Chapter 6 The Cultivation of Bacteria

OUTLINE

Except for certain ecological studies where bacterial populations are examined in their natural habitats, bacteria are usually cultivated and studied under laboratory conditions. Numerous **media** (singular, **medium**) have been developed for bacterial cultivation. Because the nutritional requirements of bacteria vary widely, there are great differences in the chemical compositions of the media used in the laboratory. Bacteria also exhibit wide differences with respect to the physical conditions favoring their growth, such as temperature, pH, and gaseous environment. The successful cultivation of bacteria requires an awareness of all of these factors.

NUTRITIONAL REQUIREMENTS

All forms of life, from microorganisms to human beings, share certain nutritional requirements for growth and normal functioning. The following observations substantiate this statement and also illustrate the great diversity of nutritional types found among bacteria.

1 All organisms require a **source of energy.** Some rely on chemical compounds for their energy and are designated as **chemotrophs.** Others can utilize radiant energy (light) and are called **phototrophs.** Both chemotrophs and phototrophs exist among bacteria (see Table 6-1 for examples).
2 All organisms require a **source of electrons** for their metabolism. Some organisms can use reduced inorganic compounds as electron donors and are termed **lithotrophs** (some may be **chemolithotrophs,** others **photolithotrophs**). Other organisms use organic compounds as electron donors and are called **organotrophs** (some are **chemoorganotrophs,** others **photoorganotrophs**). Examples appear in Table 6-1.
3 All organisms require **carbon** in some form for use in synthesizing cell compo-

Table 6-1. Nutritional Characterization of Bacteria

Bacteria	Energy		Electron Donor		Carbon for Assimilation	
	Phototrophic	Chemotrophic	Lithotrophic	Organotrophic	Autotrophic	Heterotrophic
Chromatium okenii	+		+		+	
Rhodospirillum rubrum						
(anaerobic conditions)	+			+		+
(aerobic conditions)		+		+		+
Nitrosomonas europaea		+	+		+	
Desulfovibrio desulfuricans		+	+			+
Pseudomonas pseudoflava						
(H_2 supplied)		+	+		+	
(no H_2 supplied)		+		+		+
Escherichia coli		+		+		+

nents. All organisms require at least small amounts of CO_2. However, some can use CO_2 as their major, or even sole, source of carbon; such organisms are termed autotrophs. Others require organic compounds as their carbon source and are termed heterotrophs (Table 6-1).

4 All organisms require nitrogen in some form for cell components. Bacteria are extremely versatile in this respect. Unlike eucaryotes, some bacteria can use atmospheric nitrogen. Others thrive on inorganic nitrogen compounds such as nitrates, nitrites, or ammonium salts, and still others derive nitrogen from organic compounds such as amino acids.

5 All organisms require oxygen, sulfur and phosphorus for cell components. Oxygen is provided in various forms, such as water; component atoms of various nutrients; or molecular oxygen. Sulfur is needed for synthesis of certain amino acids (cysteine, cystine, and methionine). Some bacteria require organic sulfur compounds, some are capable of utilizing inorganic sulfur compounds, and some can even use elemental sulfur. Phosphorus, usually supplied in the form of phosphate, is an essential component of nucleotides, nucleic acids, phospholipids, teichoic acids, and other compounds.

6 All living organisms require *metal ions*, such as K^+, Ca^{2+}, Mg^{2+}, and Fe^{2+} for normal growth. Other metal ions are also needed but usually only at very low concentrations, such as Zn^{2+}, Cu^{2+}, Mn^{2+}, Mo^{6+}, Ni^{2+}, B^{3+}, and Co^{2+}; these are often termed trace elements and often occur as contaminants of other components of culture media in amounts sufficient to support bacterial growth.

Not all the biological functions of metal ions are known, but Fe^{2+}, Mg^{2+}, Zn^{2+}, Mo^{6+}, Mn^{2+}, and Cu^{2+}, are known to be cofactors for various enzymes (see Chap. 9).

Most bacteria do not require Na^+, but certain marine bacteria, cyanobacteria, and photosynthetic bacteria do require it. For those members of the archaeobacteria known as the "red extreme halophiles," the requirement is astonishing: they cannot grow with less than 12 to 15 percent NaCl! They require this high level of NaCl for maintenance of the integrity of their cell walls and for the stability and activity of certain of their enzymes.

7 All living organisms contain vitamins and vitaminlike compounds. These function either as coenzymes for several enzymes (see Chap. 9) or as the building blocks for coenzymes. Some bacteria are capable of synthesizing their entire requirement of vitamins from other compounds in the culture medium, but others

cannot do so and will not grow unless the required vitamins are supplied preformed to them in the medium (see Table 6-2). Research in bacterial nutrition led to the discovery of some of the vitamins required by humans, and metabolic studies with bacteria contributed to our understanding of how these vitamins are synthesized and how they function.

8 All living organisms require water, and in the case of bacteria all nutrients must be in aqueous solution before they can enter the cells. Water is a highly polar compound that is unequaled in its ability to dissolve or disperse cellular components and to provide a suitable milieu for the various metabolic reactions of a cell. Moreover, the high specific heat of water provides resistance to sudden, transient temperature changes in the environment. Water is also a chemical reactant, being required for the many hydrolytic reactions carried out by a cell.

NUTRITIONAL TYPES OF BACTERIA

From the generalizations in the preceding paragraphs, it is apparent that bacteria can be divided into many groups on the basis of their nutritional requirements. The major separation is into two groups, phototrophs and chemotrophs.

Phototrophs

Among the phototrophic bacteria are species that use inorganic compounds as their source of electrons (i.e., photolithotrophs). For example, *Chromatium okenii* uses H_2S as its electron donor, oxidizing it to elemental sulfur:

$$H_2S \rightarrow S + 2e^- + 2H^+$$

Some phototrophic bacteria use organic compounds such as fatty acids and alcohols as electron donors and are therefore photoorganotrophs. For example, *Rhodospirillum rubrum* can use succinate as an electron donor:

$$\text{Succinate} \rightarrow \text{fumarate} + 2e^- + 2H^+$$

Certain phototrophic bacteria are not restricted to being phototrophic. As indicated before, chemotrophs rely on chemical compounds rather than light for their energy, and under some circumstances a phototrophic bacterium can grow as a chemotroph. For example, in the absence of O_2 (i.e., under anaerobic conditions) *R. rubrum* is dependent on light as its source of energy and lives as a photoorganotroph; however, in the presence of O_2 it can grow in the dark as a chemoorganotroph.

Chemotrophs

Among the chemotrophic bacteria are species that use inorganic compounds as

Table 6-2. Vitamin Requirements for Some Bacteria

Vitamin	Species Exhibiting Requirement (or Growth Stimulation)
Thiamine (B_1)	*Bacillus anthracis*
Riboflavin	*Clostridium tetani*
Niacin (nicotinic acid)	*Brucella abortus*
Pyridoxine (B_6)	*Lactobacillus* spp.
Biotin	*Leuconostoc mesenteroides*
Pantothenic acid	*Morganella morganii*
Folic acid	*Leuconostoc dextranicum*
Cobalamin (B_{12})	*Lactobacillus* spp.
Vitamin K	*Bacteroides melaninogenicus*

their source of electrons (i.e., chemolithotrophs). For example, bacteria of the genus *Nitrosomonas* use ammonia as their electron source, obtaining energy by oxidizing ammonia to nitrite:

$$NH_4^+ + 3/2\ O_2 + H_2O \rightarrow NO_2^- + 2H_3O^+$$

This reaction involves a net transfer of 6 electrons, causing a valence change of the nitrogen atom from -3 to $+3$.

Many other chemotrophic bacteria use organic compounds, such as sugars and amino acids, as electron donors and are therefore chemoorganotrophs.

Certain bacteria can grow as either chemolithotrophs or chemoorganotrophs. For example, *Pseudomonas pseudoflava* can use either the organic compound glucose or the inorganic compound H_2 as its source of electrons:

$$\underset{\text{glucose}}{C_6H_{12}O_6} + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^-$$

$$H_2 \rightarrow 2H^+ + 2e^-$$

Autotrophs and Heterotrophs

Autotrophs

As indicated before, the chemolithotrophic bacteria of the genus *Nitrosomonas* are able to oxidize ammonia to nitrite, thereby obtaining sufficient energy to assimilate the carbon of CO_2 into cell components (CO_2 fixation):

$$CO_2 + 4e^- + 4H^+ \rightarrow (CH_2O) + H_2O$$

where (CH_2O) represents carbohydrate. Organisms that can use CO_2 as their sole source of carbon for assimilation are termed autotrophs.

Until recently it was thought that all chemolithotrophic bacteria were autotrophs. Although this is true for most chemolithotrophs, a few are now recognized as being chemolithotrophic heterotrophs **(mixotrophs)**; i.e., they obtain energy by utilizing inorganic electron donors, but obtain most of their carbon from organic compounds. One such organism is *Desulfovibrio desulfuricans*, which uses electrons from H_2 for the reduction of sulfate, yet derives most of its carbon from organic compounds in the culture medium.

Some autotrophs are facultative autotrophs; i.e., they can either live as autotrophs, deriving their carbon from CO_2, or they can live as heterotrophs, deriving their carbon from organic compounds. For example, *P. pseudoflava* can live as a heterotroph, using glucose as a source of carbon for assimilation (and also as its source of electrons, as mentioned above); however, if H_2 is provided as the electron source, then it can use CO_2 as its sole carbon source and can grow as an autotroph.

Table 6-3. Medium for *Nitrosomonas europaea*

Ingredient	Amount
NH_4Cl*	0.8 g
$MgSO_4 \cdot 7H_2O$	0.2 g
K_2HPO_4	0.016 g
$CaCl_2 \cdot 2H_2O$	0.02 g
Chelated iron	0.001 g
$MnCl_2 \cdot 4H_2O$	0.0002 g
$Na_2MoO_4 \cdot 2H_2O$	0.0001 g
$ZnSO_4 \cdot 7H_2O$	0.0001 g
$CuSO_4 \cdot 5H_2O$	0.00002 g
$CoCl_2 \cdot 6H_2O$	0.000002 g
Distilled water	1,000 ml
Atmospheric CO_2†	

* The ammonium salt serves not only as the nitrogen source for this organism but also as the electron donor. The organism obtains energy by oxidizing ammonium ions to nitrite ions.

† Carbon dioxide is the sole carbon source.

Cultivation of Autotrophs. In terms of chemical complexity of nutrient substances required for growth, the autotrophic bacteria exhibit the simplest requirements. For example, a medium of the composition shown in Table 6-3 supports the growth of *Nitrosomonas europaea*. (Because this medium is composed of known chemical compounds, it is called a **chemically defined** or **synthetic medium**.) The fact that an organism can grow and reproduce in such a mixture of inorganic compounds indicates that it has an elaborate capacity for synthesis. That is, the organism can transform these compounds into the carbohydrates, proteins, nucleic acids, lipids, vitamins, and other complex organic substances that constitute the living cell.

Table 6-4. Minimal Nutritional Requirements of Some Heterotrophic Bacteria

Bacteria	Inorganic Salts	Organic Carbon	Atmospheric N_2	Inorganic Nitrogen	One Amino Acid	Two or More Amino Acids	One Vitamin	Two or More Vitamins
Azospirillum brasilense	+	+	+					
Escherichia coli	+	+		+				
Salmonella typhi	+	+		+	+			
Proteus vulgaris	+	+		+	+		+	
Staphylococcus aureus	+	+		+		+	+	
Lactobacillus acidophilus	+	+		+		+		+

Heterotrophs

Heterotrophic bacteria have been studied more extensively than the autotrophs because heterotrophs, in a sense, are of more immediate concern to us: it is here that we find all the species that cause diseases of human beings, other animals, and plants, as well as those that constitute the greater part of the microbial population in our immediate environment. However, we need to emphasize this does not mean that autotrophs are less important. On the contrary, they are of utmost importance in less conspicuous but indispensable processes in nature such as the cycling of elements through biological systems.

Cultivation of Heterotrophs. The heterotrophic bacteria, although they constitute one major nutritional group, vary considerably in the specific nutrients required for growth, particularly with respect to their organic carbon sources, nitrogen sources, and vitamin requirements. As indicated in Table 6-4, the requirements may be relatively simple or complex, depending on the species. This is shown more specifically in Table 6-5, where chemically defined media for the growth of *Escherichia coli* and lactobacilli are compared. From this table it is evident that *E. coli* has much simpler nutritional requirements than lactobacilli. Organisms such as lactobacilli that have elaborate requiremnts for specific nutrients, i.e., vitamins and other growth-promoting substances, are designated **fastidious heterotrophs.**

Obligate Parasites

Some bacteria have not yet been successfully cultivated on an artificial medium, and their nutritional and physical requirements are not understood. At present, such bacteria can be propagated only in association with a living host which, in a sense, serves as the medium. One example is the bacterium that causes leprosy, *Mycobacterium leprae*, which can be cultivated by infecting mice or armadillos. Other examples include the rickettsias, the chlamydias, and the spirochete that causes syphilis, *Treponema pallidum*.

BACTERIOLOGICAL MEDIA

Chemically defined media are needed for the cultivation of autotrophs and are also useful for defining the nutritional requirements of heterotrophs. However, for the routine cultivation of heterotrophs, chemically defined media are not generally used. Instead, certain complex raw materials such as **peptones, meat**

Table 6-5. Composition of Media Supporting Growth of Lactobacilli and *Escherichia coli* (Heterotrophic Bacteria)

MEDIUM FOR CULTIVATION OF LACTOBACILLI*	
Casein hydrolysate	5 g
Glucose	10 g
Solution A	10 ml
Solution B	5 ml
L-Asparagine	250 ml
L-Tryptophan	50 mg
L-Cystine	100 mg
DL-Methionine	100 mg
Cysteine	100 mg
Ammonium citrate	2 g
Sodium acetate (anhydrous)	6 g
Adenine, guanine, xanthine, and uracil, each	10 mg
Riboflavin, thiamine, panththenate, and niacin, each	500 μg
Pyridoxamine	200 μg
Pyridoxal	100 μg
Pyridoxine	200 μg
Inositol and choline, each	10 μg
p-Aminobenzoic acid	200 μg
Biotin	5 μg
Folic acid (synthetic)	3 μg

Make up to 1 liter with distilled water.
Solution A: K_2HPO_4 and KH_2PO_4, each 25 g, into distilled water to a volume of 250 ml
Solution B: $FeSO_4 \cdot 7H_2O$, 0.5 g; $MnSO_4 \cdot 2H_2O$, 2.0 g; NaCl, 0.5 g; and $MgSO_4 \cdot 7H_2O$, 10g
Dissolve in distilled water to a volume of 250 ml

MEDIUM FOR CULTIVATION OF *E. COLI*	
$NH_4H_2PO_4$	1 g
Glucose	5 g
NaCl	5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
K_2HPO_4	1 g
H_2O	1,000 ml

* SOURCE: M. Rogosa et al., *J Bacteriol*, 54:13, 1947.

extract, and yeast extract are used, and the resulting media support the growth of a wide variety of heterotrophic bacteria. Agar is included as a nonnutritive solidifying agent when a solid medium is desired. A description of these raw materials is given in Table 6-6. Examples of relatively simple liquid and solid media that support the growth of many common heterotrophs are nutrient broth and nutrient agar (Table 6-7). The addition of yeast extract to each of these formulas improves the nutrient quality, since yeast extract contains several of the B vitamins and other growth-promoting substances. Other complex supplements such as bovine rumen fluid, animal blood, blood serum, or extracts of plant and animal tissues may be required for the cultivation of certain fastidious heterotrophs.

Types of Media

Many special-purpose media are needed to facilitate recognition, enumeration, and isolation of certain types of bacteria. To meet these needs, the microbiologist

Table 6-6. Characteristics of Several Complex Materials Used as Ingredients of Media

Raw Material	Characteristic	Nutritional Value
Beef extract	An aqueous extract of lean beef tissue concentrated to a paste	Contains the water-soluble substances of animal tissue, which include carbohydrates, organic nitrogen compounds, water-soluble vitamins, and salts
Peptone	The product resulting from the digestion of proteinaceous materials, e.g., meat, casein, and gelatin; digestion of the protein material is accomplished with acids or enzymes; many different peptones (depending upon the protein used and the method of digestion) are available for use in bacteriological media; peptones differ in their ability to support growth of bacteria	Principal source of organic nitrogen; may also contain some vitamins and sometimes carbohydrates, depending upon the kind of proteinaceous material digested
Agar	A complex carbohydrate obtained from certain marine algae; processed to remove extraneous substances	Used as a solidification agent for media; agar, dissolved in aqueous solutions, gels when the temperature is reduced below 45°C; agar not considered a source of nutrient to the bacteria
Yeast extract	An aqueous extract of yeast cells, commercially available as a powder	A very rich source of the B vitamins; also contains organic nitrogen and carbon compounds

Table 6-7. Composition of Nutrient Broth and Nutrient Agar

Nutrient broth	
Beef extract	3 g
Peptone	5 g
Water	1,000 ml
Nutrient agar	
Beef extract	3 g
Peptone	5 g
Agar	15 g
Water	1,000 ml

has available numerous media which, on the basis of their application or function, may be classified as follows.

Selective Media

These media provide nutrients that enhance the growth and predominance of a particular type of bacterium and do not enhance (and may even inhibit) other types of organisms that may be present. For instance, a medium in which cellulose is the only carbon source will specifically select for or enrich the growth of cellulose-utilizing organisms when it is inoculated with a soil sample containing many kinds of bacteria. As an example of a different type of selective medium, the isolation of the gonorrhea-causing organism, *Neisseria gonorrhoeae*, from a clinical specimen is facilitated by the use of media containing certain antibiotics; these antibiotics do not affect *N. gonorrhoeae* but do inhibit the growth of contaminating bacteria.

Differential Media

Certain reagents or supplements, when incorporated into culture media, may allow differentiation of various kinds of bacteria. For example, if a mixture of bacteria is inoculated onto a blood-containing agar medium (blood agar), some of the bacteria may hemolyze (destroy) the red blood cells; others do not. Thus one can distinguish between **hemolytic** and **nonhemolytic** bacteria on the same medium.

Assay Media

Media of prescribed compositions are used for the assay of vitamins, amino acids, and antibiotics. Media of special composition are also available for testing disinfectants.

Media for Enumeration of Bacteria

Specific kinds of media are used for determining the bacterial content of such materials as milk and water. Their composition must adhere to prescribed specifications.

Media for Characterization of Bacteria

A wide variety of media are conventionally used to determine the type of growth produced by bacteria, as well as to determine their ability to produce certain chemical changes.

Maintenance Media

Satisfactory maintenance of the viability and physiological characteristics of a culture over time may require a medium different from that which is optimum for growth. Prolific, rapid growth may also be associated with rapid death of the cells at the end of the growth phase. For example, glucose in a medium frequently enhances growth, but acid harmful to the cells is likely to be produced. Therefore, omission of the glucose is preferable in a maintenance medium.

Solid and Semisolid Media

In addition to liquid media, solid and semisolid media are widely used for cultivation of bacteria. Solid media are useful for isolating bacteria or for determining the characteristics of colonies. The solidifying agent is usually agar, which at concentrations of 1.5 to 2.0 percent forms firm, transparent gels that are not degraded by most bacteria. Silica gel is sometimes used as an inorganic solidifying agent for autotrophic bacteria.

Semisolid media, prepared with agar at concentrations of 0.5 percent or less, have a soft, custardlike consistency and are useful for the cultivation of microaerophilic bacteria (see Gaseous Requirements later in this chapter) or for determination of bacterial motility.

Preparation of Media

Some naturally occurring substances are used for the cultivation of bacteria. Notable among these is milk, usually skimmed rather than whole. Such natural materials are merely dispensed into tubes or flasks and sterilized before use. Media of the nutrient broth or nutrient agar type are prepared by compounding the required individual ingredients or, more conveniently, by adding water to a dehydrated product which contains all the ingredients. Practically all media are available commercially in powdered form.

The preparation of bacteriological media usually involves the following steps:

1. Each ingredient, or the complete dehydrated medium, is dissolved in the appropriate volume of distilled water.
2. The pH of the fluid medium is determined with a pH meter and adjusted if necessary.
3. If a solid medium is desired, agar is added and the medium is boiled to dissolve the agar.
4. The medium is dispensed into tubes or flasks.
5. The medium is sterilized, generally by autoclaving. Some media (or specific ingredients) that are heat-labile are sterilized by filtration.

PHYSICAL CONDITIONS REQUIRED FOR GROWTH

In addition to knowing the proper nutrients for the cultivation of bacteria, it is also necessary to know the physical environment in which the organisms will grow best. Just as bacteria vary greatly in their nutritional requirements, so do they exhibit diverse responses to physical conditions such as temperature, gaseous conditions, and pH.

Temperature

Since all processes of growth are dependent on chemical reactions and since the rates of these reactions are influenced by temperature, the pattern of bacterial growth can be profoundly influenced by this condition. The temperature that allows for most rapid growth during a short period of time (12 to 24 h) is known as the **optimum growth temperature.** (It should be noted, however, that the optimum growth temperature thus defined may not necessarily be optimum for other cellular activities.)

Table 6-8 shows the optimum temperture for several bacteria and also the range of temperatures within which they will grow. It can be seen that the maximum temperature at which growth occurs is usually quite close to the optimum temperature, whereas the minimum temperature for growth is usually much lower than the optimum. On the basis of their temperature relationships, bacteria are divided into three main groups:

1 **Psychrophiles** are able to grow at 0°C or lower, though they grow best at higher temperatures. Many microbiologists restrict the term *psychrophile* to organisms that can grow at 0°C but have an optimum temperature of 15°C or lower and a maximum temperature of about 20°C; the term *psychrotroph* or *facultative psychrophile* is used for those organisms able to grow at 0°C but which grow best at temperatures in the range of about 20 to 30°C (e.g., see Fig. 6-1).

During isolation of strict psychrophiles it is usually necessary to maintain the source samples (for example, Antarctic soil samples) at cold temperatures from the time they are collected and also to chill all media before attempting isolation. This is because strict psychrophiles usually die if they are even temporarily exposed to room temperature. Even at optimum growth temperatures, it often takes two or three weeks for colonies of psychrophiles to develop.

The physiological factors responsible for the low temperature maxima for strict psychrophiles are not entirely clear, but some factors that have been implicated are heat instability of ribosomes and various enzymes, increased leakage of cell

Table 6-8. Characteristics of Several Species of Bacteria with Regard to Temperatures at Which They Grow

	Temperature of Growth, °C		
	Minimum	Optimum	Maximum
Vibrio marinus strain MP-1	-1	15	20
Vibrio psychroerythrus	0	15	19
Pseudomonas fluorescens	4	25–30	40
Staphylococcus aureus	6.5	30–37	46
Corynebacterium diphtheriae	15	37	40
Neisseria gonorrhoeae	30	35–36	38.5
Streptococcus thermophilus	20	40–45	50
Thermoactinomyces vulgaris	27–30	60	65–70
Thermus aquaticus	40	70–72	79

SOURCE: Data from R. Y. Morita, *Bacteriol Rev*, 39:144, 1975, and from *Bergey's Manual of Determinative Bacteriology*, 8th ed, Williams & Wilkins, Baltimore, 1974.

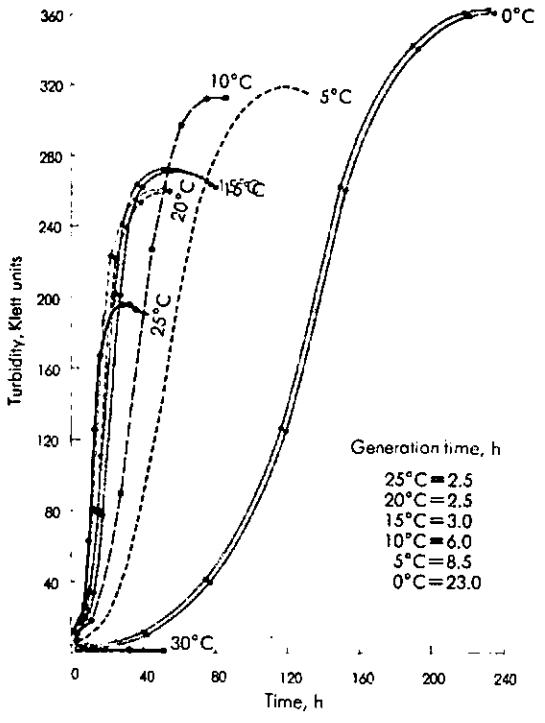


Figure 6-1. Effect of temperature on the growth of a psychrotrophic *Bacillus* sp. Note that rate of growth (measured turbidimetrically in Klett units) is more rapid at 25°C than at 0°C, although the total quantity of cells at the termination of growth is greater at the lower temperature. *(Courtesy of J. L. Stokes in Low Temperature Biology of Food Stuffs, Pergamon, New York, 1968.)*

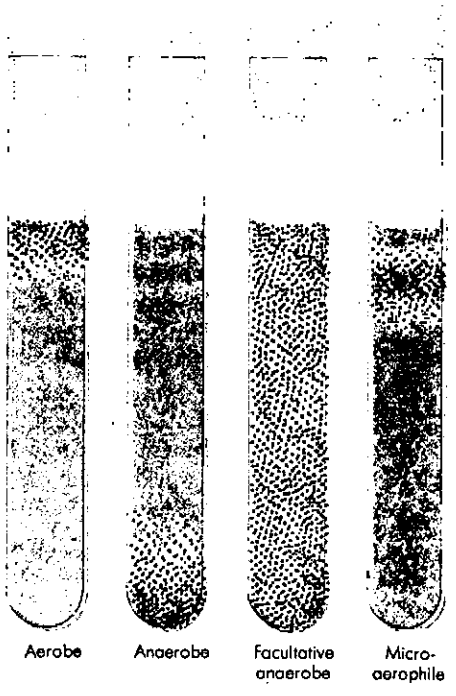


Figure 6-2. Schematic illustration of the growth of bacteria in deep agar tubes, showing differences in response to atmospheric oxygen.

components, and impaired transport of nutrients above the maximum temperature.

2 Mesophiles grow best within a temperature range of approximately 25 to 40°C. For example, all bacteria that are pathogenic for humans and warm-blooded animals are mesophiles, most growing best at about body temperature (37°C).

