of known organisms.

Enormous progress has been made in the development of automated and semiautomated instrumentation for the identification of microbial contaminants. Once an unknown bacterium or fungus is isolated in pure culture, it can be analyzed by any of a number of instruments, some capable of identifying the organism in as little as a few hours.

Examples of some of the principles on which the instruments for microbial identification are based are:

1. Biochemical/enzymatic reactions
2. Fatty acid composition
3. DNA analysis

In all cases a particular instrument develops some type of pattern or fingerprint from the organisms' responses to one of the principles listed above. The pattern is then compared with patterns stored in the instrument's digital file and possible matches are displayed on a screen or in a printed report.

In those automated identification systems that are based on biochemical/enzymatic reactions, plastic card-like devices or other carriers are used. Each card has numerous small chambers containing various differential media. The chambers are inoculated with pure cultures of the unknown organisms and placed into the instrument. The instrument incubates the cultures and over time automatically determines the reactions for each of the 30 to 95 biochemical reactions. The instrument's computer then compares the reaction patterns with the computer's library of known bacterial metabolic patterns and prints out a report that lists one or more possible genus and species matches for each unknown organism. Most identifications can be accomplished in less than 18 h, and some within 2 to 4 h. Slow-growing organisms such as yeast may take up to 4 days for an identification. Identifications are limited to those bacteria and yeasts that are in the instrument manufacturer's computer data banks, but most systems allow for updating their computer software with new strains isolated in a particular facility.

Gas chromatography is used to capture fatty acid profiles of unknown microbes. Again, pure cultures of the isolates are required. Fatty acid patterns are compared with a data bank and one or more possible identities are reported in less than 2 h. Since bacteria may contain upward of 300 fatty acids, patterns can be quite complex.

More and more attention is being focused on a variety of identification approaches involving microbial DNA. In one system, DNA is extracted from unknown cells and digested with enzymes to break it up into manageable fragments. The fragments are then separated by gel electrophoresis. The pieces are treated with DNA probes specific for bacterial ribosomal RNA. The probes hybridize or stick to any DNA fragments that have the specific sequences. Those DNA fragments that have hybridized with the probes form visible patterns that are similar to the human DNA fingerprints seen in criminal investigations. Generally, patterns are unique to each species of microorganism. The patterns can be used to identify the microorganisms provided that the patterns are in the instrument data bank. In some instances this system is said to have the sensitivity to distinguish individual strains of the same species, providing a valuable tool in tracing sources of contamination.

XI. TESTING FOR BACTERIOSTASIS AND FUNGISTASIS

It is generally assumed that viable microorganisms collected in cleanroom environments will show their presence consistently by producing growth in the form of colonies on agar media or turbidity in liquid media under appropriate culture conditions. There are several reasons why normally culturable microorganisms may fail to grow. One is because of injury,

discussed above. Another reason may be that, in the course of collecting microorganisms from environmental surfaces, remnants of inhibitory substances such as chemical cleaners may have been transferred along with the microorganisms. The presence of mere traces of inhibitory substances may prevent normal microbial growth in the medium that would normally support the organisms. If the nature of the inhibitory substances is known, neutralizing additives, as described above, may be added to the medium.

If bacteriostasis or fungistasis, as this effect of inhibitory residues is called, is suspected, the environmental surfaces can be sampled with sterile swabs. The swabs are extracted with sterile medium and the medium is then sterilized by filtration. The medium is then tested to determine if it will support the growth of a standardized inoculum of test organisms compared with normal medium treated in the same manner. Bacteriostasis or fungistasis may also occur when sampling raw materials or finished products that contain inhibitory material.

XII. DETECTION OF BIOFILMS

As covered in Chapter 2, biofilms form wherever surfaces are continually wet. The inner surfaces of purified water systems are of special concern. As explained earlier, there are sufficient traces of nutrients in so-called purified water to support the growth of microorganisms in the system. Reverse osmosis membranes, distribution pipes, storage tanks, and delivery systems are all susceptible to biofilm formation, creating breeding grounds for large numbers of bacteria and other microorganisms. It is thought that biofilms of Gram-negative bacteria in purified water systems are the principal source of endotoxins (pyrogens) in process water. The detection and elimination of biofilms are clearly a high priority.