3 Thermophiles grow best at temperatures above 45°C. The growth range of many thermophiles extends into the mesophilic region; these species are designated facultative thermophiles. Other thermophiles cannot grow in the mesophilic range; these are termed true thermophiles, obligate thermophiles, or stenothermophiles.

Factors that have been implicated in the ability to grow at high temperatures are an increased thermal stability of ribosomes, membranes, and various enzymes. Loss of the fluidity that exists within the lipid bilayer of the cytoplasmic membrane may be a factor governing the minimum temperature.

It is important to note that a bacterial species may not manifest the same characteristics in every detail when grown at different temperatures. For example, *Serratia marcescens* forms a blood-red to orange pigment when cultured at 25°C but produces little or no pigment when cultured at 37°C. Similarly, *Lactobacillus plantarum* does not require the amino acid phenylalanine for growth when cultured at 25°C but does require it at 37°C.

Gaseous Requirements

The principal gases that affect bacterial growth are oxygen and carbon dioxide. Bacteria display such a wide variety of responses to free oxygen that it is convenient to divide them into four groups on the following bases:

1 Aerobic bacteria require oxygen for growth and can grow when incubated in an air atmosphere (i.e., 21 percent oxygen).
2 Anaerobic bacteria do not use oxygen to obtain energy; moreover, oxygen is toxic for them and they cannot grow when incubated in an air atmosphere. Some can tolerate low levels of oxygen (nonstringent or tolerant anaerobes), but others (stringent or strict anaerobes) cannot tolerate even low levels and may die upon brief exposure to air.
3 Facultatively anaerobic bacteria do not require oxygen for growth, although they may use it for energy production if it is available. They are not inhibited by oxygen and usually grow as well under an air atmosphere as they do in the absence of oxygen.
4 Microaerophilic bacteria require low levels of oxygen for growth but cannot tolerate the level of oxygen present in an air atmosphere.

Figure 6-2 shows diagrammatically how these four classes can be distinguished by their patterns of growth in tubes in deep agar media where the diffusion of oxygen into the medium is a controlling factor.

Oxygen Toxicity

Oxygen is both beneficial and poisonous to living organisms. It is beneficial because its strong oxidizing ability makes it an excellent terminal electron acceptor for the energy-yielding process known as respiration. However, oxygen is also a toxic substance. Aerobic and facultative organisms have developed protective mechanisms that greatly mitigate this toxicity, but microaerophiles and anaerobes are deficient in these mechanisms and are restricted to habitats where little or no oxygen is present. The following factors are among those that have been implicated in oxygen toxicity.

1 ***Oxygen inactivation of enzymes.*** Molecular oxygen can directly oxidize certain essential reduced groups, such as thiol (-SH) groups, or enzymes, resulting in enzyme inactivation. For instance, the enzyme complex known as nitrogenase, responsible for nitrogen fixation, is irreversibly destroyed by even small amounts of oxygen.
2 ***Damage due to toxic derivatives of oxygen.*** Various cellular enzymes catalyze chemical reactions involving molecular oxygen; some of these reactions can result in addition of a single electron to an oxygen molecule, thereby forming superoxide radical ($O_2^{\cdot-}$):

$$O_2 + e^- \rightarrow O_2^{\cdot-} \qquad (1)$$

Superoxide radicals can inactivate vital cell components. However, recent studies suggest that their greatest detrimental action is through production of even more toxic substances such as hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH\cdot$) by means of the following reactions:

$$2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2 \qquad (2)$$

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{\text{chelated iron}} O_2 + OH^- + OH\cdot \qquad (3)$$

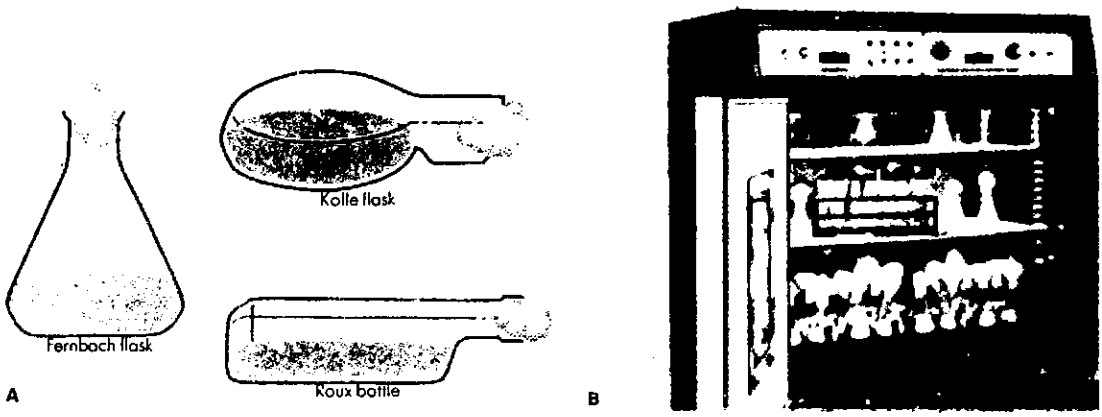


Figure 6-3. Methods for providing increased aeration during incubation. (A) Culture vessels of several designs that provide a large surface area for a shallow layer of medium. (B) An example of an incubator-shaker. The environmental chamber provides controlled conditions of temperature, humidity, and illumination. Within the chamber, flasks are fixed firmly on a platform which rotates in a circular manner, thus agitating the fluid medium constantly during incubation and exposing more culture surface to the gas phase. *(Courtesy of New Brunswick Scientific Company.)*

Hydroxyl radicals are among the most reactive free radicals known to organic chemistry and can damage almost every kind of molecule found in living cells. Hydrogen peroxide is not a free radical, but it is a powerful oxidizing agent that is highly toxic to many kinds of cells. Another toxic derivative of oxygen is an energized form known as **singlet oxygen**, $(^1\Delta_g)O_2$, which is produced in biological systems by certain photochemical reactions.

Aerobic and facultative organisms have developed various protective mechanisms against the toxic forms of oxygen. One is the enzyme known as **superoxide dismutase**, which eliminates superoxide radicals by greatly increasing the rate of reaction 2 above. The hydrogen peroxide produced by this reaction can in turn be dissipated by **catalase** and **peroxidase** enzymes:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad (4)$$

$$H_2O_2 + \text{reduced substrate} \xrightarrow{\text{peroxidase}} 2H_2O + \text{oxidized substrate} \qquad (5)$$

Note that elimination of either superoxide radicals or hydrogen peroxide can prevent the formation of the highly dangerous hydroxyl radicals, since *both reactants* are required for reaction (3).

In general, anaerobic bacteria have either no superoxide dismutase or only relatively low levels compared to aerobes. Many anaerobes are also deficient in catalase and/or peroxidase. This may help to explain, at least in part, their sensitivity to oxygen, although other factors are probably involved as well.

Cultivation of Aerobic Bacteria. To grow aerobic or facultative bacteria in tubes or small flasks, incubation of the medium under normal atmospheric conditions is generally satisfactory. However, when aerobic organisms are to be grown in large quantities, it is advantageous to increase the exposure of the medium to the atmosphere. This can be accomplished by dispensing the medium in shallow layers, for which special containers are available. Aeration can also be increased by constantly shaking the inoculated liquid cultures (Fig. 6-3).

Cultivation of Anaerobic Bacteria. Stringent anaerobes can be grown only by taking special precautions to exclude all atmospheric oxygen from the medium. Such an environment can be established by using one of the following methods:

1 ***Prereduced media.*** During preparation, the culture medium is boiled for several minutes to drive off most of the dissolved oxygen. A reducing agent, e.g., cysteine, is added to further lower the oxygen content. Oxygen-free N_2 is bubbled through the medium to keep it anaerobic. The medium is then dispensed into tubes which are being flushed with oxygen-free N_2, stoppered tightly, and sterilized by autoclaving. Such tubes can be stored for many months before being used. During inoculation, the tubes are continuously flushed with oxygen-free CO_2 by means of a cannula (Fig. 6-4), restoppered, and incubated.
2 ***Anaerobic chamber.*** This refers to a plastic anaerobic glove box (Fig. 6-5) that

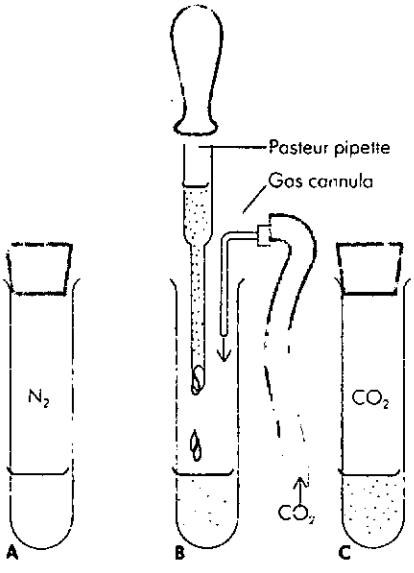
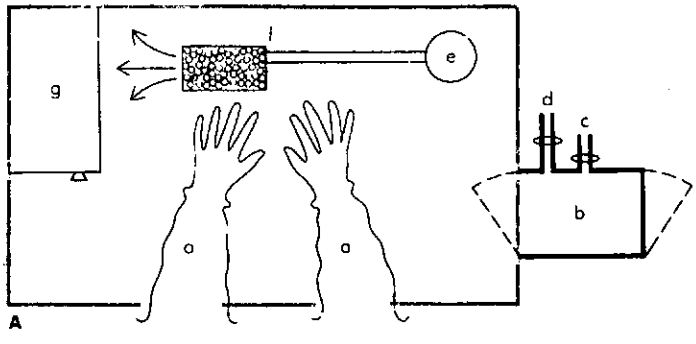


Figure 6-4. Use of prereduced media for cultivation of stringent anaerobes. (A) Tube of prereduced medium containing an atmosphere of oxygen-free N_2. (B) To inoculate, the stopper is removed and a gas cannula inserted to flush the tube continuously with oxygen-free CO_2 and maintain anaerobic conditions. The medium is inoculated with a few drops of culture by means of a Pasteur pipette. (C) After inoculation the tube is restoppered and incubated.



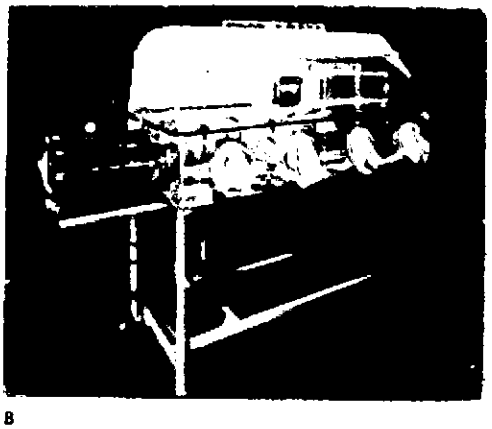


Figure 6-5. (A) Schematic diagram of the various parts of an anaerobic chamber (top view). (a) Glove ports and rubber gloves that allow the operator to perform manipulations within the chamber. (b) Air lock with inner and outer doors. Media are placed within the air lock with the inner door remaining sealed; air is removed by a vacuum pump connection (c) and replaced with N_2 through (d). The inner door is opened and the media are placed within the main chamber, which contains an atmosphere of H_2 + CO_2 + N_2. A circulator (e) circulates the gas atmosphere through pellets of palladium catalyst (f), causing any residual oxygen in the media to be used up by reaction with H_2. After media have become completely anaerobic they can be inoculated and placed in an incubator (g) located within the chamber. (B) Photograph of an anaerobic chamber. *(Courtesy of The Germfree Laboratories, Inc.)*

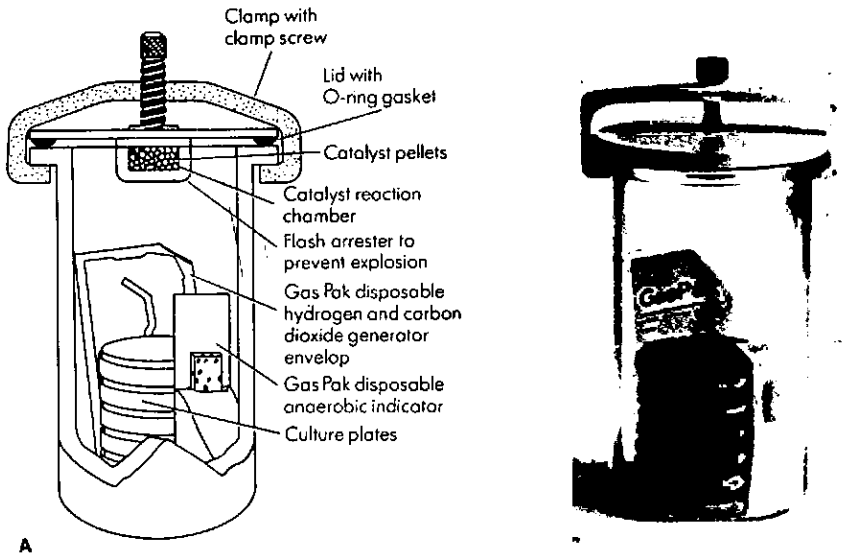


Figure 6-6. Anaerobic jar: GasPak system. (A) Media are inoculated and then placed in the jar. Water is added to the GasPak generator envelope, causing the evolution of H_2 and CO_2. The H_2 reacts with O_2 on the surface of the palladium catalyst, forming water and establishing anaerobic conditions. The CO_2 aids growth of fastidious anaerobes which sometimes fail to grow, or grow only poorly, in its absence. An anaerobic indicator strip (a pad saturated with methylene blue solution) changes from blue to colorless in the absence of oxygen. (B) The GasPak Anaerobic System with inoculated Petri dishes, the GasPak generator envelope, and the anaerobic indicator strip. *(Courtesy of BBL Microbiology Systems.)*

contains an atmosphere of H_2, CO_2, and N_2. Culture media are placed within the chamber by means of an air lock which can be evacuated and refilled with N_2. From the air lock the media are placed within the main chamber. Any O_2 in the media is slowly removed by reaction with the H_2, forming water; this reaction is aided by a palladium catalyst. After being rendered oxygen-free, the media are inoculated within the chamber (by means of the glove ports) and incubated (also within the chamber).

Nonstringent anaerobes can be cultured within an anaerobic jar such as that depicted in Fig. 6-6. Inoculated media are placed in the jar along with an H_2 + CO_2 generating system. After the jar is sealed, the oxygen present in the atmosphere within the jar, as well as that dissolved in the culture medium, is gradually used up through reaction with the hydrogen in the presence of a catalyst.

Acidity or Alkalinity (pH)

For most bacteria the optimum pH for growth lies between 6.5 and 7.5, and the limits generally lie somewhere between 5 and 9. However, a few bacteria prefer more extreme pH values for growth. For example, *Thiobacillus thiooxidans* has an optimum pH of 2.0 to 3.5 and can grow in a range between pH 0.5 and 6.0. On the other hand, an unclassified bacterium isolated from an alkaline spring in California was found to grow best at a pH of 9.0 to 9.5 and could grow within a range from 8.0 to 11.4.

When bacteria are cultivated in a medium originally adjusted to a given pH, for example, 7.0, it is very likely that this pH will change as a result of the chemical activities of the organism. If a carbohydrate is present it may be fermented or oxidized to organic acids, thus decreasing the pH of the medium. If the salt of an organic acid is supplied as a carbon source (e.g., sodium malate), its oxidation by bacteria will cause an increase in pH. Such shifts in pH may be so great that further growth of the organism is eventually inhibited.

Radical shifts in pH can be prevented by incorporating a **buffer** (i.e., a substance that resists change in pH) into the medium. A buffer is a mixture of a weak acid and its conjugate base [e.g., acetic acid (CH_3COOH) and acetate (CH_3COO^-)]. Such mixtures have maximum buffering capacity at the pH where the concentration of the acid equals that of its conjugate base. This pH value is called the pK_a and is the negative logarithm of the dissociation constant of the acid. Phosphate buffer, i.e., a combination of $H_2PO_4^-$ and HPO_4^{2-} having a pK_a of 6.8, is widely used in bacteriological media. Some of the nutritional ingredients of the medium, such as peptones, also possess some buffering capacity because the component amino acids provide weak acid/conjugate base systems (e.g., —COOH/—COO^-, —NH_3^+/—NH_2, —NH_2^+/—NH). The extent to which a medium should or may be buffered depends on its intended purpose and is limited by the buffering capacity of the compounds used. Some large fermentation apparatuses are equipped with automatic controls that maintain a constant pH.

Miscellaneous Physical Requirements

Temperature, the gaseous environment, and pH are the major physical factors to be taken into consideration in establishing the optimum conditions for the growth of most species of bacteria. However, some bacteria have additional requirements. For example, phototrophic bacteria must be exposed to a source of *illumination*, since light is their source of energy. Bacterial growth may also be influenced by *hydrostatic* pressure. Bacteria have been isolated from the deepest ocean trenches where the pressure is measured in tons per square inch, and many of these organisms will not grow in the laboratory unless the medium is subjected to a similar pressure.

CHOICE OF MEDIA AND CONDITIONS OF INCUBATION

From this brief excursion into the nutritional requirements of bacteria, it is apparent that to grow bacteria successfully the laboratory worker must provide the proper kind of medium and an appropriate set of physical conditions. A great deal of information is available to a microbiologist with respect to choice of media, preparation of media, and the physical and nutritional conditions required for the cultivation of the various genera and species of bacteria. Much of this information can be obtained by consulting the three references listed at the end of this chapter.

QUESTIONS

1 What nutritional requirements in terms of chemicals are needed by all forms of life for growth and cellular maintenance?
2 Distinguish between (a) phototrophs and chemotrophs, (b) lithotrophs and organotrophs, and (c) autotrophs and heterotrophs.
3 Suppose you want to determine the occurrence of the vitamin biotin in a batch of milk. *Leuconostoc mesenteroides* requires biotin for growth. Using this bacterium and a chemically defined medium to which a small sample of sterilized milk can be added, devise an experiment that could indicate whether biotin is present in milk. What substance should be omitted during preparation of the chemically defined medium?
4 What conditions of cultivation would allow you to grow selectively:
(a) *Thiobacillus thiooxidans* from a mixed culture of bacteria
(b) *Neisseria gonorrhoeae* from a clinical specimen

(c) The stenothermophile *Clostridium thermosaccharolyticum* from a can of spoiled corn
(d) an extreme halophile from a sample of sea salt
(e) a nitrogen-fixing bacterium from a soil sample

5 Most medically important anaerobic bacteria are nonstringent anaerobes, but some are the stringent type. Describe two methods recommended for cultivating the latter organisms.
6 Is nutrient broth a "universal" medium (that is, can it support the growth of every kind of bacteria)? Explain.
7 If you wanted to buffer a culture medium to keep the pH at 5.5, you would try to use a buffer having what pK_a? Why?
8 List some of the ways whereby you could enhance the growth of an aerobic bacterium.
9 Under what circumstances might you wish to use silica gel as a solidifying agent for a culture medium?
10 Indicate the various toxic derivatives of oxygen and explain how aerobic organisms might protect themselves against these derivatives.

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Starr, M. P., H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (eds.): *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*, Springer-Verlag, New York, 1981. *The introductory chapters of this book contain summaries of the various nutritional and physical factors that affect bacteria; other chapters provide specific information about the habitat, isolation, and cultivation of each bacterial group.*

Chapter 7 Reproduction and Growth

OUTLINE

Reproduction
Modes of Cell Division • New Cell Formation (Macromolecular Synthesis)

Growth
Normal Growth Cycle (Growth Curve) of Bacteria • Transitional Periods Between Growth Phases • Synchronous Growth • Continuous Culture

Quantitative Measurement of Bacterial Growth
Direct Microscopic Count • Electronic Enumeration of Cell Numbers • The Plate-Count Method • Membrane-Filter Count • Turbidimetric Methods • Determination of Nitrogen Content • Determination of the Dry Weight of Cells • Measurement of a Specific Chemical Change Produced on a Constituent of the Medium • The Relation of Turbidity Measurements to Direct Expressions of Growth • The Selection of a Procedure to Measure Growth • Importance of Quantitative Measurement of Growth

When bacteria are inoculated into a suitable medium and incubated under appropriate conditions, a tremendous increase in the number of cells occurs within a relatively short time. With some species the maximum population is reached within 24 h, but others require a much longer period of incubation to reach maximum growth. The term *growth* as commonly applied to bacteria and other microorganisms usually refers to *changes in the total population* rather than an increase in the size or mass of an individual organism. More frequently than not, the inoculum contains thousands of organisms; growth denotes the increase in number beyond that present in the original inoculum. Therefore, determination of growth requires quantitative measurement of the total population of cells or cell crops at the time of inoculation and again after incubation. In this chapter we will discuss how bacteria reproduce and how their growth can be measured.

REPRODUCTION

Modes of Cell Division

Binary Fission

The most common, and no doubt the most important, mode of cell division in the usual growth cycle of bacterial populations is **transverse binary fission**, in which a single cell divides after developing a transverse septum (crosswall) (Fig. 7-1 A, B, C). Transverse binary fission is an asexual reproductive process. (Infrequently, in some species, binary fission may be preceded by a mating or conjugation of cells; this sexual process is discussed in Chap. 12.)

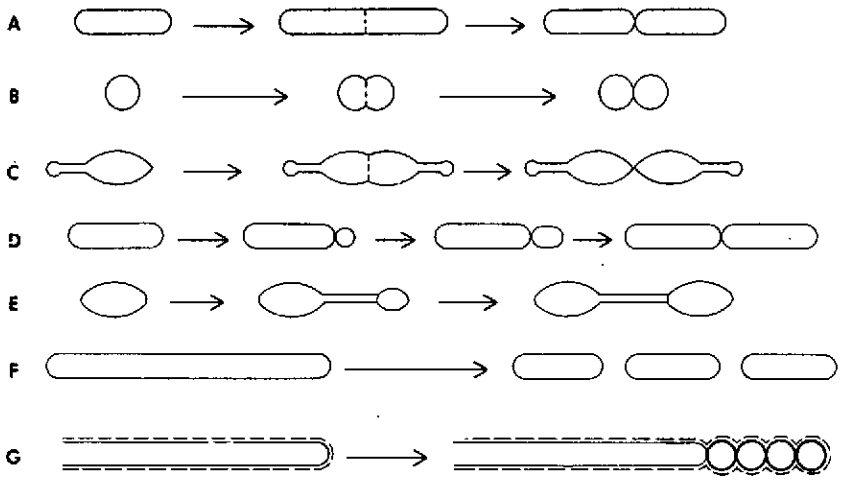


Figure 7-1. Schematic drawing of modes of cell division in various bacteria. Transverse binary fission occurs in *Bacillus subtilis* (A), *Streptococcus faecalis* (B), and the prosthecate bacterium *Prosthecobacter fusiformis* (C); in the latter species the small round area at the tip of each prostheca is a holdfast—a means of attachment to surfaces. Budding occurs in *Rhodopseudomonas acidophila* (D) and *Hyphomicrobium vulgare* (E); in the latter species the mother cell produces a prostheca on which a terminal bud forms; this bud develops into a daughter cell. (F) Fragmentation occurs in the filamentous cells of a *Nocardia* species. (G) Formation of conidiospores by a *Streptomyces* species. A hypha that gives rise to spores is covered by a sheath (represented here by a dashed line); septation occurs at the hyphal tip to produce a chain of conidiospores still enclosed by the sheath.

Budding

Some bacteria, such as *Rhodopseudomonas acidophila*, reproduce by budding, a process in which a small protuberance (*bud*) develops at one end of the cell; this enlarges and eventually develops into a new cell which separates from the parent (Fig. 7-1D). In some budding bacteria, such as *Hyphomicrobium* species, the bud may develop at the end of a prostheca (Fig. 7-1E).

Fragmentation

Bacteria that produce extensive filamentous growth, such as *Nocardia* species, reproduce by fragmentation of the filaments into small bacillary or coccoid cells, each of which gives rise to new growth (Fig. 7-1F).

Formation of Conidiospores or Sporangiospores

Species of the genus *Streptomyces* and related bacteria produce many spores per organism by developing crosswalls (septation) at the hyphal tips; each spore gives rise to a new organism (Fig. 7-1G).

New Cell Formation (Macromolecular Synthesis)

A bacterial cell inoculated into a fresh medium selectively takes up nutrients from its environment. Many biochemical syntheses then take place. The nutrients are converted into cell substance—RNA, DNA, proteins, enzymes, and

other macromolecules. Cell mass and cell size increase, and new cell wall building blocks are synthesized. Subsequently, the process of binary fission is initiated, ultimately resulting in the formation of two new cells.

Septum Formation

In transverse binary fission, septum formation does not begin until the chromosome content of the cell has been doubled; i.e., cell division is triggered by completion of DNA replication (discussed in Chap. 11). The first step is an inward growth of the cytoplasmic membrane at the middle of the cell; a mesosome is usually attached to the cytoplasmic membrane at this location, particularly in Gram-positive cells, and may have a role in the synthesis of new membrane material. The next step is the inward growth of the cell wall to form a septum that ultimately splits to allow separation of the two daughter cells.

For example, during growth of the Gram-positive coccus *Streptococcus faecalis*, all of the new wall material formed by the dividing cell is made during synthesis of the septum. Septum formation begins beneath an equatorial ridge in the cell wall (see Figs. 7-2 and 5-26). New cell-wall material is synthesized in this region and, as the septum forms, this material becomes one half of the wall of each daughter cell. Some plasticity must be present in order for the new wall to achieve its final, more or less hemispherical shape; this is believed to be due to two factors: (1) the turgor pressure of the protoplast against the newly synthesized wall and (2) a certain amount of reorganization of the peptidoglycan due to breakage of some of the chemical bonds by hydrolytic enzymes and subsequent formation of new bonds at a different location.

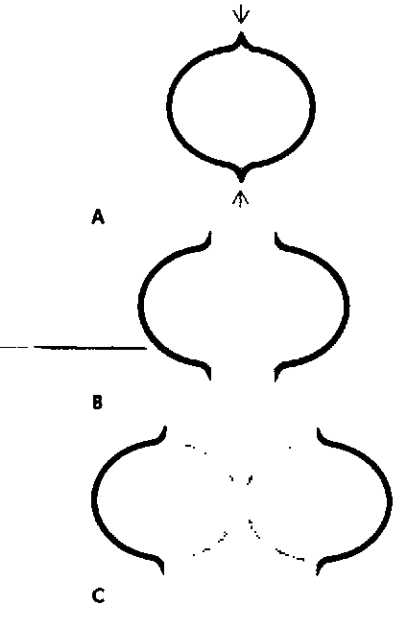


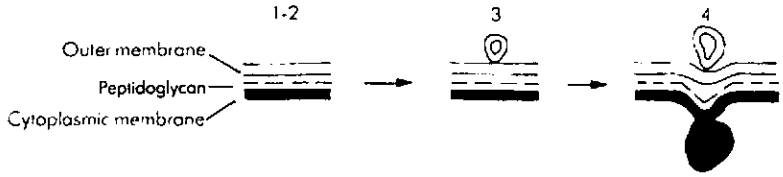
Figure 7-2. Schematic diagram illustrating septum formation in Gram-positive cocci such as *Streptococcus faecalis*. (A) Synthesis of new wall begins at the equatorial ridge (arrows). (B) From this site new wall grows peripherally, pushing apart the hemispheres of old wall. Half of the entire wall of each daughter cell is derived from the septum.

In Gram-positive rods such as *Bacillus subtilis*, the transverse septum is formed in a manner similar to that for *S. faecalis*, although no ridge is present at the middle of the cell. Moreover, only about 15 percent of the new wall of a daughter cell is derived from formation of the septum. The remainder is synthesized along the cylindrical part of the cell, since a bacillus grows mainly by *elongation* rather than just by septum formation as does a coccus. Perhaps there are just a few discrete regions where new wall is synthesized and inserted into old wall, or perhaps new wall is made and inserted into old wall all along the length of the cell, but this is a question yet unanswered. However, there is strong evidence that the youngest portion of the cylindrical wall is that layer which is immediately adjacent to the cytoplasmic membrane. During growth and extension of the wall, the older, outer layers of the wall become more thinly spread out. They are eventually destroyed by degradative enzymes located in the cell wall and are replaced from below by the newer wall material. Thus the wall is not static like a plastic coating; rather, it is in a dynamic state, with old, outer material continually being destroyed and new, inner material continually being added as the cell elongates.

The stages of septum formation in Gram-negative bacilli such as *Escherichia coli* are depicted in Fig. 7-3.

A number of basic questions remain to be answered concerning transverse binary fission:

1 What ensures that each daughter cell will receive a complete genome? That is, during septum formation in any bacterial cell, it is essential that the DNA be precisely distributed to the daughter cells so that each receives a complete genome.



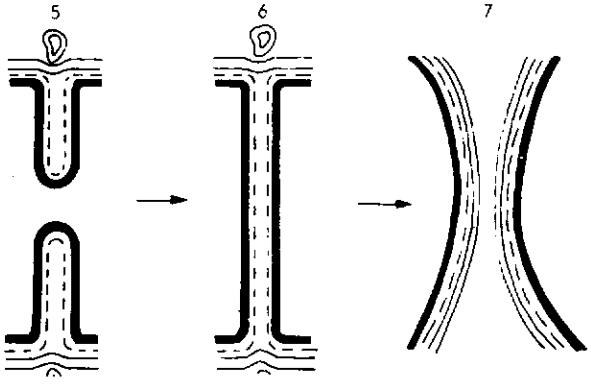


Figure 7-3. Septum formation in the Gram-negative bacterium *Escherichia coli*. In stages 3 to 6, a bleb, or fold, of the outer membrane occurs at the site where the septum will be formed; it is not evident in the final stages of septum formation (stage 7). The outer membrane does not invaginate until the final stages of septum formation (stage 7); however, the cytoplasmic membrane and the peptidoglycan layer grow inward in the early stages (stages 4 and 5). A mesosome, linked to the cytoplasmic membrane, is present during the early stages of septum formation (stage 4). *(Courtesy of I. D. J. Burdett and R. G. E. Murray, J Bacteriol* **119**:*1039, 1974.)*

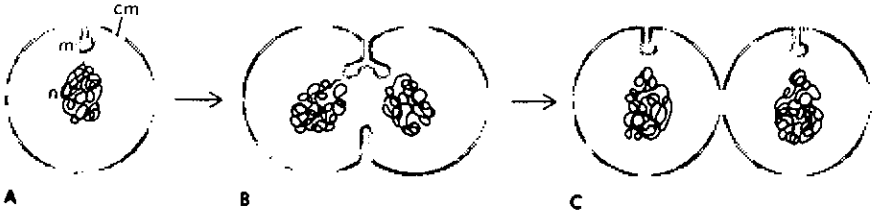


Figure 7-4. Illustration of the hypothesized role of the central mesosome in segregation of DNA into daughter bacterial cells. (A) Cell before binary fission, showing cytoplasmic membrane (cm), central mesosome (m), and the nucleoid (n). (The cell wall is not shown.) In this model the nucleoid is shown attached to the mesosome. Evidence for such attachment has been obtained by electron microscopy of thin sections of bacteria. (B) During binary fission both the DNA of the nucleoid and its attachment site to the mesosome are duplicated. The mesosome begins to divide because of synthesis of membrane between the DNA-mesosome attachment sites. (C) During binary fission each mesosome is "pushed" to the middle of a daughter cell because of synthesis of cytoplasmic membrane between the mesosomes. Because a nucleoid is attached to each mesosome, the nucleoids become properly segregated into the daughter cells.

No mitotic apparatus for this segregation of daughter DNA molecules exists in bacteria; however, the cytoplasmic membrane, or the central mesosome derived from it, may play an equivalent role (Fig. 7-4).

2 What causes the septum to form at approximately the middle of the cell—why not near one of the poles? (Indeed, some mutants of *Escherichia coli* and *B. subtilis* have been obtained which often do form the septum near a pole, resulting

in a very small daughter cell (termed a minicell) which lacks DNA and therefore cannot multiply.)

3 How does the completion of DNA replication initiate septum formation?

It is apparent from these and other questions that although transverse binary fission may be a primitive means of reproduction compared to that which occurs in eucaryotes, it is by no means a simple process; rather, it is the result of a precisely orchestrated series of interdependent events, many of which are not yet completely understood.

GROWTH

The most common means of bacterial reproduction is binary fission; one cell divides, producing two cells. Thus, if we start with a single bacterium, the increase in population is by geometric progression:

$$1 \rightarrow 2 \rightarrow 2^2 \rightarrow 2^3 \rightarrow 2^4 \rightarrow 2^5 \ldots 2^n$$

where n = the number of generations. Each succeeding generation, assuming no cell death, doubles the population. The total population N at the end of a given time period would be expressed

$$N = 1 \times 2^n \tag{1}$$

However, under practical conditions, the number of bacteria N_0 inoculated at time zero is not 1 but more likely several thousand, so the formula now becomes

$$N = N_0 \times 2^n \tag{2}$$

Solving Eq. (2) for n, we have

$$\log_{10} N = \log_{10} N_0 + n \log_{10} 2$$

$$n = \frac{\log_{10} N - \log_{10} N_0}{\log_{10} 2} \tag{3}$$

If we now substitute the value of $\log_{10} 2$, which is 0.301, in the above equation, we can simplify the equation to

$$n = \frac{\log_{10} N - \log_{10} N_0}{0.301}$$

$$n = 3.3 (\log_{10} N - \log_{10} N_0) \tag{4}$$

Thus, by use of Eq. (4), we can calculate the number of generations that have taken place, providing we know the initial population and the population after growth has occurred.

Normal Growth Cycle (Growth Curve) of Bacteria

Assume that a single bacterium has been inoculated into a flask of liquid culture medium which is subsequently incubated. Eventually the bacterium will undergo binary fission and a period of rapid growth will ensue in which the cells multiply at an exponential rate. During this period of rapid growth, if we used the theoretical number of bacteria which should be present at various intervals of time and then plotted the data in two ways (logarithm of number of bacteria and arithmetic number of bacteria versus time), we would obtain the curve shown in Fig. 7-5. Here, the population increases regularly, doubling at regular

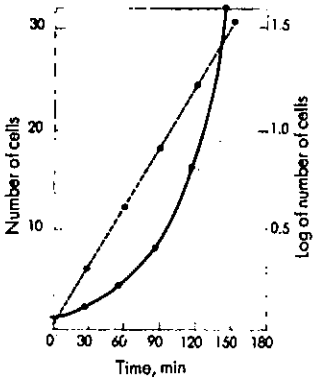


Figure 7-5. Hypothetical bacterial growth curve, assuming that one bacterial cell is inoculated into a medium and divisions occur regularly at 30-min intervals (generation time). – – – = logarithm of number of bacteria versus time; ——— = arithmetic number of bacteria versus time.

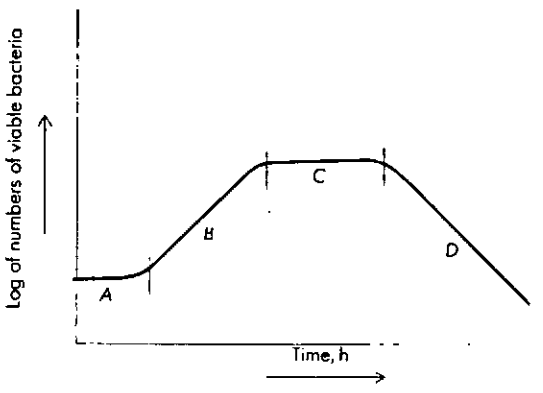


Figure 7-6. Typical bacterial growth curve. A, lag phase; B, log (logarithmic), or exponential, phase; C, stationary phase; D, death or decline phase.

time intervals (the generation time) during incubation. However, exponential growth represents only one specific portion of the growth cycle of a population. In reality, when we inoculate a fresh medium with a given number of cells, determine the bacterial population intermittently during an incubation period of 24 h (more or less), and plot the logarithms of the number of cells versus time, we obtain a curve of the type illustrated in Fig. 7-6. From this it can be seen that there is an initial period of what appears to be no growth (the lag phase), followed by rapid growth (the exponential or logarithmic phase), then a leveling off (stationary phase), and finally a decline in the viable population (death or decline phase). Between each of these phases there is a transitional period (curved portion). This represents the time required before all cells enter the new phase. Let us examine what happens to the bacterial cells during each of the phases of the growth curve.

The Lag Phase

The addition of inoculum to a new medium is not followed immediately by a doubling of the population. Instead, the population remains temporarily unchanged, as illustrated in Fig. 7-6. But this does not mean that the cells are quiescent or dormant; on the contrary, during this stage the individual cells increase in size beyond their normal dimensions. Physiologically they are very active and are synthesizing new protoplasm. The bacteria in this new environment may be deficient in enzymes or coenzymes which must first be synthesized in amounts required for optimal operation of the chemical machinery of the cell. Time for adjustments in the physical environment around each cell may be required. The organisms are metabolizing, but there is a lag in cell division.

At the end of the lag phase, each organism divides. However, since not all organisms complete the lag period simultaneously, there is a gradual increase in the population until the end of this period, when all cells are capable of dividing at regular intervals.

The Logarithmic or Exponential Phase

During this period the cells divide steadily at a constant rate, and the log of the number of cells plotted against time results in a straight line (Figs. 7-5 and 7-6). Moreover, the population is most nearly uniform in terms of chemical composition of cells, metabolic activity, and other physiological characteristics.

Table 7-1. Generation Times of Several Species of Bacteria

Bacterium	Medium	Temperature, °C	Generation Time, min
Escherichia coli	Milk	37	12.5
	Broth	37	17
Bacillus thermophilus	Broth	55	18.3
Streptococcus lactis	Milk	37	26
	Lactose broth	37	48
Staphylococcus aureus	Broth	37	27–30
Bacillus mycoides	Broth	37	28
Lactobacillus acidophilus	Milk	37	66–87
Bradyrhizobium japonicum	Mineral salts + yeast extract + mannitol	25	344–461
Mycobacterium tuberculosis	Synthetic	37	792–932
Treponema pallidum	Rabbit testes	37	1,980

SOURCE: W. B. Spector (ed.): *Handbook of Biological Data*, table 75, Saunders, Philadelphia, 1956.

The generation time g (the time required for the population to double) can be determined from the number of generations n that occur in a particular time interval t. Using Eq. (4) for n, the generation time can be calculated by the following formula:

$$g = \frac{t}{n} = \frac{t}{3.3\ (\log_{10}N - \log_{10}N_0)} \tag{5}$$

Not all bacteria have the same generation time; for some, such as *E. coli*, it may be 15 to 20 minutes; for others it may be many hours (see Table 7-1). Similarly, the generation time is not the same for a particular species under all conditions. It is strongly dependent upon the nutrients in the medium and on prevailing physical conditions, such as those outlined in Chap. 6.

During exponential growth, the **growth rate** (i.e., the number of generations per hour), termed R, is the reciprocal of the generation time g. It is also the slope of the straight line obtained when the log number of cells is plotted against time:

$$R = \frac{3.3(\log_{10} N - \log_{10}N_0)}{t} \tag{6}$$

You may ask how this growth rate can remain constant during the logarithmic phase of growth even though the concentration of substrate (i.e., some essential nutrient in the culture medium, usually the carbon and energy source) is continually decreasing through utilization by the organisms. To understand this, one must recognize that the relationship between R and substrate concentration is not a simple linear relationship, as shown in Fig. 7-7. When the substrate concentration is high, a change in the concentration has very little effect on the growth rate. It is only when the substrate concentration becomes quite low that the growth rate begins to decrease significantly. Since bacteria are commonly "overfed" in laboratory culture, (i.e., are supplied with far greater substrate concentrations then they need), they can multiply at a constant exponential rate for many generations before the substrate level becomes low enough to affect this rate.

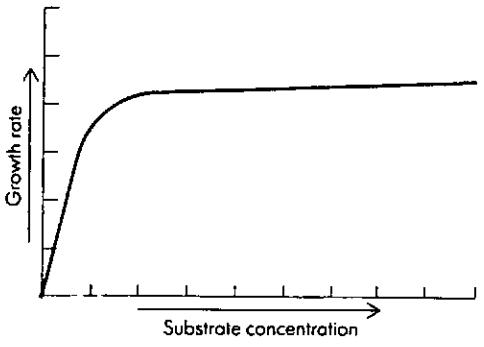


Figure 7-7. The effect of nutrient (substrate) concentration upon the growth rate of a bacterial culture. The level of substrate commonly provided in a bacterial culture is sufficiently high (right portion of curve) so that, even though the bacteria use up some substrate during the log phase of growth, the growth rate does not decrease appreciably. It is only when substrate levels become very low (left portion of curve) that the growth rate begins to be severely affected.

A microbiologist must be able to calculate growth rates and generation times. For example, it is often essential to predict how long it will take a certain population to grow to a given level. An appreciation of the full meaning of the normal growth curve is also necessary; it must be understood that during some phases of growth the cells are young and actively metabolizing while during others they are dying, so that there may be enormous structural and physiological differences between cells harvested at different times. Physical conditions and chemical substances may also affect organisms differently during different phases. Because, in general, cells in the logarithmic phase of growth are the most uniform and are in a more clearly defined condition than in any other phase, log-phase cultures are commonly used for studies of microbial metabolism.

The Stationary Phase

The logarithmic phase of growth begins to taper off after several hours, again in a gradual fashion represented by the transition from a straight line through a curve to another straight line, the stationary phase, as shown in Fig. 7-6. This trend toward cessation of growth can be attributed to a variety of circumstances, particularly the exhaustion of some nutrients, and, less often, the production of toxic products during growth. The population remains constant for a time, perhaps as a result of complete cessation of division or perhaps because the reproduction rate is balanced by an equivalent death rate.

The Phase of Decline or Death

Following the stationary phase the bacteria may die faster than new cells are produced, if indeed some cells are still reproducing. Undoubtedly a variety of conditions contribute to bacterial death, but the most important are the depletion of essential nutrients and the accumulation of inhibitory products, such as acids. During the death phase, the number of viable cells decreases exponentially, essentially the inverse of growth during the log phase. Bacteria die at different rates, just as they grow at different rates. Some species of Gram-negative cocci die very rapidly, so that there may be very few viable cells left in a culture after 72 h or less. Other species die so slowly that viable cells may persist for months or even years.

Transitional Periods Between Growth Phases

Note that a culture proceeds gradually from one phase of growth to the next (Fig. 7-6). This means that not all the cells are in an identical physiological

condition toward the end of a given phase of growth. Time is required for some to catch up with others.

Synchronous Growth

There are many aspects of microbiological research where it would be desirable to relate the various aspects of bacterial growth, organization, and differentiation to a particular stage of the cell division cycle. It is not feasible to analyze a single bacterium because of its small size. However, if all the cells in a culture were to be in the same stage of the division cycle, the result from analysis of the cell crop could be interpreted as that for a single cell. There are several laboratory techniques by which we can manipulate the growth of cultures so that all the cells will be in the same stage of their growth cycle, i.e., growing synchronously.

The synchrony generally lasts only a few generations, since even the daughters of a single cell soon get out of phase with one another. A population can be synchronized by manipulating the physical environment or the chemical composition of the medium. For example, the cells may be inoculated into a medium at a suboptimal temperature; if they are kept in this condition for some time, they will metabolize slowly but will not divide. When the temperature is subsequently raised, the cells will undergo a synchronized division. The most common method of synchronization takes advantage of the fact that the smallest cells in a log-phase culture are those which have just divided. When these cells are separated out by filtration or by differential centrifugation, they are reasonably well synchronized with each other. Figure 7-8 shows the growth pattern of a population of synchronous cells.

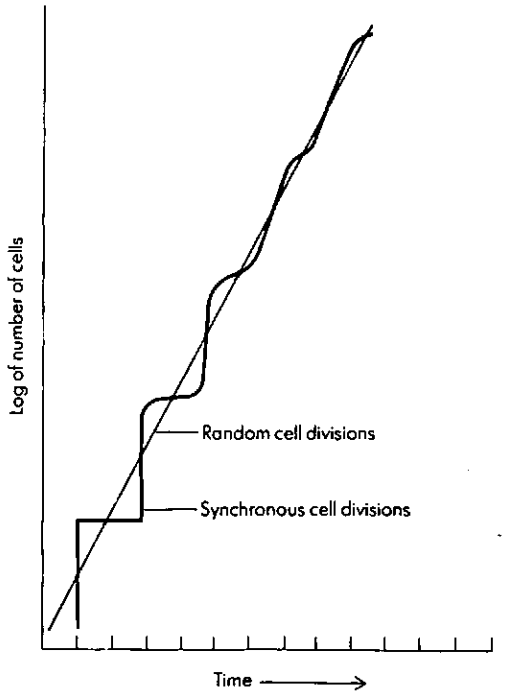


Figure 7-8. Synchronous growth of bacteria. The steplike growth pattern indicates that all the cells of the population divide at about the same time.

Continuous Culture

In both experimental research and in industrial processes, it is often desirable to maintain a bacterial population growing at a particular rate in the exponential or log phase. This condition is known as steady-state growth. The culture volume and the cell concentration are both kept constant by allowing fresh sterile medium to enter the culture vessel at the same rate that "spent" medium, containing cells, is removed from the growing culture (see Fig. 7-9). Under these conditions, the rate at which new cells are produced in the culture vessel is exactly balanced by the rate at which cells are being lost through the overflow from the culture vessel.

One type of system that is widely employed for continuous cultivation is the chemostat. This system depends on the fact that the concentration of an essential nutrient (substrate) within the culture vessel will control the growth rate of the cells. The concentration of substrate within the culture vessel is in turn controlled by the dilution rate, i.e., the rate at which fresh medium is being added to the culture (flow rate) divided by the volume of the culture vessel. Therefore, by adjusting the dilution rate we can control the growth rate. For example, suppose that the dilution rate is very low. The cells reach a high density because they are leaving the culture vessel at a very slow rate; moreover, they have time to use the substrate almost completely. Therefore, the substrate concentration is maintained at a low level within the vessel. This low substrate concentration permits the cells to grow at only a slow rate. On the other hand, if the dilution rate is high, the cell density is low because the cells are leaving the vessel at a

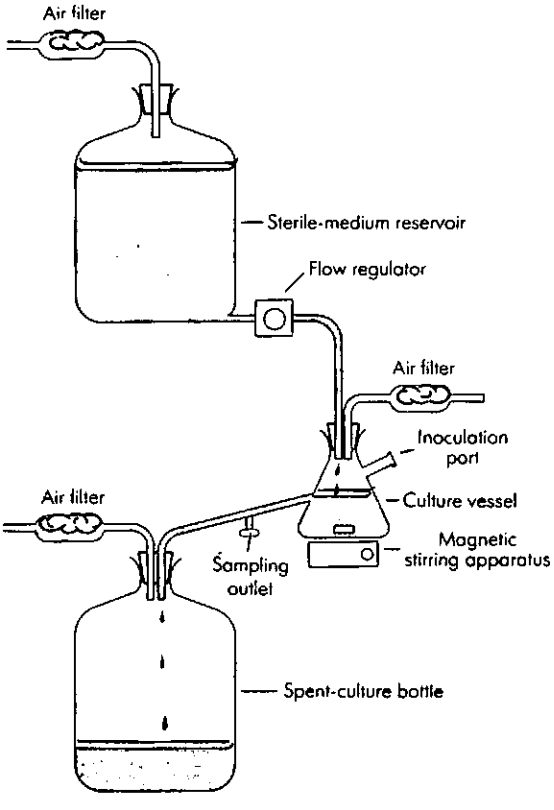


Figure 7-9. Apparatus for continuous cultivation of bacteria. The system can be regulated for continuous addition of fresh sterile medium to and removal of spent medium (and cells) from the culture vessel.

high rate; moreover, they have little time to utilize the substrate that is entering the vessel, and therefore the substrate concentration is maintained at a high level within the vessel (but still less than that in the sterile-medium reservoir). This high concentration allows the cells to grow at a high rate. In each case the growth rate automatically adjusts to match the dilution rate. However, if the dilution rate is increased to the point where it exceeds the maximum growth rate of the cells, then **washout** occurs; that is, the cells cannot grow as fast as the rate at which the culture is being diluted by fresh medium, and they are soon eliminated from the culture vessel.

A second type of continuous culture apparatus is the **turbidostat**. Here a photoelectric device continuously monitors the cell density within the culture vessel and controls the dilution rate to maintain the cell density at a constant value. If the density becomes too high the dilution rate is increased; if the density becomes too low, the dilution rate is decreased.

QUANTITATIVE MEASUREMENT OF BACTERIAL GROWTH

We have seen that the term *growth* as commonly applied in microbiology refers to the magnitude of the total population. Growth in this sense can be determined by numerous techniques based on one or more of the following types of measurement:

1 ***Cell count.*** Directly by microscopy or by using an electronic particle counter, or indirectly by a colony count
2 ***Cell mass.*** Directly by weighing or by a measurement of cell nitrogen, or indirectly by turbidity
3 ***Cell activity.*** Indirectly by relating the degree of biochemical activity to the size of the population

Certain specific procedures will illustrate the application of each type of measurement.

Direct Microscopic Count

Bacteria can be counted easily and accurately with the Petroff-Hausser counting chamber. This is a special slide accurately ruled into squares that are 1/400 mm^2 in area; a glass cover slip rests 1/50 mm above the slide, so that the volume over a square is 1/20,000 mm^3 (1/20,000,000 cm^3). A suspension of unstained bacteria can be counted in the chamber, using a phase-contrast microscope. If, for example, an average of five bacteria is present in each ruled square, there are $5 \times 20{,}000{,}000$, or 10^8, bacteria per milliliter. Direct microscopic counts can be made rapidly and simply with a minimum of equipment; moreover, the morphology of the bacteria can be observed as they are counted. Very dense suspensions can be counted if they are diluted appropriately; however, suspensions having low numbers of bacteria, e.g., at the beginning of a growth curve, cannot be counted accurately.

Electronic Enumeration of Cell Numbers

In this method, the bacterial suspension is placed inside an electronic particle counter, within which the bacteria are passed through a tiny orifice 10 to 30 μm in diameter. This orifice connects the two compartments of the counter which contain an electrically conductive solution. As each bacterium passes through the orifice, the electrical resistance between the two compartments increases momentarily. This generates an electrical signal which is automatically

counted. Although this method is rapid, it requires sophisticated electronic equipment; moreover, the orifice tends to become clogged.

The main disadvantage of direct counting of cell numbers is that there is no way to determine whether the cells being counted are *viable*. To determine the viable count of a culture, we must use a technique that allows viable cells to multiply, such as the plate-count method or the membrane-filter method.

The Plate-Count Method

This method, illustrated in Fig. 7-10, allows determination of the number of cells that will multiply under certain defined conditions. A measured amount of the bacterial suspension is introduced into a Petri dish, after which the agar medium (maintained in liquid form at 45°C) is added and the two thoroughly mixed by rotating the plate. When the medium solidifies, the organisms are trapped in the gel. Each organism grows, reproducing itself until a visible mass of organisms—a colony—develops; i.e., one organism gives rise to one colony. Hence, a colony count performed on the plate reveals the viable microbial population of the inoculum. The original sample is usually diluted so that the number of colonies developing on the plate will fall in the range of 30 to 300. Within this range the count can be accurate, and the possibility of interference of the growth of one organism with that of another is minimized. Colonies are usually counted by illuminating them from below (dark-field illumination) so that they are easily visible, and a large magnifying lens is often used (see Fig. 7-11A). Various electronic techniques have been developed for the counting of colonies (Fig. 7-11B).