There is little correlation between numbers of suspended microorganisms in a purified water system and the extent of biofilm formation. That is, low counts taken from sampling ports or point-of-use taps do not necessarily indicate an absence of biofilms in the system. However, unexplained spikes in bacterial levels in a water system can indicate the possible presence of biofilms somewhere in the system. These spikes are caused by hydrodynamic forces that occasionally dislodge fragments of a biofilm. The fragments may consist of clumps of very large numbers of cells that become part of the suspended population. To detect biofilms, internal surfaces of the system must be examined. If surfaces within the water system are accessible, viable surface sampling methodology as described above may be used. System components that can be disassembled can be removed to the sterility suite and examined by biological or optical methods. Biofilms can be avoided by proper maintenance of a purified water system.

XIII. STERILE MEDIA FILL TESTS

Filter-sterilized product added to presterilized containers and sealed with sterile closures under stringent contamination controls is known as aseptic filling. One important method used to assess the effectiveness of an aseptic filling operation is to substitute sterile liquid growth medium for the product. The containers are then sealed and incubated. Any viable contamination that finds its way into the filling operation will be evidenced by the development of microbial growth in one or more of the containers. Media fill tests must be carried out with a normal environmental background and under circumstances that simulate actual production conditions, including duration of operations, the number of personnel present, and the occurrence of interventions. Personnel should rotate positions during a media fill test to validate each operator. Occasional tests should be conducted under stressful situations that might reveal a weak link in the process.

XIV. ALERT AND ACTION LEVELS

An environmental sampling program is useless unless appropriate action is taken whenever excessive contamination is detected. Maximum levels of contamination should be established by company policy, which, when exceeded, trigger specific action. Such levels are generally conservative and based on long historical records of the facility. An alert level is designated that, if exceeded, alerts appropriate personnel to a possible deviation from normal operating conditions. Action may or may not be necessary at this stage, depending on company policy. Action might involve increasing disinfection frequencies, tightening personnel practices, or recertification of filters and other contamination control equipment. A higher level of contamination, designated the action level, if exceeded, is answered by appropriate action that would be directed toward discovering the nature and source of the contamination and controlling it. If necessary, more drastic action such as shutting down a filling operation or delaying release of product may be required.

Before any action is taken as the result of action level exceedances, the situation should be fully analyzed. Is it a true positive test or is there evidence it may be the result of a laboratory error? The organism(s) in the positive test should be speciated and compared with isolates from various sites around the facility. The time when the sample was taken should be correlated with unusual incidents at the sampling site such as power failures, machinery interventions, shift changes, or introduction of new batches of raw materials.

XV. THE STERILITY SUITE

The sterility suite is a laboratory specifically dedicated to processing and analyzing products that are labeled as sterile. The microbiological quality of the sterility suite must equal or exceed the strictest levels of the cleanroom facility; anything less would compromise test results. The contamination of products during testing produces false-positive results that could create enormous problems. It has been reported that the majority of positive sterility tests can be traced to samples that were contaminated through laboratory errors. To avoid such errors, it is becoming more common for microbiological testing of finished sterile products to be conducted in isolators (see Chapter 4), where workers manipulate the tests in half-suits. This approach appears to have essentially eliminated false positive tests.

XVI. DISPOSAL OF CULTURES

All liquid and solid cultures from environmental and other samplings are classified as biomedical waste and should be treated as biohazardous material. That is, prior to discarding such material into normal trash receptacles it must be sterilized in an autoclave certified for biomedical waste for at least 1 h (as a safety margin), destroyed in an approved incinerator, or collected by a biohazardous waste disposal service company. When autoclaving laboratory waste in biohazard bags, the bags should be unsealed to allow steam to enter, or at least 250 ml of water should added to each bag before sealing. This will provide sufficient moisture to produce the 100% humidity required for moist sterilization.

XVII. SUMMARY

A principal purpose of a cleanroom in a biomedical research or manufacturing setting is to reduce levels of microbial contamination. This is achieved through a combination of architectural features, materials quality control, and personnel practices. Since microorganisms are not visible to the naked eye, there are only two ways to determine if the contamination control purpose of the cleanroom is satisfied: product failure and monitoring. Obviously, the latter approach is the preferred one.

A variety of methods are available to the microbiologist to measure levels of microbial contamination in the cleanroom. Such methods should be used on a regular basis and the results maintained and displayed in a manner that allows for trend analysis that can quickly alert workers of possible breakdowns in control operations. Company policy should include action levels, which are contamination levels that, when exceeded,

trigger specific corrective action to eliminate the identified cause. The facility microbiologist carries an enormously important burden in maintaining the success of the operation of the biocleanroom, and ultimately the success of the company. It is the viable population that potentially can cause the greatest damage, whether it is fouling a water system, causing a spoiled product, or creating a health hazard in a supposedly sterile medical product.

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