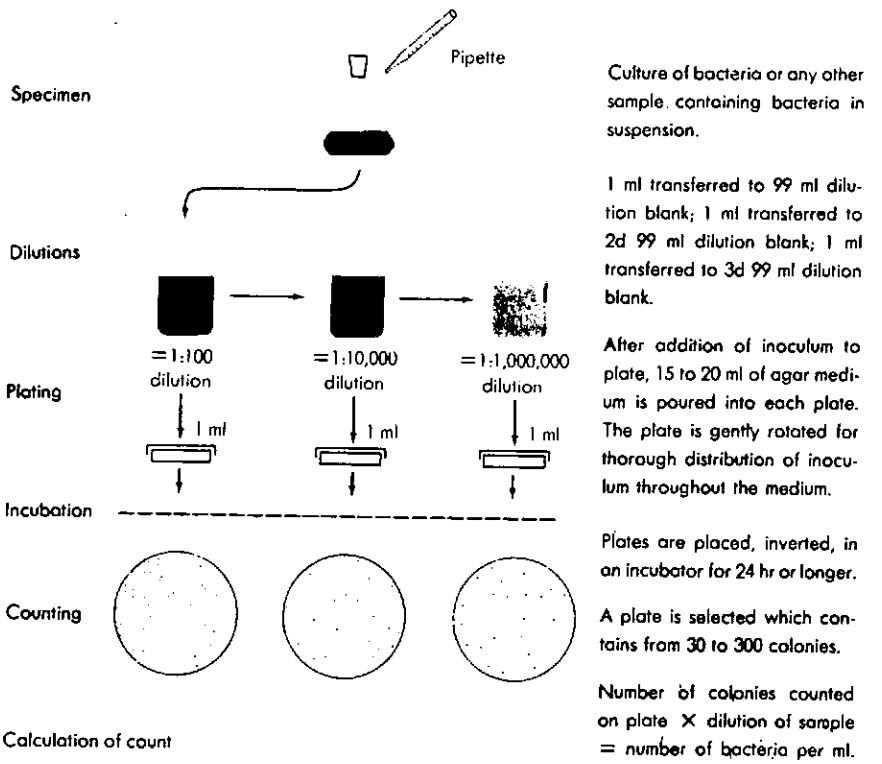


Figure 7-10. The plate-count technique, in which the sample is diluted quantitatively and measured amounts of the dilutions are cultured in Petri dishes.

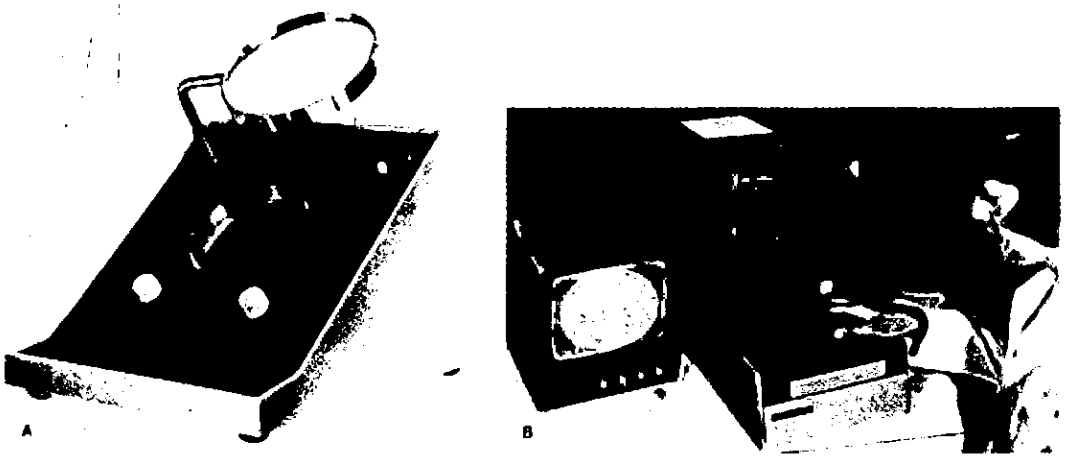


Figure 7-11. Bacterial colony counters. (A) Quebec colony counter. A Petri dish fits into the recess in the platform. The Petri dish is illuminated from beneath while the lens provides X1.5 magnification. *(Courtesy of American Optical Corporation.)* (B) An electronic colony counter. The Petri dish is placed on the illuminated stage, the count bar is depressed, and the precise number of colonies is instantly displayed on a digital readout. *(Courtesy of New Brunswick Scientific Company, Inc.)*

One limitation of the plate-count technique is that the only bacteria that will be counted are those which can grow on the medium used and under the conditions of incubation provided. This can be an important consideration if a mixture of bacteria is to be counted. Another limitation is that each viable organism that is capable of growing under the culture conditions provided may not necessarily give rise to one colony. The development of one colony from one cell can occur when the bacterial suspension is homogeneous and no aggregates of cells are present; however, if the cells have a tendency to aggregate, e.g., cocci in clusters (staphylococci), chains (streptococci), or pairs (diplococci), the resulting counts will be lower than the number of individual cells. For this reason the "counts" are often reported as **colony-forming units** per milliliter rather than number of bacteria per milliliter.

The plate-count technique is used routinely and with satisfactory results for the estimation of bacterial populations in milk, water, foods, and many other materials. It is easy to perform and can be adapted to the measurement of populations of any magnitude. It has the advantage of sensitivity, since very small numbers of organisms can be counted. Theoretically, if a specimen contains as few as one bacterium per milliliter, one colony should develop upon the plating of 1 ml.

Membrane-Filter Count

A very useful variation on the plate-count technique is based on the use of molecular or membrane filters. These filters have a known uniform porosity of predetermined size sufficiently small to trap microorganisms. This technique is particularly valuable in determining the number of bacteria in a large sample that has a very small number of viable cells; e.g., the bacteria in a large volume of air or water can be collected simply by filtering them through an assembly as illustrated in Fig. 7-12A. The membrane, with its trapped bacteria, is then placed in a special plate containing a pad saturated with the appropriate medium. Special media and dyes can be used to make it easier to detect certain types of organisms than with the conventional plate count. During incubation,

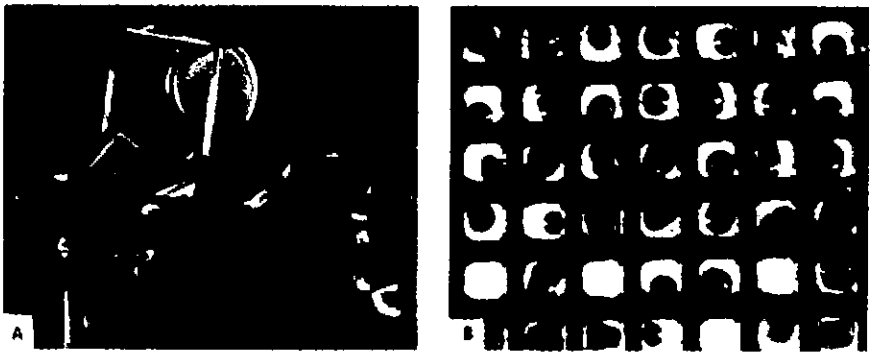


Figure 7-12. (A) Filtration apparatus for use with a membrane filter. After placing the filter on the support, the hinged upper part of the apparatus clamps it in place. A known volume of the bacteria-containing sample is then passed through the filter. (B) The filter is then incubated on a suitable culture medium. In the particular type of filter shown, a grid divides the filter into 1,600 small square compartments, and colony growth is restricted to these compartments. This greatly facilitates the counting of the colonies. *(Courtesy of New Brunswick Scientific Company, Inc.)*

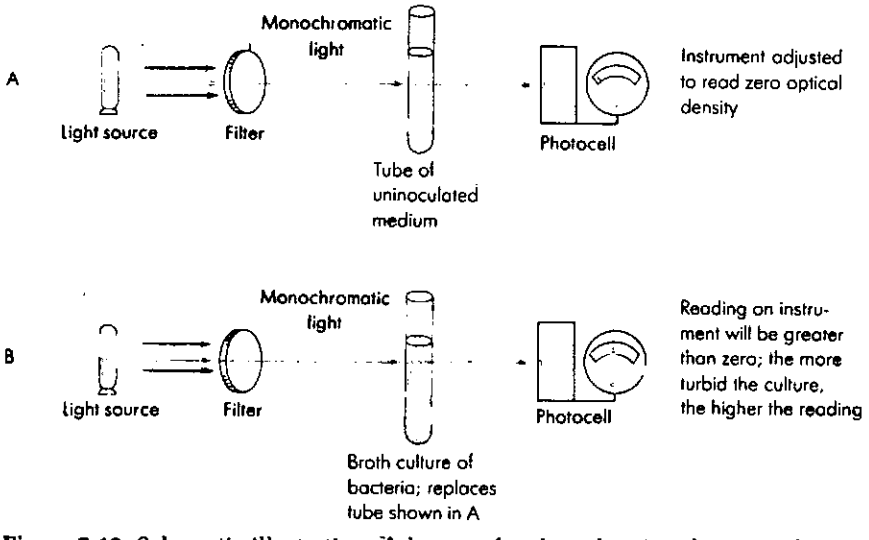


Figure 7-13. Schematic illustration of the use of a photoelectric colorimeter for measuring bacterial populations. The instrument measures optical density (also termed absorbance), a function of light intensity which is almost linearly proportional to cell mass. (A) Adjustment of instrument. A glass tube (cuvette) filled with uninoculated culture medium is used to set the instrument to give a basal optical density reading of 0. (B) The "blank" cuvette is replaced by a similar cuvette containing the broth culture (i.e., medium + cells), and the increase in optical density is recorded.

the organisms grow into colonies which appear on the membrane surface (see Fig. 7-12B).

Turbidimetric Methods

Anyone who has tried to see through a fog realizes that visibility is reduced in proportion to the density of the fog and the distance between the observer and the object that he or she is looking at. This is because each droplet of water in the fog absorbs and scatters the light passing through it, and the more droplets in the light path, the less one can see. Similarly, bacteria in a suspension absorb and scatter the light passing through them, so that a culture of more than 10^7 to 10^8 cells per milliliter appears turbid to the naked eye. A spectrophotometer or colorimeter can be used for turbidimetric measurements of cell mass (see Fig. 7-13). Turbidimetry is a simple, rapid method for following growth; however, the culture to be measured must be dense enough to register some turbidity on the instrument. Moreover, it may not be possible to measure cultures grown in deeply colored media or cultures that contain suspended material other than bacteria. It must also be recognized that dead as well as living cells contribute to turbidity.

Determination of Nitrogen Content

The major constituent of cell material is protein, and since nitrogen is a characteristic part of proteins, one can measure a bacterial population or cell crop in terms of bacterial nitrogen. Bacteria average approximately 14 percent nitrogen on a dry-weight basis, although this figure is subject to some variation introduced by changes in cultural conditions or differences between species. To measure growth by this technique, you must first harvest the cells and wash them free of medium and then perform a quantitative chemical analysis for nitrogen. Bacterial nitrogen determinations are somewhat laborious and can be performed only on specimens free of all other sources of nitrogen. Furthermore, the method is applicable only for concentrated populations. For these and other reasons, this procedure is used primarily in research.

Determination of the Dry Weight of Cells

This is the most direct approach for quantitative measurement of a mass of cells. However, it can be used only with very dense suspensions, and the cells must be washed free of all extraneous matter. Moreover, dry weight may not always be indicative of the amount of living material in cells. For example, the intracellular reserve material poly-β-hydroxybutyrate can accumulate in *Azotobacter beijerinckii* at the end of the log phase of growth and during the stationary phase and finally can comprise up to 74 percent of the dry weight of the cells; thus, the dry weight may continue to increase without corresponding cell growth. Yet, for many organisms the determination of dry weight is an accurate and reliable way to measure growth and is widely used in research.

Measurement of a Specific Chemical Change Produced on a Constituent of the Medium

As an example of this method of estimating cell mass, we may take a species that produces an organic acid from glucose fermentation. The assumption is that the amount of acid produced, under specified conditions and during a fixed period of time, is proportional to the magnitude of the bacterial population. Admittedly, the measurement of acid or any other end product is a very indirect approach to the measurement of growth and is applicable only in special circumstances.

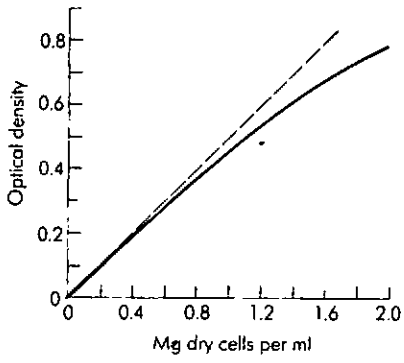


Figure 7-14. Turbidity of a culture serves as a convenient indirect measure of the dry weight of bacterial cells in the culture, as shown in this typical curve (——); however, some deviation from a theoretical linear relationship (- - -) does occur, particularly at optical densities greater than 0.4.

The Relation of Turbidity Measurements to Direct Expressions of Growth

It is frequently desirable to relate measurements of growth made by an indirect method, e.g., turbidity, to a direct measurement, e.g., dry weight of the cell crop. This can be done conveniently by measuring the bacterial suspension simultaneously by the two methods and establishing a relationship between the values obtained, as in the following example. Samples are removed from a cell suspension and dried under predetermined conditions, and the weight of cells per milliliter is determined. From the same cell suspension, dilutions are prepared and turbidity measurements are made. We can calculate the weight of the bacteria in each dilution, since the weight of cells per milliliter of the original sample was determined. Two sets of data will be obtained, which can then be plotted (cell weight against turbidity), as illustrated in Fig. 7-14, to obtain a standard curve. For practical purposes, and within a certain range of concentrations, a nearly linear relationship exists. When the standard curve has been

Table 7-2. Summary of Methods for Measuring Bacterial Growth

Method	Some Applications	Manner in Which Growth Is Expressed
Microscopic count	Enumeration of bacteria in vaccines and cultures	Number of cells per ml
Electronic enumeration	Same as for microscopic count	Same as for microscopic count
Plate count	Enumeration of bacteria in milk, water, foods, soil, cultures, etc.	Colony-forming units per ml
Membrane filter	Same as plate count	Same as plate count
Turbidimetric measurement	Microbiological assay, estimation of cell crop in broth, cultures, or aqueous suspensions	Optical density (absorbance)
Nitrogen determination	Measurement of cell crop from heavy culture suspensions to be used for research in metabolism	Mg nitrogen per ml
Dry weight determination	Same as for nitrogen determination	Mg dry weight of cells per ml
Measurements of biochemical activity, e.g., acid production by cultures	Microbiological assays	Milliequivalents of acid per ml or per culture

established, we can measure the turbidity of a bacterial suspension and convert this value to bacterial weight. Similarly, we can prepare a standard curve correlating other direct measurements (numbers of bacteria or bacterial nitrogen) with turbidity. Thus it is possible to use the convenient indirect measurement (turbidity) and convert the value to a direct expression of growth.

The Selection of a Procedure to Measure Growth

Table 7-2 summarizes the methods described above for measuring bacterial growth. Each has its particular advantages and limitations, and no one method can be recommended universally. The best procedure for your work can be selected only after these factors are considered in relation to the problem at hand. The colony count is the most widely used procedure for general microbiological work, and complete familiarity with this technique, both in principle and practice, is essential. It should be emphasized that the colony count is theoretically the only technique that reflects the viable population. Furthermore, it is not at all unlikely that discrepancies may occur in results of growth of a bacterial population when measured by two different methods. For example, a microscopic count of a culture in the stationary phase would include all cells, viable and nonviable, whereas the colony count would reveal only the viable population.

Importance of Quantitative Measurement of Growth

Before we can evaluate or interpret growth responses of bacteria in different media or under various conditions, growth must be expressed in quantitative terms. In microbiology the term *growth* is used in several ways. For example, we may judge a certain set of conditions as being good because the bacteria grow rapidly, but the final total cell crop may not be as large as under another set of conditions where the growth proceeds at a slower rate but continues to increase over a longer time period. Such a situation is shown schematically in Fig. 7-15, where the growth of the same bacterial species is compared in two different media. If we measured growth at time *A*, we should conclude that growth is best in medium II; measured at time *B*, growth would be equally good in both media; and at time C, growth would be better in medium I. If we were primarily interested in a large cell crop, we should select medium I. In any

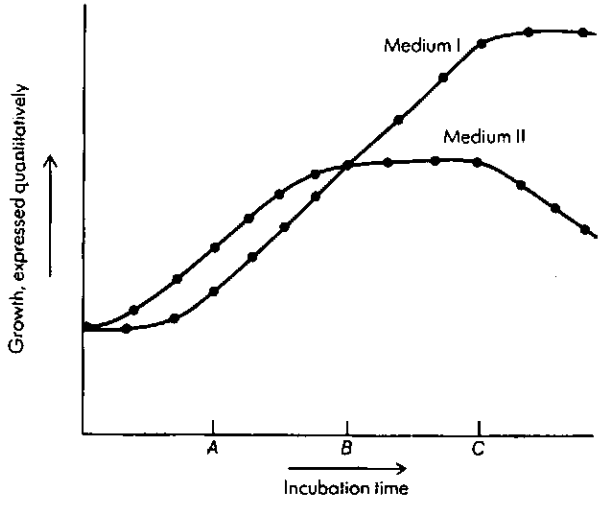


Figure 7-15. Quantitative measurement of growth is significant for interpretation of various growth responses. Hypothetical growth response of same bacterium in media of two different compositions. Compare the cell crops, or amount of growth, at times *A*, *B*, and C.

event, we must have knowledge of growth in quantitative terms to make the correct choice.

QUESTIONS

1 How does the term *growth* as used in microbiology differ from the same term as applied to higher plants and animals?
2 Describe the differences between the various modes of cell division in bacteria.
3 How is it that the septum formation in *Streptococcus faecalis* accounts for all the new cell wall material of newly formed daughter cells, yet in *Bacillus subtilis* it accounts for only 15 percent?
4 In a culture of bacteria that is actively multiplying by transverse binary fission, are there any "old" cells? Explain.
5 During log-phase growth of a bacterial culture, a sample is taken at 8:00 a.m. and found to contain 1,000 cells per milliliter. A second sample is taken at 5:54 p.m. and is found to contain 1,000,000 cells per milliliter. What is the generation time in hours?
6 Would you expect generation time to be a constant characteristic of a bacterial species? Explain.
7 In the lag phase of growth the number of bacteria remains constant. Does this mean the cells are dormant and inert? Explain.
8 Draw a typical bacterial growth curve and label the various phases. Discuss those factors which determine the beginning and end of each phase.
9 When the appropriate data are plotted for the log-phase growth of a bacterial culture, how is it that we obtain a steady increase in the number of cells rather than a series of stepwise increases? Explain.
10 How can synchronous growth of a bacterial culture be obtained? In what way could a synchronously growing culture be useful for the electron microscopist who is trying to determine the cytological changes associated with bacterial growth?
11 What is steady-state growth and what advantages does it offer? Describe how steady-state growth at a certain rate could be obtained by use of a chemostat.
12 Compare the direct and indirect methods for estimating bacterial populations on the basis of *(a)* practical applications, *(b)* advantages and *(c)* limitations of use.

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Chapter 8 Pure Cultures and Cultural Characteristics

OUTLINE **Natural Microbial Populations (Mixed Cultures)**

Selective Methods
Chemical Methods of Selection • Physical Methods of Selection • Biological Methods of Selection • Selection in Nature

Pure Cultures
Methods of Isolating Pure Cultures

Maintenance and Preservation of Pure Cultures
Methods of Maintenance and Preservation • Culture Collections

Cultural Characteristics
Colony Characteristics • Characteristics of Broth Cultures

In natural environments a single kind of bacterium, i.e., a bacterial species, usually occurs as only one component of a large and complex population containing many other species. To study the characteristics of one species, that species must be separated from all the other species, i.e., it must be **isolated** in pure culture. However, before attempting isolation, it is often helpful to use a **selective method** first. Such a method can increase the relative proportion of the desired species in the population so that it can be more easily isolated. Once obtained, a pure culture can be maintained or preserved in a **culture collection.** Different species of bacteria growing on the same kind of medium may appear quite different; thus knowledge of the appearance, or the **cultural characteristics,** of a species is useful for the recognition of certain kinds of bacteria and may also serve as an aid to the identification of species. In this chapter we shall describe methods for selection, isolation, and preservation of bacteria, as well as their cultural characteristics on various media.

NATURAL MICROBIAL POPULATIONS (MIXED CULTURES)

The microbial population in our environment is large and complex. Many different microbial species normally inhabit various parts of our bodies, such as the oral cavity, the intestinal tract, and the skin. These microbes may be present in extremely large numbers. For example, a single sneeze may disperse from 10,000 to 100,000 bacteria. One gram of feces may contain 10^{11} bacteria. Our environment—air, soil, water—likewise consists of mixed populations of

bacteria plus other microbes. In fertile garden soil, microorganisms may number several billions per gram and include many species of bacteria, fungi, algae, and protozoa. A study of the microorganisms in these habitats requires knowledge of the specific microbes present. This, in turn, requires unraveling the complex mixed population into pure cultures of separate, distinct species.

SELECTIVE METHODS

A particular bacterial species is often present in small numbers compared to the total population of a mixed culture. Moreover, the species may be one that grows less rapidly on ordinary culture media than other species. In order to achieve its isolation into pure culture, it is helpful—and often necessary—to first achieve an increase in the relative number of the species, preferably to the point where the species becomes the numerically dominant component of the population. This can be accomplished by the use of selective methods. These methods favor the growth of the desired species while discouraging, or even killing, the other organisms present in the mixed culture. Chemical, physical, or biological methods are used in order to achieve selection of a particular kind of bacterium.

Chemical Methods of Selection

Use of a Special Carbon or Nitrogen Source

One type of chemical method is to provide in the culture medium a substrate, i.e., a single carbon or nitrogen source, that can be used only by the species being sought (Fig. 8-1). This particular kind of selection is often referred to by a special name, enrichment. For example, if we wish to isolate, from soil, bacteria capable of utilizing a very complex organic compound like α-conidendrin, a constituent of wood, we find that when we inoculate a medium such as nutrient agar directly with the soil sample, our chances of finding α-conidendrin–utilizing bacteria will be very limited. There are so many other rapidly growing bacteria present that the more slowly growing kind we wish to obtain will be soon overgrown. Consequently, we prepare a liquid enrichment medium in which α-conidendrin is the sole source of carbon. Under these conditions, only organisms capable of utilizing this compound will be able to grow well. However, it is important to recognize that other bacteria may be able to grow to some extent by utilizing organic compounds made by the conidendrin-utilizing

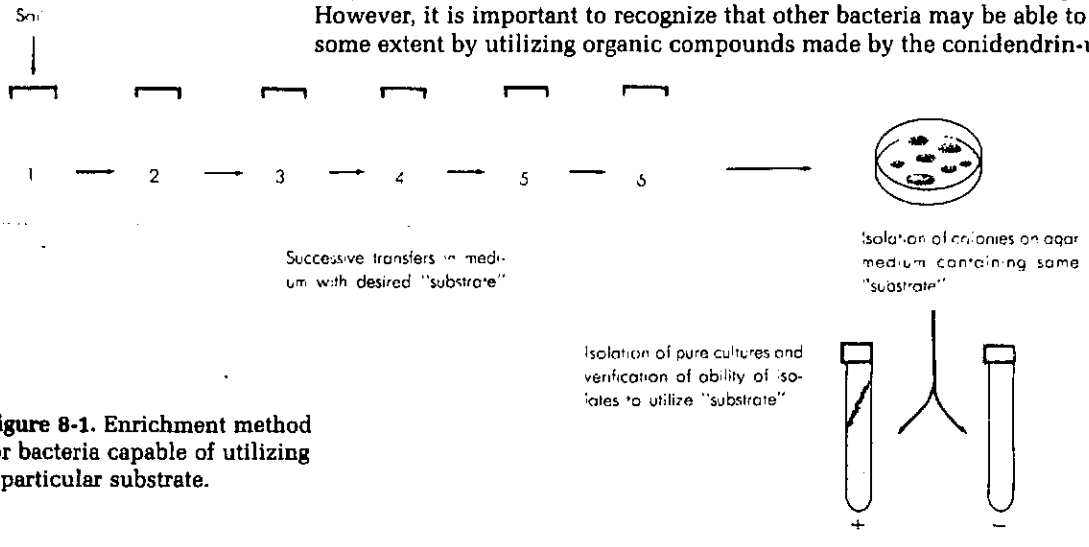


Figure 8-1. Enrichment method for bacteria capable of utilizing a particular substrate.

organisms and that the method is not completely specific. As another example, if we wish to select for nitrogen-fixing bacteria, nitrogen gas (N_2) can be supplied as the sole nitrogen source, since only nitrogen-fixing bacteria will be able to grow well. Other bacteria may grow, but to a lesser degree, by using the nitrogenous products made by the nitrogen-fixers.

Use of Dilute Media

Certain aquatic bacteria, such as *Caulobacter* species, are capable of growing with very low levels of carbon or nitrogen sources. Consequently, one way to select for such bacteria is to inoculate a mixed culture into a very dilute medium, e.g., a broth containing only 0.01 percent peptone. The medium must have low enough levels of nutrients that other kinds of organisms will not be able to grow well in it.

Use of Inhibitory or Toxic Chemicals

The addition of low levels of certain chemicals, such as dyes, bile salts, salts of heavy metals, or antibiotics, to culture media can be useful for the selection of certain kinds of bacteria. The following are examples of this type of selection:

1 Many Gram-negative bacteria can grow in the presence of low concentrations of various dyes that inhibit the growth of Gram-positive bacteria. Similarly, intestinal bacteria can grow in the presence of bile salts such as sodium deoxycholate, whereas nonintestinal bacteria are usually inhibited. Consequently, a medium containing crystal violet dye plus sodium deoxycholate will allow Gram-negative intestinal bacteria to grow but will inhibit most other kinds of bacteria. An example of such a medium is MacConkey agar, which is widely used to select for Gram-negative intestinal pathogens such as *Salmonella* and *Shigella* species.
2 *Campylobacter jejuni* is a frequent cause of diarrhea in humans, yet diarrheic stool samples contain many other kinds of bacteria that interfere with the isolation of this species. By incorporating certain antibiotics or other chemotherapeutic agents, such as vancomycin, polymyxin, and trimethoprim, into the culture medium, most of these contaminants can be inhibited without affecting the growth of *C. jejuni*.

Physical Methods of Selection

Heat Treatment

To select for endospore-forming bacteria, a mixed culture can be heated to 80°C for 10 min before being used to inoculate culture media. Vegetative cells will be killed by this treatment but endospores will survive and subsequently germinate and grow.

Incubation Temperature

To select for psychrophilic or psychrotrophic bacteria, cultures are incubated at low temperatures, e.g., 0 to 5°C. For selection of thermophiles, a high incubation temperature is used, e.g., 55°C.

pH of the Medium

To select for acid-tolerant bacteria, a low-pH medium can be used. For example, to select for the lactobacilli present in cheddar cheese, the pH of the medium is maintained at 5.35 with an acetic acid/acetate buffer; other organisms in the cheese cannot grow well at such a low pH. Similarly, to select for alkali-tolerant organisms, a high-pH medium can be used. For example, to select for the cholera-causing bacterium, *Vibrio cholerae*, from a stool sample, we can use a medium with a pH of 8.5; most other intestinal bacteria are unable to grow at this pH.

Cell Size and Motility

We can sometimes make use of a small cell diameter or of bacterial motility to achieve selection. For instance, *Treponema* species from the human oral cavity can be selected by taking advantage of both of these properties. A membrane filter having a pore size of 0.15 μm is placed on the surface of an agar plate and gingival scrapings are placed on the filter. The unusually small size of treponemes allows them to penetrate the pores of the filter to reach the underlying agar. Moreover, treponemes have the ability to swim through solid agar media; consequently, they migrate away from the filter and grow to form a hazy zone within the agar, from which they can be subcultured. Other bacteria from the oral cavity are either too large to penetrate the membrane filter or, if they can penetrate it, are unable to migrate away through the agar.

Biological Methods of Selection

A disease-producing species occurring in a mixed culture can often be selected by taking advantage of its pathogenic properties. For example, a sputum sample containing *Streptococcus pneumoniae* is ordinarily contaminated by many other bacterial species. However, laboratory mice are extremely susceptible to infection by *S. pneumoniae*, and if the sputum sample is injected into a mouse the pathogen will multiply extensively. Nonpathogenic bacteria present in the sample will either be inhibited or killed by the defense mechanisms of the animal. In a sense, the animal serves as the selective medium.

How can the microbiologist know what selective media or conditions to use for a given species? Many selective methods are given in the references at the end of this chapter. Moreover, you can often devise a satisfactory selective procedure by comparing the characteristics of the species sought with those of the accompanying contaminants. Differences in these characteristics, e.g., in susceptibility to certain antibiotics, can provide the basis for a suitable selective procedure.

Selection in Nature

It is important to realize that the principle of selection is not limited to the laboratory; it also commonly operates in nature. For instance, the occurrence of high salt concentrations in bodies of water such as the Dead Sea selects for extreme halophiles such as those of the genus *Halobacterium*. In lakes, the anaerobic, sulfide-containing zone on or above the sediment mud provides conditions that often favor the mass development of green or purple sulfide-oxidizing, phototrophic bacteria. The nodules that occur on the roots of leguminous plants contain bacteria of the genus *Rhizobium*, which are uniquely suited for nitrogen fixation in association with these plants. In many types of natural infections of humans or animals, a single, uncontaminated pathogenic bacterial species can often be obtained from a blood sample; blood from a healthy animal or human is normally free of bacteria. Numerous other examples of selective conditions in nature exist.

PURE CULTURES

If the bacterial species being sought comprises a suitably high proportion of the mixed population, it can be isolated in pure culture. The descendents of a single isolation in pure culture comprise a **strain**. A strain is usually made up of a succession of cultures and is often derived from a single colony; however, the number of bacteria which gave rise to the original colony is usually unknown. If a strain is derived from a single parent cell, it is termed a **clone**. Each strain

is designated by an identifying number and its history is recorded (the source from which the isolation was made, the name of the person who made the isolation, the date of the isolation, and the culture collection in which the strain is maintained and from which it can be obtained for study).

A variety of techniques have been developed whereby isolation into pure culture can be accomplished. Each technique has certain advantages and limitations, and there is no one method that can be used for all bacteria.

Methods of Isolating Pure Cultures

The Streak-Plate Technique

By means of a transfer loop, a portion of the mixed culture is placed on the surface of an agar medium and streaked across the surface. This manipulation "thins out" the bacteria on the agar surface so that some individual bacteria are separated from each other. Figure 8-2 illustrates a nutrient-agar plate culture that has been streaked to provide isolated colonies. When streaking is properly performed, the bacterial cells will be sufficiently far apart in some areas of the plate to ensure that the colony developing from one cell will not merge with that growing from another. Figure 8-3 illustrates a modification known as the **roll-tube technique** that is used for the isolation of stringent anaerobes.

Figure 8-2. Streak-plate culture showing areas of isolated colonial growth. Note that where the colonies are sparse they are larger than when crowded together. *(Courtesy of Naval Biological Laboratory.)*

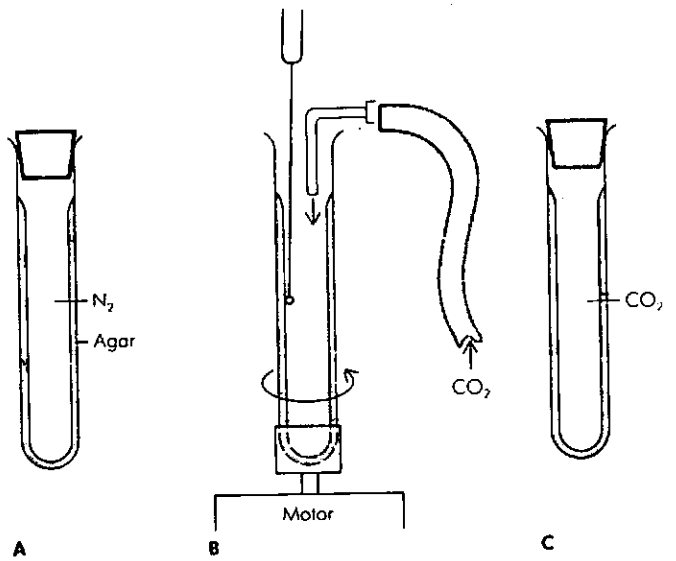


Figure 8-3. Roll-tube method for isolating stringent anaerobes. (A) Stoppered anaerobic culture tube whose inner walls have been coated with a prereduced agar medium. The tube contains an atmosphere of oxygen-free N_2. (B) When the stopper is removed the tube is kept anaerobic by continuously flushing it with oxygen-free CO_2 from a gas cannula. Inoculation is done with a transfer loop held against the agar surface as the tube is being rotated by a motor. By starting at the bottom and drawing the loop gradually upward, the inoculum becomes "thinned" to the point where well-isolated colonies can develop. (C) After inoculation the tube is restoppered and incubated.

The assumption is often made that a colony is derived from a single cell and, therefore, that the colony is a clone. However, this is not necessarily true. With species in which the cells form a characteristic grouping during cell division (for example, clumps of staphylococci or chains of streptococci), the colony may develop from a group of cells rather than from a single cell. Although not a clone, such a colony is nevertheless a pure culture if it contains only one kind of organism.

One should recognize that subculturing a colony from a single streak plate does not automatically assure purity. The colony may have been derived from two or more different kinds of bacteria. For example, when we attempt to isolate slime- or chain-producing bacteria, contaminants may be found to have adhered to the slime or to have been enmeshed in the network of chains, thereby resulting in impure colonies. The use of selective media can also lead to impure colonies. Although the growth of contaminants is inhibited on selective media, low numbers of viable cells may still be present, and such cells can be subcultured along with a colony. For these reasons, it is advisable to streak a culture several times in succession, preferably on nonselective media, in order to ensure purity.

The Pour-Plate and Spread-Plate Techniques

In both of these methods the mixed culture is first diluted to provide only a few cells per milliliter before being used to inoculate media. Since the number of bacteria in the specimen is not known beforehand, a *series of dilutions* is made so that at least one of the dilutions will contain a suitably sparse concentration of cells.

In the **pour-plate method** the mixed culture is diluted directly in tubes of liquid (cooled) agar medium (see Fig. 8-4). The medium is maintained in a liquid state at a temperature of 45°C to allow thorough distribution of the inoculum. The inoculated medium is dispensed into Petri dishes, allowed to solidify, and then incubated. A series of agar plates showing decreasing numbers of colonies resulting from the dilution procedure in the pour-plate technique is shown in Fig. 8-4. The pour-plate technique has certain disadvantages. For

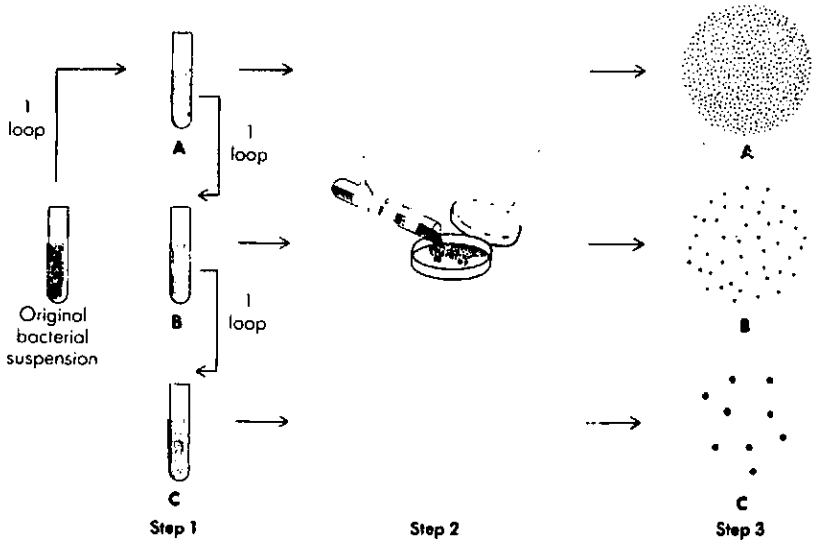


Figure 8-4. Pour-plate technique is used for isolation of pure cultures of bacteria. Step 1: One loopful of original suspension is transferred to tube A (liquid, cooled agar medium). Tube A is rolled between the hands to effect thorough mixing of inoculum. Similar transfers are made from A to B to C. Step 2: Contents of each tube are poured into separate Petri dishes. Step 3: After incubation, plates are examined for the one which contains well-separated colonies. From this plate, pure cultures of bacteria can be isolated by transferring a portion of a colony to a tube of sterile medium.

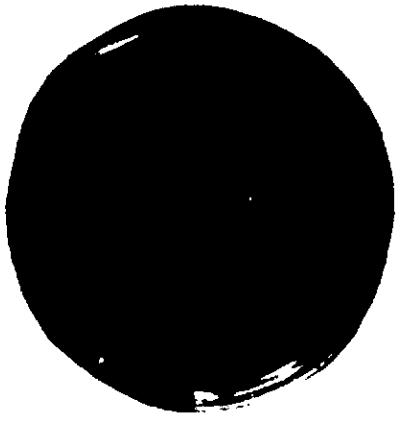


Figure 8-5. Spread plate showing colonies of two different bacterial species. A dilution of the mixed culture was spread over the surface with a glass rod. The large, dark colonies are *Serratia marcescens*, which has a brick-red pigment, and the smaller, light colonies are *Micrococcus luteus*, which has a lemon-yellow pigment. *(Courtesy of Naval Biological Laboratory.)*

instance, some of the organisms are trapped beneath the surface of the medium when it gels, and therefore both *surface* and *subsurface* colonies develop. The subsurface colonies can be transferred to fresh media only by first digging them out of the agar with a sterile instrument. Another disadvantage is that the organisms being isolated must be able to withstand temporary exposure to the 45°C temperature of the liquid agar medium; for instance, the pour-plate method would be unsuitable for isolating psychrophilic bacteria.

In the spread-plate method the mixed culture is not diluted in the culture medium; instead it is diluted in a series of tubes containing a sterile liquid, usually water or physiological saline. A sample is removed from each tube, placed onto the surface of an agar plate, and spread evenly over the surface by means of a sterile, bent glass rod. On at least one plate of the series the bacteria will be in numbers sufficiently low as to allow the development of well-separated colonies (see Fig. 8-5). In contrast to the pour-plate technique, only surface colonies develop; moreover, the organisms are not required to withstand the temperature of liquid agar.

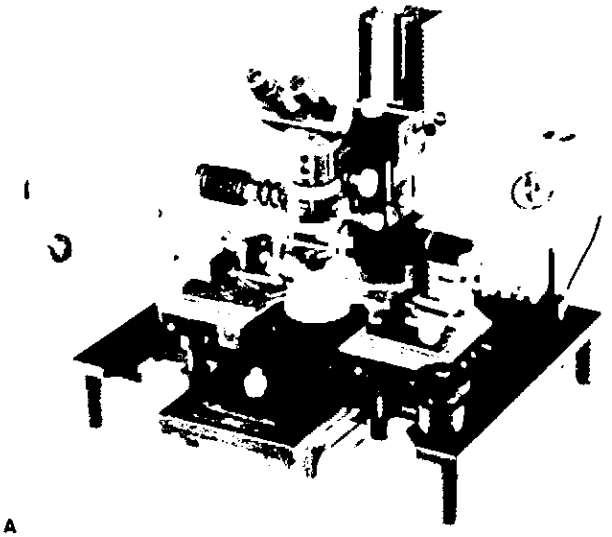
Unlike the streak-plate technique, the pour-plate and the spread-plate techniques may be performed in a *quantitative* manner to determine the number of bacteria (of a particular type) present in a specimen (see Chap. 7).

Micromanipulator Techniques

A device called the **micromanipulator** can be used in conjunction with a microscope to pick a single bacterial cell from a mixed culture. The micromanipulator permits the operator to control the movements of a micropipette or a microprobe (a fine needle) so that a single cell can be isolated (see Fig. 8-6). This technique requires a skilled operator and is reserved for studies in which a clone must be obtained unequivocally.

MAINTENANCE AND PRESERVATION OF PURE CULTURES

Most microbiology laboratories maintain a large collection of strains, frequently referred to as a **stock-culture collection.** These organisms are needed for laboratory classes and research work, as test agents for particular procedures, or as reference strains for taxonomic studies. Most major biological companies maintain large culture collections. The strains are used for screening of new, potentially effective chemotherapeutic agents; as assay tools for vitamins and amino



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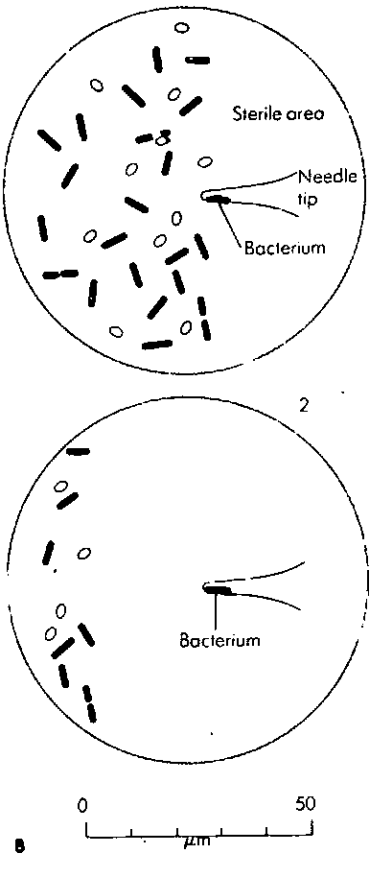

Figure 8-6. Isolation of single bacterial cells. (A) Micromanipulator equipment and microscope. The micromanipulator is equipped with probes that can position on objects a few μm in size. The manipulation of the probes is done while viewing the specimen through the microscope. *(Micromanipulator Company.)* (B) Schematic illustration showing isolation of a single bacterium from a mixture of cells on an agar gel. The microscope field (1) shows the point of the microprobe touching the agar gel near a bacterial cell, causing the organism to float in the small amount of water exuded from the gel. The microscope stage is then moved to the left (2) while the microprobe remains fixed; this causes the bacterium to follow the probe and become separated from the other cells. The agar block is subsequently dissected and the small piece containing the isolated bacterium is transferred to a sterile culture medium. *(Courtesy of K. I. Johnstone, Manipulation of Bacteria, Churchill Livingstone, Edinburgh, 1973.)*

acids; as agents for the production of vaccines, antisera, antitumor agents, enzymes, and organic chemicals; and as reference cultures that are cited in company patents. For these and other purposes it is extremely important to have properly identified and cataloged strains of bacteria available. Consequently, a considerable amount of research has been performed to develop methods whereby bacterial strains can be preserved and stored until they are needed. Several different methods have been developed, since not all bacteria respond in a similar manner to a specific method. Moreover, there are various practical considerations such as the amount of labor involved and the amount of storage space required. However, all the methods which we will now describe have the same objective: to maintain strains alive and uncontaminated and to prevent any change in their characteristics.

Methods of Maintenance and Preservation

Strains can be maintained by periodically preparing a fresh stock culture from the previous stock culture. The culture medium, the storage temperature, and the time interval at which the transfers are made vary with the species and must

Periodic Transfer to Fresh Media

be ascertained beforehand. The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible. Many of the more common heterotrophs remain viable for several weeks or months on a medium like nutrient agar. The transfer method has the disadvantage of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

Preservation by Overlaying Cultures with Mineral Oil

Many bacteria can be successfully preserved by covering the growth on an agar slant with sterile mineral oil. The oil must cover the slant completely; to ensure this, the oil should be about ½ in above the tip of the slanted surface. Maintenance of viability under this treatment varies with the species (1 month to 2 years). This method of maintenance has the unique advantage that you can remove some of the growth under the oil with a transfer needle, inoculate a fresh medium, and still preserve the original culture. The simplicity of the method makes it attractive, but changes in the characteristics of a strain can still occur. Figure 8-7 illustrates a culture collection maintained by this technique.

Preservation by Lyophilization (Freeze-Drying)

Most bacteria die if cultures are allowed to become dry, although spore- and cyst-formers can remain viable for many years. However, freeze-drying can satisfactorily preserve many kinds of bacteria that would be killed by ordinary

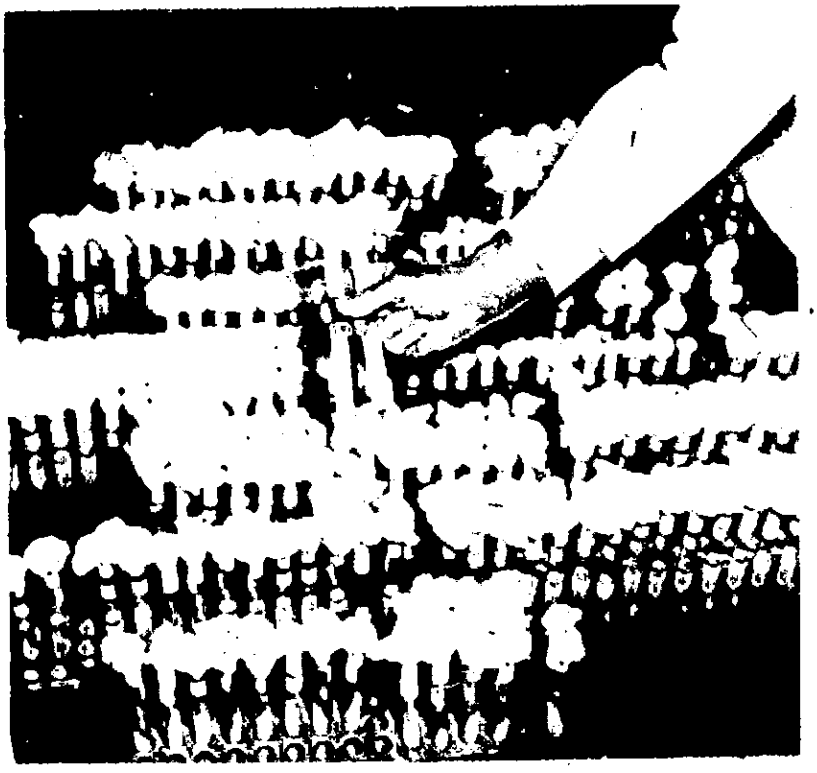

Figure 8-7. A culture collection maintained by overlaying cultures with mineral oil. *(Courtesy of U.S. Department of Agriculture.)*

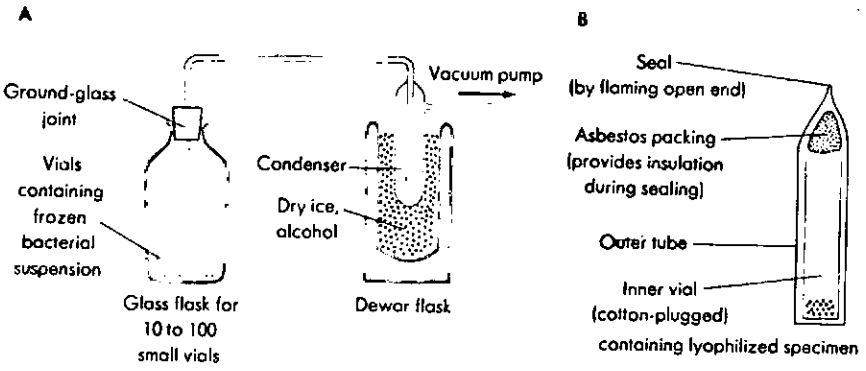

Figure 8-8. Lyophilization process for preservation of cultures. (A) A simple apparatus for lyophilization. Small cotton-plugged vials containing frozen suspensions of bacteria are placed in the glass flask, which is attached to a condenser. The condenser is connected to a high-vacuum pump. The bacteria become desiccated as the ice in the frozen suspension sublimes directly to water vapor. The vapor is trapped on the cold surface of the condenser, thereby preventing it from entering the vacuum line and contaminating the pump oil. (B) After desiccation of the cultures as in (A), the vials are removed and each is placed in a larger tube. After insulating the vial with a plug of glass wool packing, the outer tube is hermetically sealed under a vacuum by means of torch. *(Courtesy of American Type Culture Collection.)*

drying. In this process a dense cell suspension is placed in small vials and frozen at −60 to −78°C. The vials are then connected to a high-vacuum line. The ice present in the frozen suspension sublimes under the vacuum, i.e., evaporates without first going through a liquid water phase. This results in dehydration of the bacteria with a minimum of damage to delicate cell structures. The vials are then sealed off under a vacuum and stored in a refrigerator. One arrangement of equipment employed to lyophilize cultures is shown in Fig. 8-8. Many species of bacteria preserved by this method have remained viable and unchanged in their characteristics for more than 30 years. Only minimal storage space is required; hundreds of lyophilized cultures can be stored in a small area. Furthermore, the small vials can be sent conveniently through the mail to other microbiology laboratories when packaged in special sealed mailing containers. Lyophilized cultures are revived by opening the vials, adding liquid medium, and transferring the rehydrated culture to a suitable growth medium.

Storage at Low Temperatures

The ready availability of liquid nitrogen has provided the microbiologist with another very useful means for long-term preservation of cultures. In this procedure cells are prepared as a dense suspension in a medium containing a cryoprotective agent such as glycerol or dimethyl sulfoxide (DMSO), which prevents cell damage due to ice crystal formation during the subsequent steps. The cell suspension is sealed into small ampoules or vials and then frozen at a controlled rate to −150°C. The ampoules or vials are then stored in a liquid nitrogen refrigerator (essentially a large tank having vacuum-insulated walls; see Fig. 8-9) either by immersion in the liquid nitrogen (−196°C) or by storage in the gas phase above the liquid nitrogen (−150°C). The liquid nitrogen method has been successful with many species that cannot be preserved by lyophilization, and most species can remain viable under these conditions for 10 to 30 years or more without undergoing change in their characteristics. However, the method is relatively expensive, since the liquid nitrogen in the refrigerators must be replenished at regular intervals to replace the loss due to evaporation.

Culture Collections

When microbiologists first began to isolate pure cultures, each microbiologist kept a personal collection of those strains having special interest. Subcultures of some strains were often sent to other microbiologists; other subcultures were received and added to the scientist's own collection. Certain strains had tax-

onomic importance because they formed the basis for descriptions of species and genera. Others had special properties useful for various purposes. However, many important strains became lost or were inadequately maintained. Thus, it became imperative to establish large central collections whose main purpose would be the acquisition, preservation, and distribution of authentic cultures of living microorganisms.

Many countries have at least one central collection. As examples, in France a collection of bacteria is maintained at the Institut Pasteur in Paris; in England the National Collection of Type Cultures is in London; the Federal Republic of Germany maintains the Deutsche Sammlung von Mikroorganismen in Darmstadt; and Japan maintains a large collection at the Institute for Fermentation in Osaka. Many other such collections exist.

In the United States the major collection is the American Type Culture Collection (ATCC), located in Rockville, Maryland. In 1980 the collection included the following numbers of strains: bacteria, 11,500; bacteriophages, 300; fungi and fungal viruses; 13,700; protozoa, 720; algae, 130; animal-cell cultures, 500; animal viruses, rickettsiae, and chlamydiae, 1,135; and plant viruses, 220. More than 1 million ampoules of lyophilized or frozen living strains are inventoried and stored at the ATCC. Other large collections in the United States are more specialized in scope. For example, the Northern Utilization Research and Development Division, USDA, at Peoria, Illinois, maintains a collection of yeasts, molds, and bacteria especially for use in fermentations. The Quartermaster Research and Development Center, U.S. Army, Natick, Massachusetts, maintains a collection of microbial strains that are associated with deterioration processes. A number of smaller collections of a specialized nature also exist, such as the collection of anaerobic bacteria maintained by the Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg.

One of the major functions of a large national collection is the preservation of type strains. As discussed in Chap. 3, the type strain of a species has great taxonomic importance because it is the "name-bearer" strain, or permanent example, of the species. Microbiologists who propose a new species are expected to deposit the type strain with one or more national collections so that it can be preserved and so that subcultures can be distributed to other workers for study and comparison with other microorganisms.

Figure 8-9. (A) Liquid-nitrogen refrigerators used for preservation of bacteria. Each container holds many thousands of ampoules. *(Courtesy of American Type Culture Collection.)* (B) Preservation of bacterial cultures in the gas phase (−150°C) of liquid-nitrogen refrigerators. For preservation, a bacterial suspension is placed in a vial which is then sealed. This picture shows six vials, attached to a metal cane, being removed from storage. *(Courtesy of Alma Dietz, The Upjohn Company.)*

CULTURAL CHARACTERISTICS

One of the major features of a bacterial strain is its appearance following growth on various media. Such commonplace characteristics as the abundance of the growth, the size of the colonies, and the color (or chromogenesis) of the colonies provide useful clues for identification.

To determine the growth characteristics of a bacterial strain, it is customary to observe the features of colonies and broth cultures. Inoculation of agar plates to obtain isolated colonies has already been described. Tubes of broth can be inoculated with the transfer needle or the loop; generally the loop is used when the inoculum is a liquid.

After inoculation of the medium and subsequent incubation, the cultural characteristics can be determined. The main features can be summarized as follows.

Colony Characteristics

Size. Colonies range in size from extremely small (**pinpoint**), measuring only a fraction of a millimeter in diameter, to **large** colonies measuring 5 to 10 mm in diameter. Although the colonies of a given species have a characteristic diameter, one must be aware of certain factors affecting colony diameter. For instance, only well-separated colonies should be measured, since such colonies tend to have a larger diameter than those which are crowded together (for example, see Fig. 8-2). This is because widely separated colonies are subject to less competition for nutrients and less inhibition by toxic products of metabolism. Moreover, young colonies are smaller than older colonies; therefore the time at which measurements are made must be stated. There is generally an upper limit to the final size of the colonies of a given species; i.e., a point is reached where further incubation no longer results in a corresponding increase in size. However, some bacteria (e.g., certain species of *Proteus* and *Bacillus*) can spread across the entire agar surface, and the colony size is limited only by the dimensions of the Petri dish!

Margin or Edge. The periphery of bacterial colonies may take one of several different patterns, depending on the species. It may be evenly circular like the edge of a droplet or it may show irregularities such as rounded projections, notches, and threadlike or rootlike projections.

Surface Texture. Depending on the species, the colony surface may be **smooth** (shiny, glistening); **rough** (dull, granular, or matte); or **mucoid** (slimy or gummy). Certain species have colonies posessing a highly **wrinkled** surface.

For a pure culture, all the colonies on the plate should have a similar type of surface; however, you should bear in mind that some pure cultures may exhibit surface variation. One of the commonest variations is known as the **S → R variation.** This is due to the presence of mutant cells that give rise to some rough (R) colonies in a population that otherwise produces smooth (S) colonies. Some R mutants produce rough colonies because they lack the ability to make capsules, or, if the species is Gram-negative, they may no longer be able to form O antigens.

For several species of pathogenic bacteria, the surface texture of colonies may bear a relation to virulence. For instance, S colonies of *S. pneumoniae* or of *Salmonella* species are usually virulent, whereas R colonies are not. On the other hand, for strains of *Mycobacterium tuberculosis* a rough surface showing serpentine cords is usually a good indicator of virulence.

Elevation. Depending on the species, colonies may be **thin** to **thick**, and the surface may be **flat** or it may exhibit varying degrees of **convexity**.

Consistency. This can be determined by touching a transfer needle to the colony. Some bacterial species form colonies having a **butyrous** or butterlike consistency. Others may form colonies that are **viscous**, **stringy**, or **rubbery**; in the latter type the whole colony, rather than just a portion of it, may come off the agar surface with the transfer needle. Still other species may form **dry**, **brittle**, or **powdery** colonies that break up when touched with the needle.

Optical features. Colonies may be **opaque**, **translucent**, or **opalescent**.

Chromogenesis or Pigmentation. Some bacterial species produce and retain water-insoluble pigments intracellularly, thus causing the colonies to become colored (pigmented). Some species which form pigmented colonies are:

Flectobacillus major	Pink
Serratia marcescens	Red
Chromobacterium violaceum	Violet
Staphylococcus aureus	Gold
Micrococcus luteus	Yellow
Derxia gummosa	Brown
Bacteroides melaninogenicus	Black

Some colonies produce pigments that are **water-soluble**; these diffuse into the surrounding agar and stain it. For instance, *Pseudomonas aeruginosa* forms a blue water-soluble pigment called pyocyanin. Some pigments are only sparingly water-soluble and may precipitate in the medium. For example, *Pseudomonas chlororaphis* forms a pigment called chlororaphin which accumulates in the form of green crystals around the colonies.

Certain water-soluble pigments are **fluorescent**; i.e., the agar medium around the colonies glows white or blue-green when exposed to ultraviolet light. For example, *P. aeruginosa* produces not only the nonfluorescent pigment pyocyanin but also a fluorescent pigment, pyoverdin.

For a bacterial strain to exhibit its characteristic pigmentation, special media, incubation temperatures, or other conditions may be required. For instance, *Mycobacterium kansasii* forms a characteristic yellow pigment (β-carotene) only when the colonies are exposed to light.

Several types of bacterial colonies are shown in Fig. 8-10.

Characteristics of Broth Cultures

1 ***Amount of growth.*** Scanty, moderate, or abundant.
2 ***Distribution and type of growth.*** The growth may be uniformly distributed throughout the medium (evenly turbid). Alternatively, it may be confined to the surface of the broth as a scum or film (pellicle), or it may accumulate as a sediment, which may be granular or viscous.

The scheme for interpreting the appearance of bacterial growth has been described in some detail to emphasize the fact that many differences in cultural characteristics do occur among bacteria. With experience, familiarity with such characteristics becomes very helpful as a guide for the recognition of major groups of bacteria. Too often students pay little attention to these features of

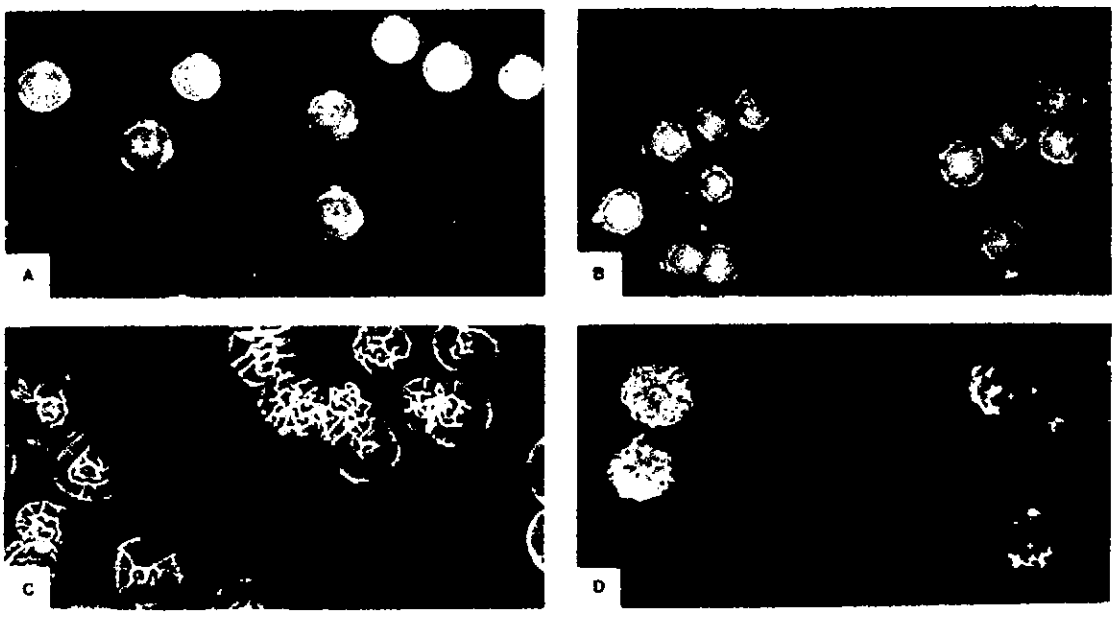

Figure 8-10. Bacterial colonies illustrating differences in characteristics. (A) Circular, raised, smooth surface; (B) circular, raised, finely granular surface; (C) irregular edge, flat, elevated folds in surface; (D) undulate edge, raised, irregularly elevated surface. *(Courtesy of Naval Biological Laboratory.)*

bacterial growth and thus deprive themselves of much useful information in the laboratory study of cultures.

To further emphasize the importance of cultural characteristics, suppose that we have prepared plate and broth cultures of an unidentified strain designated as strain 24. The colonies are irregular and raised and appear dry, with a roughened, granular surface. When we touch a colony with a transfer needle it proves to be brittle, and when a portion of the colony is removed it will not emulsify easily when spread in a drop of water. Growth in broth cultures occurs mainly in the form of a heavy surface pellicle, and the medium below the pellicle is only slightly turbid. Familiarity with the cultural appearance of bacteria would suggest that strain 24 might be an acid-fact bacterium *(Mycobacterium)*. Additional tests must be performed to verify this possibility, but the cultural characteristics have provided a clue to the type of organism we are working with.

QUESTIONS

1 Devise an enrichment procedure for an aerobic bacterial species that can use methane gas as a sole carbon and energy source.

2 During an epidemic of meningitis caused by *Neisseria meningitidis*, many people become healthy carriers (i.e., harbor the organism in their nasopharynx but do not have meningitis) and can spread the organism by coughing and sneezing. Suppose you are given the task of determining how many people in the epidemic region are healthy carriers. You soon discover that the human nasopharynx is inhabited by many different kinds of microorganisms. However, you learn from selected references that *N. meningitidis*

is resistant to the chemotherapeutic agents vancomycin, colistin, and nystatin but is susceptible to penicillin. On the basis of this information devise a selective medium that could help you in your task.

3 What selective procedure would you use in the process of isolating from a soil sample *(a)* an endospore-forming organism, *(b)* a nonsporeforming gliding organism, *(c)* a psychrophilic organism?

4 Give three examples of selective mechanisms occurring in nature that lead to the predominance of particular kinds of bacteria.

5 Distinguish between the meanings of the terms mixed culture, pure culture, clone, and strain.

6 Compare the advantages and disadvantages of the various techniques for the isolation of microorganisms in pure culture.

7 What are the advantages and disadvantages of the various methods for preservation of pure cultures?

8 Why have organizations been established to maintain pure cultures? Of what use are such collections?

9 What difficulty might exist in subculturing the colony of a desired organism from a selective agar medium? What additional steps should be taken to help assure culture purity?

10 How could you acquire a subculture of the type strain of *Streptococcus lactis* so that you could compare its characteristics with another strain that you have isolated from milk?

11 What general categories of pigments are produced by bacteria? For each category give an example of an organism that makes such a pigment.

12 Give several reasons why industrial biological companies maintain large stock-culture collections.

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PART THREE MICROBIAL PHYSIOLOGY AND GENETICS

The many parts of a cell

Lewis Thomas, in his fascinating book *The Lives of a Cell*, describes the interesting protozoan *Myxotricha paradoxa* that lives within the digestive tract of Australian termites. Therein it thrives symbiotically, enzymatically degrading the tough woody cellulose down to assimilable carbohydrate for the termites. It is also a very motile creature, able to swim from place to place by means of surface appendages. A cursory examination would deduce these appendages to be cilia or flagella. However, upon careful scrutiny, these locomotor organelles turn out to be fully formed, whole bacterial spirochetes that have attached themselves over the entire surface of the protozoan. It is the sum total of the individual movements of the spirochetal cells that contributes to the locomotion of the protozoan. Furthermore, close to the point of attachment of the spirochetes and embedded in the surface of the protozoan, as well as elsewhere in the protozoan cytosol, are oval organelles. Under the electron microscope, these turn out to be true bacteria existing symbiotically with the spirochetes and the protozoan and probably helping in the degradation of cellulose by the excretion of enzymes. Thus these various creatures thrive together in an ecosystem (the termite digestive tract), each type contributing in its own way to the benefit of the whole.

It now appears that the existence of a creature like *Myxotricha paradoxa* is not so strange after all. There is a theory in biology called the **endosymbiotic theory.** It ascribes the origin of the eucaryotes to a series of endosymbioses, with the endosymbionts becoming the organelles of the host cell. One of the most articulate proponents of this theory is Lynn Margulis of Boston University. According to this theory, the "modern" eucaryotic nucleated cell is the result of the "coming together" of endosymbionts and their host in the evolutionary process. There was presumed, in primordial time, a moderately large, amoeboid, heterotrophic, anaerobic procaryote that customarily fed on smaller procaryotes. Some of the latter were aerobic organisms that were not digested: they became stabilized as endosymbionts and continued to function as aerobes within an anaerobic host. In this capacity they conferred the advantages of aerobic respiration upon the anaerobic host. With time, they became so well integrated with the host cell that they became the mitochondria of the host. In the same way, the photosynthetic cyanobacteria integrated so well with their procaryotic host cell in evolutionary time to become the chloroplasts of the first plant eucaryotic cell. Similarly, flagellated procaryotes, like the ones attached to *M. paradoxa*, became integrated with the host cell to become its locomotor organelles. The centrioles and centromeres of the mitotic process also presumably had similar origins (much of the symbiont parts having been lost except for the basal portions including the DNA).

Much of the credence of the endosymbiotic theory rests on two contemporary findings: demonstrations that many organelles like the mitochondrion have their own DNA, and the widespread occurrence of symbiosis, including endosymbiosis. The metabolic and genetic processes carried out by procaryotic cells—progenitors of the nucleated cell—form the subject matter of this part of the text.

Preceding page. DNA content of a T2 phage appears as a single thread in this electron photomicrograph. Measurements of the DNA thread system indicate a length of 49 ± 4 μ. The phage "ghost" (the flask-shaped object at the center) is about 0.1 μ long. *(From A. K. Kleinschmidt et al., in Biochim Biophys Acta, 61:857–864, 1962.)*

Chapter 9 Enzymes and Their Regulation

OUTLINE

A cell must be capable of performing a multitude of chemical changes in order to stay alive, grow, and reproduce. It may have to alter complex nutrients in the medium before they can enter the cell. It must effect additional changes on the nutrients once they are in the cell. These nutrients are broken down chemically to provide energy for the cell and simple precursors for the synthesis of cell material. The chemical changes involved are exceedingly complex, considering the diversity of materials used as foods on the one hand and the variety of substances synthesized into cell constituents on the other. How does the cell accomplish these changes? The answer lies in the activity of **enzymes**, substances present in the cell in minute amounts and capable of speeding up chemical reactions associated with life processes. Any impairment of enzyme activity is reflected by some change in the cell, or even by death. There can be no life without enzymes.

Within a living cell, enzymes function in sequences of reactions called **pathways**. For a cell to grow normally, it is essential that the flow of chemical substances, or **metabolites**, through these pathways be under a high degree of regulation or control. This regulation ensures that no products are deficient or in excess, and is exerted either on enzyme activity or on enzyme synthesis. Our purpose in this chapter is to describe some of the major characteristics of enzymes and how the enzymes are regulated.

SOME CHARACTERISTICS OF ENZYMES

Certain substances in small amounts have the unique capacity of speeding up chemical reactions without themselves being altered after the reaction; they accelerate the velocity of the reaction without necessarily initiating it. Substances that behave in this manner are called **catalysts** or **catalytic agents**. For example, hydrogen and oxygen do not combine to any appreciable extent under normal atmospheric conditions. If, however, the two gases are allowed to touch colloidal platinum, they react instantaneously to produce water. The platinum greatly increases the speed at which this reaction takes place without being used up in the reaction. Nor do these substances ordinarily have any effect on the equilibrium of a reversible chemical reaction; they merely speed it up until it reaches equilibrium. They also exhibit specificity: a given substance will only affect a certain type of reaction. Enzymes are catalysts. However, unlike platinum, which is inorganic, enzymes are organic compounds produced by living organisms. Thus we may define an enzyme as an organic catalyst produced by a living cell. For example, in the cell the oxidation of a fatty acid to carbon dioxide and water takes place smoothly and rapidly within a narrow range of temperature and pH because of enzymes. Without these organic catalysts, as in a test tube, this same process requires extremes of pH, high temperatures, and corrosive chemicals.

Although all enzymes are initially produced in the cell, some are secreted through the cell wall and function in the cell's environment. Thus we recognize two types of enzymes on the basis of site of action: **intracellular enzymes**, or **endoenzymes** (functioning in the cell), and **extracellular enzymes**, or **exoenzymes** (functioning outside the cell). The principal function of the extracellular enzymes is to perform necessary changes on the nutrients in the medium to allow them to enter the cell. Intracellular enzymes synthesize cellular material

The Origin of the Word *Enzyme*

The word *enzyme* was coined in 1878 by Kühne from a Greek term meaning "in yeast." Earlier, enzymes were referred to as "ferments" because their actions were similar to yeast fermentation. A major controversy on this subject occurred between Pasteur and the great German chemist Liebig. Liebig maintained that fermentations were caused by chemical substances not associated with living cells, but Pasteur held that the fermentation process was inseparable from living cells. As we know today, neither position was strictly wrong. The Pasteur-Liebig controversy was resolved in 1897 by Büchner, who demonstrated that a cell-free juice, prepared from yeasts by filtration, contained active enzymes.

and also perform catabolic reactions which provide the energy required by the cell.

The general characteristics of enzymes are the same whether the enzymes are produced by the cells of microbes, by people, or by other forms of life. In fact, cells from organisms that are very different may contain some enzymes that are identical or at least have identical functions.

In any one cell there are a thousand or more different enzymes. The enzymes present in a microbial cell are determined by the environmental conditions and by the cell's genetic constitution. This means that at any one moment, the enzyme content of a microbe is a reflection of the manner in which that cell copes with the environment.

CHEMICAL AND PHYSICAL PROPERTIES OF ENZYMES

Enzymes are proteins or proteins combined with other chemical groups. Enzymes therefore possess the properties characteristic of proteins: they are denatured by heat, are precipitated by ethanol or high concentrations of inorganic salts like ammonium sulfate, and do not dialyze (pass through semipermeable membranes).

Many enzymes consist of a protein combined with a low-molecular-weight organic molecule called a coenzyme. The protein portion in this instance is referred to as the apoenzyme. When united, the two form the complete enzyme, identified as the holoenzyme, as shown below:

Apoenzyme	+ coenzyme	→ holoenzyme
Inactive	Inactive	Active
Protein	Organic molecule	
High molecular weight	Low molecular weight	
Nondialyzable	Dialyzable	

The integral part of some coenzymes is a vitamin. Several of the B vitamins, as listed in Table 9-1, have been identified as the main components of coenzymes.

A specific example of the relation of a vitamin to a coenzyme (vitamin B_6 in pyridoxal phosphate and pyridoxamine phosphate) is shown in Fig. 9-1.

In some instances the nonprotein portion of an enzyme may be a metal, e.g., iron in the enzyme catalase. The metal may be tightly bound to the protein or loosely bound and easily dissociable, depending on the specific enzyme. Many enzymes require the addition of metal ions (Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, etc.) in order to be "activated." It is assumed that these metal ions function in combination with the enzyme protein, and they are regarded as inorganic coenzymes, or cofactors. Sometimes both a cofactor and a coenzyme (organic) are required before an enzyme becomes active.

A large number of enzymes have been extracted from cells and, by a combi-

Table 9-1. Some vitamins and their coenzyme forms

Vitamin	Coenzyme
Thiamine (B_1)	Cocarboxylase
Riboflavin (B_2)	Riboflavin adenine dinucleotide
Niacin	Nicotinamide adenine dinucleotide
Pyridoxine (B_6)	Pyridoxal phosphate
Folic acid	Tetrahydrofolic acid

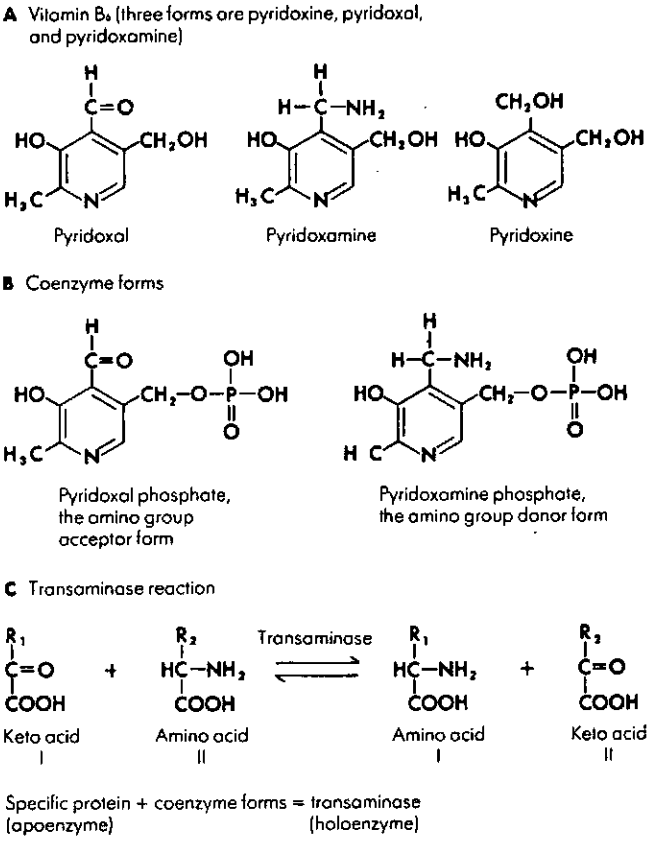


Figure 9-1. (A) Vitamin B_6 exists in three forms. (B) Coenzyme forms of the vitamin. (C) The transaminase reaction.

nation of physical and chemical techniques, have been obtained in chemically pure form. Urease was the first enzyme isolated in crystalline form; J. B. Sumner of Cornell University received the Nobel Prize in 1947 for this achievement. The protein nature of enzymes was accepted widely only after John Northrop and his colleagues, in the 1930s, crystallized pepsin and trypsin and found them also to be proteins. Enzymes are very large protein molecules; molecular weights from approximately 10,000 to 1 million have been determined for different enzymes. The enzyme catalase, which splits hydrogen peroxide into water and oxygen, has a molecular weight of 250,000. Hydrogen peroxide, on the other hand, has a molecular weight of 34.

Enzyme molecules are exceedingly efficient in accelerating the transformation of **substrate** (substance acted on by enzymes) to end product. A single enzyme molecule can effect the change of as many as 10,000 to 1 million molecules of substrate per minute. This ability, together with the fact that the enzyme is not consumed or altered in the reaction, reveals why very small quantities of enzymes are sufficient for cellular processes.

Enzymes are vulnerable to various environmental factors. Their activity may be significantly diminished or destroyed by a variety of physical or chemical

conditions, as will be shown later, but great differences exist among enzymes in this respect. Some may become inactivated by very minor alterations in the environment. The destruction of enzymes by physical or chemical agents results in a loss to the cell of the functions performed by the enzymes—a further revelation of their essential nature.

The two most striking characteristics of enzymes are (1) their high catalytic efficiency and (2) their high degree of specificity for substrates. One ounce of pure crystalline pepsin, for instance, can digest nearly two tons of egg white in only a few hours; whereas it would take 10 to 20 tons of strong acid 24 to 48 h at elevated temperatures without the enzyme. An enzyme molecule may transform 10^2 to 10^6 molecules of substrate per minute. A single enzyme may react with only a single substrate or, in some instances, with a particular chemical grouping on chemically related substrates. Essentially, this means that cells usually produce different enzymes for every compound they metabolize. Furthermore, each enzyme causes a one-step change in the substrate. For example, yeasts ferment glucose to alcohol and carbon dioxide. The initial reactants and the final products of the reaction are shown in the following equation:

$$\underset{\text{Substrate}}{\text{Glucose}} + \underset{\text{Source of enzymes}}{\text{yeast cells}} \rightarrow \underset{\text{End products}}{\text{alcohol} + \text{carbon dioxide}}$$

This transformation is accomplished not by a single enzyme but by a group of enzymes, an **enzyme system**. More than a dozen single enzymes work in sequence, each performing a single specific change in the product formed by the preceding enzyme reaction. The last reaction in the system yields the final products. (Examples of enzyme systems will be found in Chap. 10.)

Today over 1,000 different enzymes are known, and well over 150 have been crystallized. Many more remain to be discovered. Exciting new areas of enzyme involvement have been discovered in recent years, including the self-regulating nature of many enzyme systems, the genetic control of enzyme function and synthesis, and the role of enzymes in development and differentiation. E.g., Thomas Cech and his colleagues at the University of Colorado reported in the journal *Cell* (31:147–157, 1982) the discovery of a biochemical reaction mediated by RNA in the absence of protein. The notion of catalytic RNA unfolds new perspectives on the basic components required for life, and also on the evolution of life. This finding has been described as "one of the most exciting discoveries of the decade."

NOMENCLATURE OF ENZYMES

In 1956 the International Union of Biochemistry established an international Commission on Enzymes to work on a systematic arrangement and nomenclature for the large and rapidly increasing number of enzymes. Its recommendations were first published in 1961, and they have been adapted and are universally used (see References). Briefly, these recommendations follow.

How Enzymes Are Named and Classified

Except for a few of the originally studied enzymes such as pepsin, renin, and trypsin, most enzyme names end in *ase*, e.g., succinate dehydrogenase. When naming a complex of several enzymes on the basis of the overall reaction catalyzed by it, the word *system* should be used. e.g., the succinate oxidase

system, which catalyzes the oxidation of succinate by O_2 and consists of succinate dehydrogenase, cytochrome oxidase, and several intermediate carriers. For classification, only single enzymes, and not enzyme systems, are considered.

The type of chemical reaction catalyzed is the basis for the classification and naming of enzymes, because it is this specific property that distinguishes one enzyme from another. Two names are recommended for each enzyme: a working or trivial name and a systematic name. The trivial name is shorter and more convenient to use; in many cases, it is the name already in current use. The systematic name is formed in accordance with definite rules, identifies the substrate or substrates, and specifies the type of reaction catalyzed. Furthermore, each enzyme is given an identifying classification number in addition to its trivial and systematic names. For example, E.C.1.1.1.1 is the Enzyme Commission number for alcohol dehydrogenase (trivial name); its systematic name is alcohol:NAD oxidoreductase. According to international classification, enzymes are grouped into six major classes (see Table 9-2). These groupings are based on the type of chemical reaction the enzymes catalyze.

Many enzymes occur in different structural forms but possess identical (or nearly so) catalytic properties. Such enzymes are called isozymes or isoenzymes. The present nomenclatural system makes no provision for structural diversity with similar catalytic function.

THE NATURE AND MECHANISM OF ENZYME ACTION

Most enzyme reactions may be represented by the following overall reaction:

$$\text{Enzyme E} + \text{substrate S} \leftrightarrows \text{enzyme-substrate complex ES} \leftrightarrows \text{product P} + \text{enzyme E}$$

The enzyme E and substrate S combine to give an enzyme-substrate complex ES, which then breaks up to yield the product P. The enzyme is not used up in the reaction but is released for further reaction with another substrate molecule. This process may be repeated many times until all the available substrate molecules are consumed. However, equilibrium, a steady-state condition, is reached when the forward reaction rates equal the backward reaction rates. This is the basic equation upon which most enzymatic studies are based.

Table 9-2. Major classes of enzymes

Class No.	Class	Catalytic Reaction
1	Oxidoreductases	Electron-transfer reactions (transfer of electrons or hydrogen atoms)
2	Transferases	Transfer of functional groups (functional groups include phosphate, amino, methyl, etc.)
3	Hydrolases	Hydrolysis reactions (addition of a water molecule to break a chemical bond)
4	Lyases	Addition to double bonds in a molecule as well as nonhydrolytic removal of chemical groups
5	Isomerases	Isomerization reactions (reactions in which one compound is changed into an isomer, i.e., a compound having the same atoms but differing in molecular structure)
6	Ligases	Formation of bonds with cleavage or breakage of ATP (adenosine triphosphate)

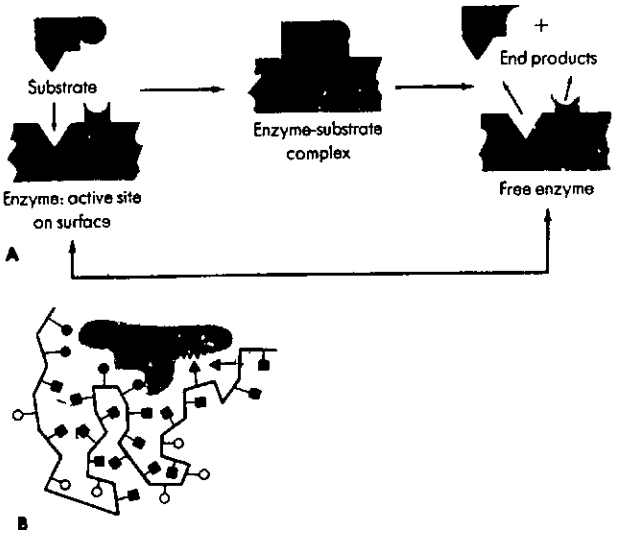


Figure 9-2. (A) Enzyme-substrate reaction, depicted schematically. The substrate is attracted to some site on the surface of the enzyme molecule. The chemical groupings of the substrate are strained by this attraction, and cleavage results. The cleavage products are released from the enzyme, and the enzyme is free to combine with more substrate and continue the process. (B) The drawing shows a schematic representation of an active site. Solid circles represent "contact" amino acid residues whose fit with substrate determines specificity; triangles are catalytic residues acting on substrate bond, indicated by a jagged line; open circles are nonessential residues on the surface; squares are residues whose interaction with each other maintains the three-dimensional structure of the protein. (*Redrawn from D. E. Koshland, Jr., "Correlation of Structure and Function in Enzyme Action," Science,* **142:**1533–1541, 1963.)

There is a high chemical affinity of the substrate for certain areas of the enzyme surface called the active sites. A strain or distortion is produced at some linkage in the substrate molecule, making it labile (unstable), and it undergoes a change determined by the particular enzyme. The altered substrate molecule then lacks affinity for the active site and hence is released. The enzyme is then free to combine with more substrate to repeat the action (see Fig. 9-2). Almost all intracellular enzymes have more than one active site per molecule. For example, lactate dehydrogenase has four. In contrast, α-chymotrypsin, like other secreted extracellular enzymes, has only one active site.

The main function of an enzyme is to lower the activation energy barrier to a chemical reaction. Activation energy refers to the amount of energy required to bring a substance to the reactive state. The enzyme combines with the substance (substrate) to produce a transition state requiring less activation energy for the chemical reaction to proceed. Figure 9-3 illustrates this concept.

The above discussion applies to substrates undergoing degradation and being utilized for energy. The same type of explanation could be used to describe synthesis, or building up of complex compounds from simpler ones. For example, two different molecules might become attached to adjacent sites on the enzyme surface. A unique activation by the enzyme would lead to establishment of a bond between the two molecules, thereby creating a new compound from the original two substrates. The product has little affinity for the active sites and hence is released, thereby freeing the active sites to repeat the process with another two molecules of substrate.

The active site on an enzyme surface is actually a very small area, which means that large regions of the enzyme protein (which has hundreds of amino acids) do not contribute to enzyme specificity or enzyme action (see Fig. 9-2). Thus only a relatively few amino acids are directly involved in the catalytic process—perhaps even less than five! It should also be emphasized that the "fit" between an active site of the enzyme surface and the substrate is not a static

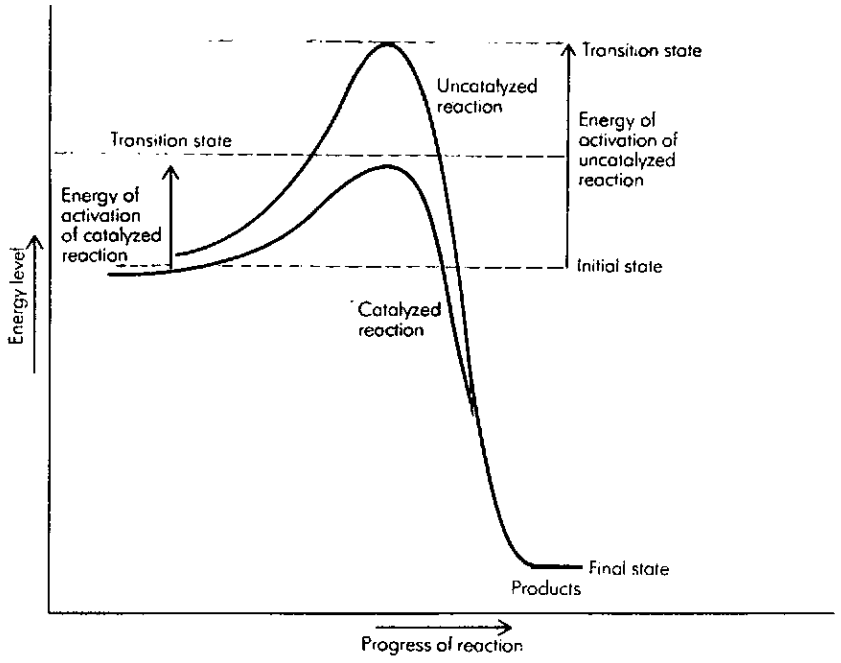


Figure 9-3. The main function of an enzyme is to lower the activation energy, the energy necessary to attain the transition state of a chemical reaction. As can be seen, an enzyme-catalyzed reaction has a lower activation energy and so requires less energy for the reaction to proceed.

one. Rather it is a dynamic interaction in which the substrate induces a structural change in the enzyme molecule, as a hand changes the shape of a glove. This phenomenon is illustrated in Fig. 9-4.

CONDITIONS AFFECTING ENZYME ACTIVITY

Among the conditions affecting the activity of an enzyme are the following:

1 Concentration of enzyme
2 Concentration of substrate
3 pH
4 Temperature

Generally speaking, there is an optimum relation between the concentrations of enzyme and substrate for maximum activity. But in order to study the effect of increasing enzyme concentrations upon the reaction rate, the substrate concentration must be in *excess*. This means that the reaction must be independent of the substrate concentration so that any variation in the amount of product formed is a function only of the enzyme concentration present. This is illustrated in Fig. 9-5.

If the amount of enzyme is kept *constant* and the substrate concentration is then gradually increased, the velocity of reaction will increase until it reaches a maximum. Any further increases in substrate concentration will not increase the reaction velocity. This is shown in Fig. 9-6.

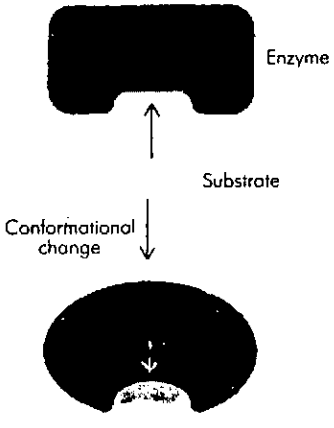


Figure 9-4. Induced fit of the active site of the enzyme molecule to the substrate molecule.

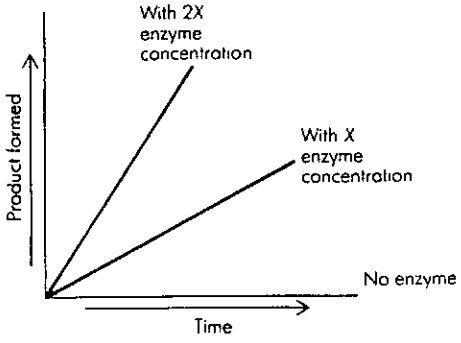


Figure 9-5. Effect of enzyme concentration on rate of enzyme activity (in the presence of excess substrate). These reactions shown are said to be "zero order" because the rates are *independent* of substrate concentration. The formation of product proceeds at a rate which is linear with time (e.g., doubling the assay time results in doubling the amount of products formed).

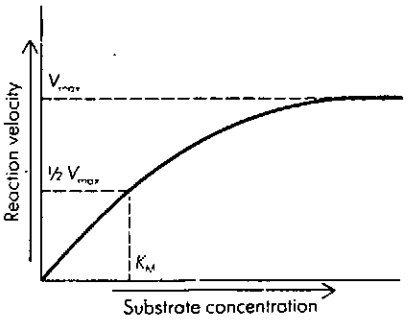


Figure 9-6. Effect of substrate concentration on enzyme activity. When the maximum velocity has been reached, all of the available enzyme has been converted to ES, the enzyme-substrate complex (the point is designated V_{max} on graph). K_m shown refers to the Michaelis constant and is defined as the substrate concentration at one-half the maximum velocity.

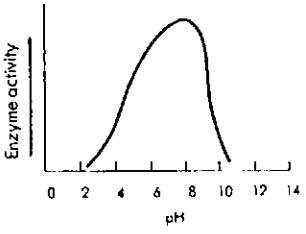


Figure 9-7. Effect of pH on enzyme activity. Maximum activity occurs at a particular pH, and deviations from it result in decreased activity. (Not all enzymes exhibit optimum activity at the same pH.)

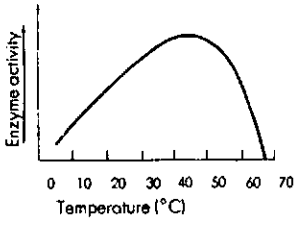


Figure 9-8. Effect of temperature on enzyme activity. Starting at a low temperature, the enzyme activity increases with increasing temperature until the optimum activity is reached. Further increases in temperature result in decreased enzyme activity and eventual destruction of the enzyme. (Not all enzymes exhibit optimum activity at the same temperature.)

Each enzyme functions optimally at a particular pH and temperature (Figs. 9-7 and 9-8). From these illustrations, it is clear that deviations from the optimal conditions result in significant reduction of enzyme activity. This is characteristic of all enzymes. Extreme variations in pH can even destroy the enzyme, as can high temperatures; boiling for a few minutes will **denature** (destroy) most enzymes. Extremely low temperatures, for all practical purposes, stop enzyme activity but do not destroy the enzymes. Many enzymes can be preserved by holding them at temperatures around 0°C or lower.

We know that the growth of microorganisms is influenced by a variety of physical and chemical conditions. Since enzymes are responsible for catalyzing the reactions associated with life processes, it follows that the conditions mentioned above affect the enzymes and thereby the growth response. Just as there is an optimal pH and temperature for growth, there is also an optimal pH and temperature for the activity of each enzyme and for the production of each enzyme by the cell. This does not mean, however, that the values are the same for each enzyme in the same cell; they may not be. The reason for the differences is that during growth, activity or response is measured in terms of the total activities required for growth when all enzymes and enzyme systems are functioning harmoniously in the cell. Optimum conditions must be estimated in terms of what is best for the entire cell. In assessing the activity of an isolated and purified specific enzyme, the situation is entirely different. The enzyme is no longer in its normal environment and thus is not influenced by, or integrated into, the multitude of reactions which occur within the cell. Hence, the optimum conditions for activity of any one enzyme in vitro are not necessarily optimum for the same enzyme in vivo.

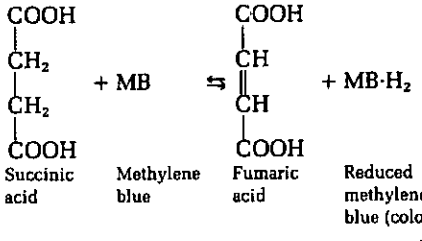
INHIBITION OF ENZYME ACTION

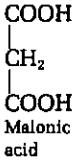
The activity of an enzyme can be inhibited (slowed down or stopped) by chemical agents in several different ways. We are concerned here with chemical substances that inhibit in a more subtle fashion than denaturation (destruction) of the protein portion of the enzyme. Specific information about how these chemical agents (naturally occurring or otherwise) exert their detrimental action contributes to our knowledge of how enzymes work, suggests new types of chemicals which may be useful for inhibiting microorganisms, and in other ways contributes to our understanding of life processes.

Enzyme inhibition may be classified as either nonreversible or reversible. Nonreversible inhibition usually involves the modification or inactivation of one or more functional groups of the enzyme so that it is no longer active.

There are two major types of reversible inhibition, namely, competitive inhibition and noncompetitive inhibition. Competitive inhibition can be reversed by increasing the substrate concentration, whereas noncompetitive inhibition cannot. We will present a few examples.

The enzyme succinic dehydrogenase accomplishes the transfer of hydrogen atoms from succinic acid to a suitable acceptor compound (in the reaction below, the acceptor, methylene blue, is a compound that does not occur naturally within a cell):





This reaction can be inhibited by chemical compounds which have a structure similar to that of succinic acid. One of these inhibitors is malonic acid, which has the structure shown in the margin. Because of its structural similarity to succinic acid, malonic acid attaches itself to the enzyme active site where succinic acid normally attaches. The succinic acid is "blocked out" by the malonic acid, but since the malonic acid is not activated by the enzyme, there is no reaction. In this example there is *competition* for the same active site by two different molecules (see Fig. 9-9). This type of enzyme inhibition is referred to as competitive inhibition and can be relieved simply by increasing the substrate concentration.

Certain chemical substances, such as cyanide, have a high affinity for metal

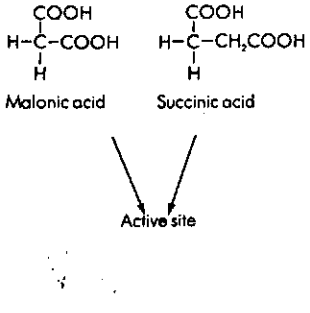


Figure 9-9. Competitive inhibition (schematic) between malonic acid and succinic acid. Note that each molecule has a structurally similar fragment. Since this portion of either molecule can fit or combine with the active site on the enzyme surface, there is competition between the two substrates for this site. Because this enzyme is specific for succinic acid, if the malonic acid occupies the site, further activity is blocked, as malonic acid is not changed by this enzyme.

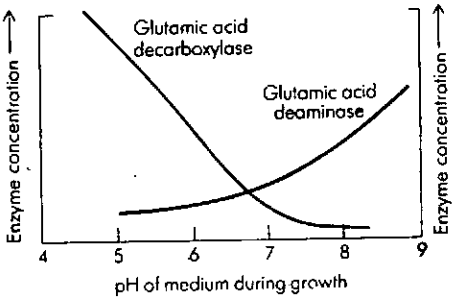


Figure 9-10. Variations in the concentrations of glutamic acid decarboxylase and glutamic acid deaminase present in *Escherichia coli* with variations in the pH of the medium during growth. Note that at a low pH (acidic medium) glutamic acid decarboxylase predominates and at a high pH (basic medium), glutamic acid deaminase predominates.

ions, and form complexes with the metal. As already mentioned, many enzymes require a metal ion for their activity. Cyanide is a strong inhibitor of iron-containing enzymes because it "ties up" the iron, depriving the enzyme of an essential component. Similarly, fluoride inhibits enzymes which require calcium or magnesium by binding these metals. (It may be noted that the relatively small amount of fluoride which we find in tooth enamel as a result of water fluoridation does not exert this inhibitory action in vivo.) But the most important noncompetitive inhibitors are naturally occurring metabolic intermediates that can combine reversibly with specific sites on certain regulatory enzymes, thus changing the activity of their catalytic sites. This type of enzyme inhibition is referred to as noncompetitive inhibition since the inhibitor is not competing with the substrate for an active site on the enzyme surface and therefore is not reversed by increasing substrate concentration.

CONDITIONS AFFECTING ENZYME FORMATION

The enzymatic content of animal-tissue cells is relatively constant, since they are in an environment which in terms of physical and chemical conditions is subjected to little change. But the bacterial cell is exposed to an ever-changing environment. For example, *Escherichia coli* may grow in an acid or alkaline medium (from pH 4.5 to pH 9.5), at room temperature or above body temperature, aerobically or anaerobically. Cells of *E. coli* grown at the extremes of these conditions do not contain the same kinds or amounts of enzymes (see Fig. 9-10). But the fact that the environment influences the formation of enzymes should not be misconstrued to mean that there is no consistent enzyme pattern for a given organism. Organisms manifest changes in reaction to environment only within certain limits. It is important to recognize their capacity for undergoing these changes when examining organisms. When organisms are studied physiologically, these studies must be performed under certain established conditions, which include the composition of the medium in which the cells are grown as well as the physical conditions during incubation.

DETERMINATION OF ENZYME ACTIVITY

The enzymatic activity of bacteria (or of any cells or tissues) can be determined by a variety of techniques. Some procedures require special, elaborate instruments; others require only a test tube and a few reagents. All are based on a few simple principles. In order to carry out a quantitative assay of enzyme activity, it is necessary to know the following:

1 The nature of the reaction catalyzed
2 What cofactors and coenzymes are required
3 The required concentrations of both substrate and cofactor or coenzyme
4 The optimum pH
5 The optimum temperature
6 A simple analytical method for determining the disappearance of substrate or the appearance of products of the reaction

The substrate concentration should be above the saturation level so that the initial reaction rate is proportional to enzyme concentration alone. Coenzymes and cofactors should also be added in excess. Doing this ensures that the true limiting factor is the enzyme concentration (at its optimum pH and temperature). Generally, measurement of reaction product formation is more accurate than measurement of the disappearance of substrate. Such determinations of enzymatic activity have provided us with a wealth of knowledge concerning cells and their chemical reactions.

ENZYME PREPARATIONS

The source of the enzymes used in any microbiological experiment may be one of the following: (1) cells in a growing culture, (2) cells removed from a growing culture and resuspended in a nonnutrient solution (resting cells, or nongrowing but viable cells), and (3) enzymes extracted from cells (cell-free enzyme preparation). Cell-free enzyme preparations may be of varying degrees of purity. They may be very crude extracts, as in the case when the material contains all the cellular contents recovered after fracturing the cell walls. However, this crude extract can be refined to obtain some particular fraction, e.g., ribosomal material, soluble cytoplasmic substance, or material of a certain molecular size range. In the last-mentioned instance, the objective may be to discover which subcellular entities house all the enzymes (enzyme system) responsible for certain cellular functions.

The manner in which each of these is used is as follows:

GROWING-CULTURE TECHNIQUE

1 Inoculation of bacteria into medium containing substrate.
2 Incubation of bacteria (1 or more days of growth).
3 Examination for change or disappearance of substrate and presence of end products.

RESTING-CELL TECHNIQUE

1 Growth of bacteria in a suitable medium.
2 Preparation of a resting-cell suspension. The cells are harvested from the medium by centrifugation and resuspended in a nonnutrient solution. The process is repeated to free the cells of all material from the medium and is known as **washing the cells.** The final suspension is referred to as a **resting-cell suspension.**
3 Addition of resting cells and substrate to special testing unit, e.g., Warburg apparatus or Thunberg tube.
4 Incubation.
5 Examination (or testing) for disappearance of substrate and appearance of end products.

CELL-FREE ENZYME TECHNIQUE

1 Preparation of concentrated resting-cell suspension.
2 Disintegration of cells by special techniques to release enzymes, e.g., grinding, sonic treatment. (Any remaining whole cells are removed by centrifugation or filtration to obtain cell-free enzymes.)
3 Addition of cell-free enzymes and substrate to special testing unit.
4 Incubation.
5 Examination (or testing) for disappearance of substrate and appearance of end products.

The growing-culture technique is used routinely for the characterization of the enzymatic activities of microorganisms. Results of such tests provide information necessary for their identification. The resting-cell technique and cell-free enzyme preparations are principally used in research work where the object is to determine how the organism accomplishes each specific change. They provide favorable tools for the meticulous examination of the events that occur when a substrate undergoes change.

REGULATION OF ENZYMES

A living cell contains upward of a thousand different enzymes, each of which is an effective catalyst for some chemical reaction. But these enzymes act together in a coordinated manner so that all the chemical activities in a living cell are integrated with one another. One consequence of this enzyme coordination is that the living cell synthesizes and degrades materials as required for normal growth and metabolism.

The control of cellular metabolism ultimately is accomplished by the regulation of enzymes. In a microbial cell, such as a bacterium, the existence of cellular regulatory mechanisms is all the more important because of the absence of supracellular controls, such as neural and hormonal controls, which are present in the tissue cells of higher organisms. Microorganisms have evolved a variety of enzyme regulatory mechanisms which accommodate the changing needs of the microbial cell in a changing environment.

MECHANISMS OF REGULATION OF ENZYMES

Within the cell, there are two different regulatory mechanisms: the regulation of enzyme activity and the regulation of enzyme synthesis. Both mechanisms share the following properties:

1 They are mediated or governed by low-molecular-weight compounds molecules). These are either formed in the cell during metabolism or are found in the environment.
2 They involve the participation of a special class of control proteins. Such proteins are mediators of metabolic change as directed by the small effector molecules. There are generally two types of control proteins, namely **allosteric enzymes** and **regulatory proteins.**

The activities of allosteric enzymes are enhanced or inhibited by combination with their effector molecules. (Allosteric enzymes are so called because the site on the enzyme molecule where an effector molecule acts *is different from* the

catalytic site. It is called the allosteric site, and it regulates the activity of the enzyme. Allosteric enzymes are generally larger and more complex than simple enzymes.) Regulatory proteins have no catalytic activity, but they modulate the biosynthesis of enzymes by attaching to the bacterial chromosome at specific sites and thus regulate gene expression. This attachment is also affected by the binding of small effector molecules.

REGULATION OF ENZYME ACTIVITY

Metabolic pathways that supply energy, precursor substances (building blocks), and reduced compounds for other biosynthetic processes are usually controlled by modulation of enzyme activity (rather than by control of enzyme synthesis). However, not every enzyme in each pathway needs to be controlled. But in each enzyme system (group of enzymes working together to carry out a given metabolic process) there is at least one enzyme that is controlled and sets the rate of the overall sequence. Different effector molecules modulate an enzyme's activity depending on the need of the particular organism either for energy or for precursors used in biosynthesis.

Energy-Link Control

In this type of regulation the effector molecules involved are adenylates, such as adenosine triphosphate (ATP), or other purine or pyrimidine nucleotides. Some enzymes appear sensitive to the absolute concentrations of ATP, adenosine diphosphate (ADP), or adenosine monophosphate (AMP); others seem to respond to the ratio of a given two of these nucleotides. (Such compounds are discussed in Chap. 10.) In general, enzymes responsible for energy production are inhibited by energy charge (e.g., high concentration of ATP), while certain key biosynthetic enzymes are stimulated. Such regulation is very important in balancing energy production and energy utilization.

Feedback Inhibition

For a biosynthetic pathway with one major end product, such as an amino acid, control is exerted by the final concentration of the product in the cell. This product, the effector molecule, typically inhibits the activity of the enzyme in the first reaction of the biosynthetic pathway. This method of regulation is called feedback or end-product inhibition. By this device, microorganisms and other cells prevent the overproduction of low-molecular-weight intermediates such as amino acids and purine and pyrimidine nucleotides. Glutamine synthetase, for example, is influenced by feedback inhibition from nine different compounds.

Precursor Activation

Sometimes the precursor, or the first metabolite of a pathway, is the effector molecule. It activates, or stimulates the activity of, the last or later enzyme in the sequence of reactions of a pathway. This is called precursor activation.

A scheme showing the above three mechanisms in the regulation of enzyme activity is given in Fig. 9-11. Two techniques that have been especially useful in elucidating these mechanisms are the analysis of the properties of isolated enzymes (enzymes removed from cells) and observation of the behavior of mutant bacteria with specific enzyme defects. Mutant studies have been important in establishing that the regulatory mechanisms postulated from isolated enzyme studies do indeed function with the cell.

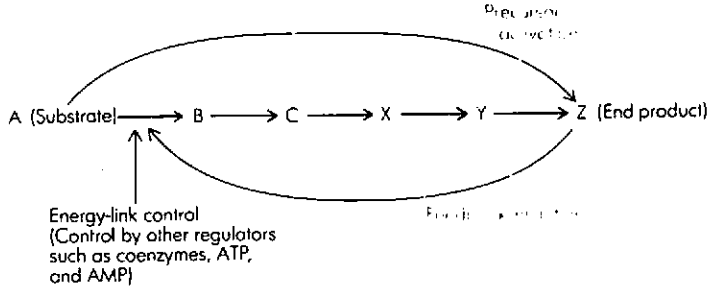


Figure 9-11. Some mechanisms for the regulation of enzyme activity by direct control through a coupling of the catalytic mechanism with other processes. Feedback inhibition, precursor activation, and energy-link control are shown. See text for fuller explanation.

General Processes Regulating Enzyme Activity

The cell can regulate enzyme activity by *less specific* or *more general* processes than those just described. These controls do not require the participation of effector molecules. The following cases illustrate this.

The cell membrane is a barrier to most hydrophilic molecules, but it has systems for the transport of specific compounds. Most of these systems require metabolic energy to function and therefore can be controlled by the availability of ATP.

The enzymatic reaction rate can be controlled by substrate concentration. As the substrate concentration increases, the reaction rate increases until a limiting value is reached when all the enzymes are saturated. And as the product accumulates, the reaction rate decreases. In addition, the concentrations of coenzymes and cofactors can exert controlling influences.

Control can also be effected by compartmentalization within the cell. Enzymes may be bound to various internal structures, especially membranes and macromolecules, so that enzymes and substrates are not in direct contact. The limiting physical access of enzymes to their substrates is more evident in the eucaryotic cell. Substrates can exist in separate pools because of their location within various membrane-bound organelles.

In some microbes, highly specific proteolytic (protein-degrading) enzymes, or proteases, break down other enzymes which are no longer required for metabolic reactions.

In a few instances, alteration in enzyme activity is brought about by a phenomenon called covalent modification of the regulatory enzyme molecule itself so that it can switch back and forth from an active to an inactive form. This modification is accomplished by the action of other enzymes. For example, the activity of phosphorylase *a* is increased by the hydrolytic action of phosphorylase phosphatase:

$$\underset{\text{(More active)}}{\text{Phosphorylase } a} + 2H_2O \xrightarrow{\text{phosphorylase phosphatase}} \underset{\text{(Less active)}}{\text{phosphorylase } b} + 2P_i$$

In turn, phosphorylase *b* can be changed back into the more active form, phosphorylase *a*, by another enzyme in the following manner:

$$\underset{\text{(Less active)}}{\text{Phosphorylase } b} + 2ATP \xrightarrow{\text{phosphorylase kinase}} \underset{\text{(More active)}}{\text{phosphorylase } a} + 2ADP$$

Figure 9-12 indicates the general processes involved in the regulation of enzyme activity.

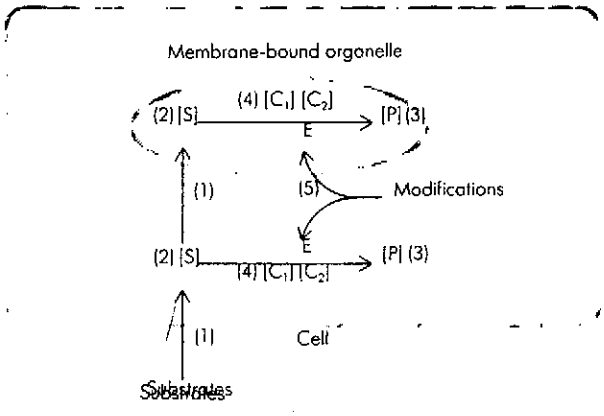


Figure 9-12. General processes for control of enzyme activity in a cell. (1) Membrane barrier controlled by energy (ATP) supply (*active transport*). (2) Substrate concentration [S]. (3) Product concentration [P]. (4) Concentrations of coenzymes [C_1] and cofactors [C_2]. (5) Modifications to enzymes (E) by other enzymes, e.g., proteases.

The regulation of enzyme activity provides a continuous control of metabolite concentration and is more responsive to fluctuations in a cell's environment than is regulation of enzyme synthesis, which we will now discuss.

REGULATION OF ENZYME SYNTHESIS

When the product of an enzymatic pathway is no longer required by a cell, the enzymes that catalyze the reactions of the pathway become unnecessary. Control mechanisms that modulate the enzymatic composition of the cell can come into play. This regulation is effected at the level of gene expression.

Induction and Repression of Enzyme Synthesis

Enzymes may be divided into two groups as follows:

1 ***Constitutive enzymes.*** These are always produced by the cell. Some of the enzymes of *glycolysis*, or sugar breakdown, are constitutive enzymes. They are found in essentially the same amounts regardless of the concentrations of their substrates in the medium.

2 ***Inducible enzymes.*** These are produced by the cell only in response to the presence of a particular substrate; they are produced, in a sense, only when needed. The process is referred to as **enzyme induction**, and the substrate (or a compound structurally similar to the substrate) responsible for evoking formation of the enzyme is an **inducer**. An example of an inducible enzyme is β-galactosidase; its inducer is the sugar lactose.

In reality, the distinction presented by these definitions is more operational than literal. Inducible enzymes are believed to exist in noninduced cells (in the absence of an inducer) but in relatively low quantities. Likewise, production of constitutive enzymes can often be enhanced when their specific substrates are present. The technique of evoking new enzyme formation through use of inducers has been extensively exploited in research designed to elucidate the mechanism of enzyme formation.

Induction is the process that occurs when an **inducer** (the effector molecule), which is either the substrate or a compound related to the substrate of the enzyme-catalyzed reaction, is required for enzyme synthesis to occur.

sion is the process that takes place when a regulatory protein, the **repressor**,

binds to a specific segment on the DNA called the **operator**, thereby preventing or repressing the synthesis of specific enzymes. Effector molecules, either products or related compounds of the particular reaction, act as **corepressors** in preventing synthesis of the enzyme. Corepressors function by combining with the repressor to form an active complex which combines with the operator gene to prevent messenger ribonucleic acid (mRNA) synthesis by the **structural genes** (see below). The repressor is also capable of combining with an inducer to form an inactive complex incapable of binding to the operator gene, in which case synthesis of mRNA can proceed. (The essential role of mRNA in protein synthesis is discussed in Chap. 11.) It is seen that the operator gene is one of the **regulator genes** on a deoxyribonucleic acid (DNA) chromosome. As discussed above, the operator gene prevents gene expression by **negative control** (see Fig. 9-13). In other cases, it can enhance gene expression by **positive control**. In this case, the repressor binds to the inducer, undergoes a conformational change, and is converted into an activator, which triggers gene expression.

To synthesize a specific protein, an organism must also have structural genes for that protein on the chromosome. A structural gene determines the amino acid sequence of a protein molecule (see Chap. 11). Unlike the regulator genes, structural genes do not control the rate at which enzymes are produced. Genetic control of the rate of enzyme synthesis is directed by the regulator genes.

In many bacteria the structural genes governing the biosynthesis of proteins are positioned in the exact order of the sequence of reactions in the particular metabolic pathway. This means that the ordering of the sequential reactions in the metabolic pathway is directed by the chromosome. A group of such consecutive genes forming an operational unit was named an **operon** by Francois Jacob and Jacques Monod. The operon includes both the structural and associated regulator genes. The regulator genes function primarily at the level of **transcription** (enzymatic process whereby the genetic information in DNA is used to specify a complementary sequence of bases in a mRNA chain) and not at the level of **translation** (process in which the genetic information present in a mRNA molecule directs the sequence of amino acids during protein synthesis). Clus-

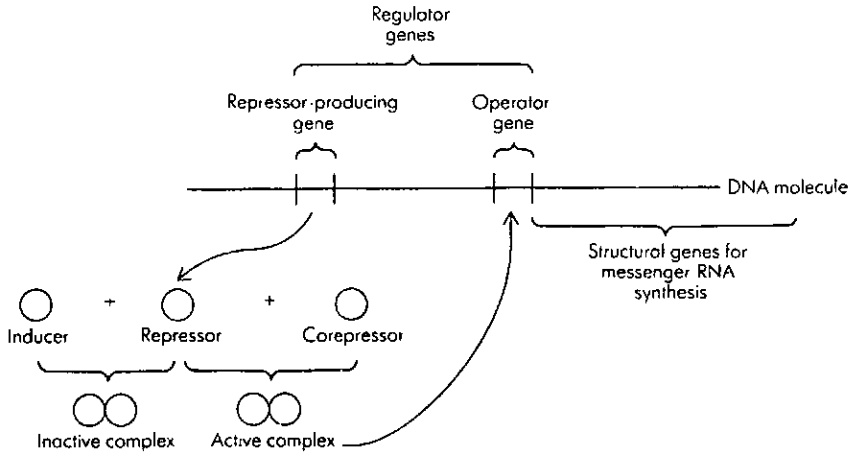


Figure 9-13. Regulation of enzyme synthesis by negative control.

tering of related genes provides a simple way of coordinating the response to a particular environmental change.

End-Product Repression

In biosynthetic pathways, the important metabolite in overall regulation is the end product and not the substrate of the pathway. In many bacteria, the addition of such an end product (e.g., an amino acid) to the culture medium results in inhibition of the *synthesis* of the enzymes of the particular pathway. This process is termed **end-product repression** or **feedback repression**.

Enzymes that are usually subject to end-product repression can be derepressed when the intracellular concentration of the end product falls to a low level.

We see then that biosynthetic pathways are subject to two types of feedback control: feedback inhibition of enzyme activity discussed previously, and end-product repression of enzyme synthesis. Both mechanisms are highly complementary, bringing about highly efficient regulation of biosynthetic pathways.

Catabolite Repression

Induction and end-product repression of enzyme synthesis are *specific* responses to a particular metabolite or closely related group of metabolites which we have called effector molecules. Two examples are induction of the lactose utilization system by some β-galactosides (see Chap. 12), and end-product repression of the enzymes of arginine biosynthesis by arginine. There are other important controls that are more *general* in their action and affect many operons. These controls allow cells to use the substrate that supports the most rapid rate of growth (the "preferred" substrate) in the presence of several others. A good example of this is the **glucose effect** (see Fig. 9-14). In a medium containing both glucose and lactose, *E. coli* uses glucose preferentially. Lactose is not metabolized until all the glucose is used up. The enzymes for catabolism of lactose are not synthesized (even though the substrate, or inducer, is present) until the glucose in the medium is exhausted. This type of regulation is called **catabolite repression** of enzyme synthesis and takes place whenever rapidly metabolizable energy sources are available in the presence of energy sources that are more slowly metabolized. Further, catabolite repression is not restricted to carbon sources such as glucose and lactose; enzymes degrading nitrogen-

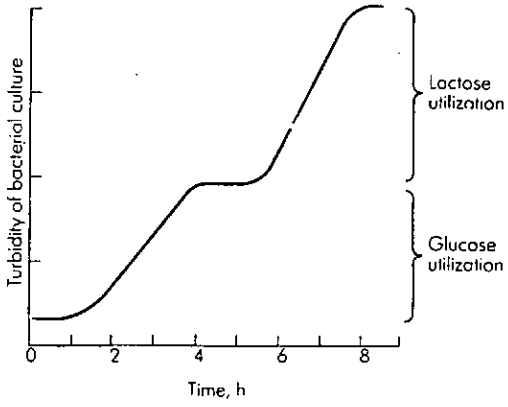


Figure 9-14. Glucose repression of lactose utilization. *E. coli* was inoculated into a simple salts medium containing equal amounts of glucose and lactose. Glucose was preferentially used first; its presence repressed the synthesis of enzymes for lactose utilization. The step-like curve with an intermediate lag phase illustrates the phenomenon known as *diauxy*.

containing metabolites are also subject to catabolite repression if preferred nitrogen sources such as ammonium ion or glutamine are present. It is apparent that catabolite repression allows cells to save energy by not expending it on the synthesis of enzymes used in less efficient pathways.

SOME DIFFERENCES BETWEEN PROCARYOTIC AND EUCARYOTIC ENZYME REGULATION

There are some aspects of enzyme regulation that are not the same between procaryotes and eucaryotes. For example, the difference between constitutive (basal) and fully induced enzyme levels is usually less for eucaryotes than it is for procaryotes. In the eucaryotic yeast *Saccharomyces cerevisiae* arginase is induced about 10-fold over the basal level while in *E. coli* the enzyme can be induced to about 100 times over basal level. In addition, in those eucaryotic organisms examined so far in sufficient genetic detail, there is no significant gene clustering into operons. Instead, the structural genes for enzymes in a specific biosynthetic pathway generally are scattered over many chromosomes and are not linked to each other. However, regulator genes can act at separate regulatory sites to coordinate enzyme synthesis.

QUESTIONS

1 Why are enzymes important to a cell?
2 Define the following terms: apoenzyme, holoenzyme, coenzyme, cofactor, and pathway.
3 Describe two most striking characteristics of enzymes.
4 What is meant by an enzyme system?
5 Distinguish between a trivial name and a systematic name in enzyme nomenclature.
6 What are the six major classes of enzymes and their catalytic reactions?
7 What is the main function of an enzyme and how is it accomplished?
8 What is an active site of an enzyme?
9 Discuss the conditions that affect the activity of an enzyme.
10 Distinguish between the following types of inhibition of enzyme action. nonreversible, reversible, competitive, and noncompetitive inhibition.
11 Differentiate between constitutive and induced enzymes.
12 Describe the technique you would use to identify a microorganism by its enzymatic activities.
13 What advantages might possibly be gained by performing studies of enzyme action by means of resting cells in preference to a growing culture? Cell-free extracts in preference to whole cells?
14 Why is the existence of cellular regulatory mechanisms more important in a microbial cell than in a cell of a higher organism?
15 Describe the two different regulatory mechanisms of enzymes in a cell and the two general properties that they share.
16 What are allosteric enzymes?
17 Describe three specific types of regulation of enzyme activity.
18 Explain what is meant by the negative control of enzyme induction.
19 Give the meaning of the term operon.
20 What are the two types of feedback control in biosynthetic pathways?
21 How does the phenomenon of diauxy reflect the meaning of catabolite repression?

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Chapter 10 Microbial Metabolism: Energy Production

OUTLINE

The multiplicity of processes performed by all biological systems can be traced, directly or indirectly, to certain chemical reactions. Even the shape of a bacterium depends on such reactions: shape is determined by the geometric structure of the rigid peptidoglycan component of the cell wall, the peptidoglycan in turn being determined by the series of chemical reactions involved in its synthesis. The term **metabolism** denotes all the organized chemical activities performed by a cell, which comprise two general types, **energy production** and **energy utilization.** Energy is the ability to do work, and the work of a bacterial cell is extensive and varied. Energy is utilized for the construction of the physical parts of the cell such as wall or membrane; it is required for synthesis of enzymes, nucleic acids, polysaccharides, and other chemical components; it is required for repair of damage and mere maintenance of the status quo, as well as for growth and multiplication; it is required for accumulating certain nutrients in high concentration in the cell and for keeping certain other substances out of the cell; and it is required for motility. To support such extensive activities, vast amounts of energy must be provided. Under certain optimal conditions,

some bacteria have been found capable of metabolizing an amount of nutrient equivalent to their own weight every few seconds to provide such energy! A bacterial cell can be pictured as a dynamo of tremendous energy production. In this chapter, some basic principles of energetics will be discussed, followed by descriptions of just a few of the energy-producing mechanisms used by bacteria. Many of these mechanisms are also used by other microorganisms and by higher organisms, including human beings.

SOME PRINCIPLES OF BIOENERGETICS

Most cells obtain energy by carrying out chemical reactions which liberate energy. Some cells are able to use light as a source of their energy, but even here the light energy must be converted into chemical energy to be in a form useful for the work of the cell.

In the course of any chemical reaction, energy available for the performance of *useful* work is either released or absorbed. The amount of energy liberated or taken up during the course of a reaction is referred to as the **free-energy change** (ΔG) of the reaction. Thus free-energy change can be defined as useful energy. ΔG is expressed in terms of calories; however, this is merely a convenience since the free energy is not always in the form of heat but can, instead, be in the form of chemical energy. If the ΔG of a chemical reaction has a negative value (such as -8000 cal), the reaction releases energy (an **exergonic** reaction). If the ΔG of a reaction has a positive value (such as $+3000$ cal), the reaction requires energy (an **endergonic** reaction).

Concentration of reactants affects the value of ΔG for a chemical reaction, and to make valid comparisons between the energetics of various reactions, a basis of reference must be used. For purposes of comparison, it is assumed that the concentration of all reactants is 1.0 *M* in the steady state; this is referred to as **standard concentration.**

Under conditions of standard concentration, the free-energy change (ΔG) of a reaction is referred to by a special term, $\Delta G°$. In other words, $\Delta G°$ is the amount of free energy released (or absorbed) when one mole of the reactant is converted to one mole of product at 25°C and one atmosphere of pressure, and, under (hypothetical) conditions, where all reactants and products are maintained at 1 *M* concentration.

The $\Delta G°$ or standard free-energy change is related to the equilibrium constant, K_{eq}, of a chemical reaction by the equation

$$\Delta G° = -RT \ln K_{eq}$$

where R is the gas constant and T is the absolute temperature. If $\Delta G°$ is a negative value, the K_{eq} is greater than 1.0 and the formation of products is favored. If $\Delta G°$ is positive in value, K_{eq} is less than 1.0 and the chemical reaction tends to proceed in the reverse direction.

The value of $\Delta G°$ for a reaction can be calculated from the equilibrium constant of the reaction by using the above equation. For example, let us calculate $\Delta G°$ for the reaction catalyzed by phosphoglucomutase at 25°C:

$$\text{Glucose-1-P} \rightleftharpoons \text{glucose-6-P}$$

$$K_{eq} \text{ of } \frac{\text{glucose-6-P}}{\text{glucose-1-P}} = 17$$

The equation $\Delta G^\circ = -RT \ln K_{eq}$ may be rewritten as:

$$\Delta G^\circ = -2.303RT \log K_{eq}$$

Substituting values for these terms, we get

$$\Delta G^\circ = -2.303RT \log 17$$
$$\Delta G^\circ = -2.303(1.987)\,(298) \log 17$$
$$\Delta G^\circ = -1{,}680 \text{ cal/mole}$$

Since the value of ΔG° is negative, the reaction can proceed from left to right under standard conditions.

Let us now calculate ΔG under physiological conditions. (ΔG is the actual free-energy change of a given chemical reaction under the conditions of concentration, pH, and temperature actually prevailing during the reaction, which are not necessarily the standard conditions as defined above.) Suppose that at 38°C the concentrations of glucose-6-P is 1×10^{-4} M and glucose-1-P are 3×10^{-5} M. We substitute these values in the following equation:

$$\Delta G = \Delta G^\circ + 2.303RT \log K_{eq}$$

We get

$$\Delta G = -1{,}680 + 2.303(1.987)\,(311) \log \frac{(1 \times 10^{-4})}{(3 \times 10^{-5})}$$
$$\Delta G = -1{,}680 + 1{,}423 \log 3.3$$
$$\Delta G = -1{,}680 + 740,$$
$$\Delta G = -940 \text{ cal/mole}$$

Thus, under physiological conditions, the reaction still proceeds from left to right.

It is also possible to obtain energy from a chemical reaction in the form of electric potential. Conversely, it is possible to use electric potential energy to drive a chemical reaction. Electric energy is generated when oxidations occur by the removal of electrons (as we will soon see). When the electrons fall through a potential difference or drop, energy is produced. The relationship between an oxidation-reduction potential difference and the standard free-energy change is

$$\Delta G^\circ = -nFE^\circ$$

where n is the number of moles of electrons transferred in the reaction, F is Faraday's constant (23,061 cals/V per equivalent), and E° is the standard oxidation-reduction potential difference.

For example, let us calculate ΔG° for the reaction in which cytochrome c is oxidized by oxygen from the ferrous to the ferric state with $E^\circ = +0.56$V:

$$2 \text{ Cytochrome c–}Fe^{2+} \text{ (reduced)} + \tfrac{1}{2}O_2 \rightleftharpoons 2 \text{ cytochrome c–}Fe^{3+} \text{ (oxidized)} + O^{2-}$$
$$\Delta G^\circ = -2(23{,}061)\,(0.56)$$
$$\Delta G^\circ = -25{,}828 \text{ cal/mole}$$

In order for life to continue, it is essential that the energy released from exergonic reactions be used to drive endergonic reactions, and living organisms have developed characteristic ways of *coupling* exergonic reactions with endergonic reactions. The basic principle involved is that there be a *common reactant*. This can be best understood by the following example.

Consider the two general reactions

$$A \rightarrow B \qquad \Delta G^{\circ\prime} = -10{,}000 \text{ cal (at pH 7)} \qquad (1)$$

$$C \rightarrow D \qquad \Delta G^{\circ\prime} = +5{,}000 \text{ cal (at pH 7)} \qquad (2)$$

(ΔG° at pH 7 is designated $\Delta G^{\circ\prime}$.) The energy liberated by the first reaction (exergonic) can be used to drive the second reaction (endergonic) by coupling the two reactions in the following manner.

$$A + X \rightarrow B + Y \qquad \Delta G^{\circ\prime} = -2{,}000 \text{ cal} \qquad (3)$$

$$C + Y \rightarrow D + X \qquad \Delta G^{\circ\prime} = -3{,}000 \text{ cal} \qquad (4)$$

Here, Y is a reactant common to both reactions. In the first reaction (3), the overall $\Delta G^{\circ\prime}$ of $-2{,}000$ cal indicates that 8,000 of the original 10,000 cal was used for the conversion of X to Y. In the second reaction, Y was converted back to X, thereby releasing the previously trapped 8,000 cal to drive the endergonic conversion of C→D. Thus, the overall $\Delta G^{\circ\prime}$ of the second reaction (4) is $+5{,}000 - 8{,}000$, or $-3{,}000$ cal. The common reactant Y is referred to as an energy-rich or energy-transfer compound.

The common reactants of greatest use to the cell are those capable of transferring large amounts of free energy, called high-energy-transfer compounds. A variety of such compounds exists in cells; and although such compounds may possess no more total energy than other compounds, the energy is distributed in such a way within the molecule that one portion opposes another, resulting in considerable molecular strain. Triggering the breakdown of the molecule (by the catalytic action of an enzyme) causes release of the energy no longer held in check. A high-energy-transfer molecule is analogous to a mousetrap. When set, the trap has great energy, but the energy of the spring is opposed by the action of the catch. Tripping the catch is analogous to catalyzing the breakdown of the transfer molecule, with subsequent release of energy of the spring.

Table 10-1 lists some of the high-energy-transfer compounds found in cells, of which ATP is by far the most important. Just as money constitutes a common medium of exchange in our society, so ATP constitutes the "energy currency" of the cell in the exchange of energy between exergonic and endergonic reactions. It should be noted that all the compounds in Table 10-1 can transfer their energy directly or indirectly to ATP synthesis; for example:

1,3-Diphosphoglyceric acid + ADP → 3-phosphoglyceric acid + ATP

Energy is released from ATP by hydrolysis (Fig. 10-1). The amount of energy so

Table 10-1. Some High-Energy-Transfer Compounds Found in Cells with Their Standard Free-Energy Changes upon Hydrolysis

Compound	$\Delta G^{\circ\prime}$, kcal mol^{-1}
Adenosine triphosphate (ATP)	−7.3
Guanosine triphosphate (GTP)	−7.3
Uridine triphosphate (UTP)	−7.3
Cytidine triphosphate (CTP)	−7.3
Acetyl phosphate	−10.1
1,3-Diphosphoglyceric acid	−11.8
Phosphoenolpyruvic acid (PEP)	−14.8

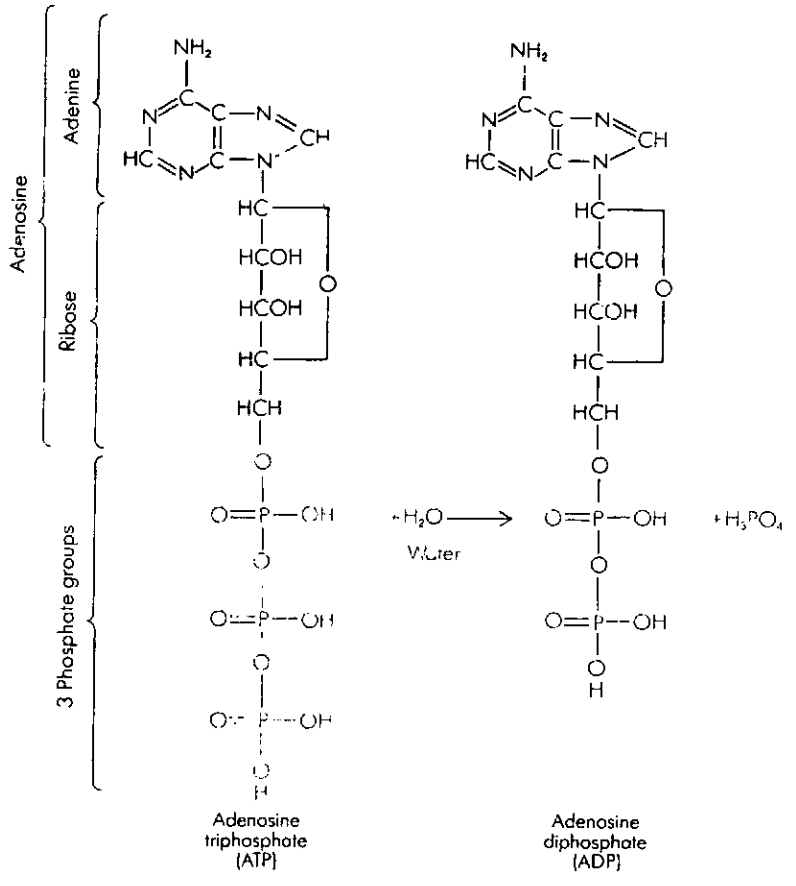


Figure 10-1. Hydrolysis of adenosine triphosphate.

Overall reaction:
$ATP + H_2O \rightarrow ADP + H_3PO_4$; $\Delta G^{\circ\prime} = -7.3$ kcal mol^{-1}

released is a measure of the transfer energy capacity of ATP. It should be remembered, however, that the $\Delta G^{\circ\prime}$ of -7.3 kcal for ATP is not necessarily that existing in the intact cell. The pH and the concentrations of ATP, ADP, Mg^{2+} etc., in the cell are not identical to the standard conditions employed for determining $\Delta G^{\circ\prime}$. If appropriate corrections are made, the free energy of hydrolysis within the cell is closer to -12.5 kcal, although this value can vary depending upon intracellular concentrations of various materials. However, for consistency and comparison, thermodynamic calculations of biological energy exchanges must be carried out under arbitrarily defined standard conditions.

The compound ADP (adenosine diphosphate) is also a high-energy-transfer compound, since its hydrolysis also liberates a large quantity of energy:

$$ADP + H_2O \rightarrow AMP + H_3PO_4 \qquad \Delta G^{\circ\prime} = -7.3 \text{ kcal}$$

where AMP stands for adenosine monophosphate. AMP, however, is a low-

energy compound; its hydrolysis yields only a small quantity of energy:

$$AMP + H_2O \rightarrow \text{adenosine} + H_3PO_4 \qquad \Delta G^{\circ\prime} = -2 \text{ kcal}$$

Several types of chemical reactions are involved in energy production, but oxidation-reduction is probably the commonest. A discussion of some of the basic aspects of oxidation-reduction reactions will clarify their relationship to energy production.

OXIDATION-REDUCTION REACTIONS

Oxidation is the loss of electrons; reduction is the gain of electrons. Frequently, oxidation reactions are dehydrogenations (reactions involving the loss of hydrogen atoms); since a hydrogen atom consists of a proton plus an electron, a compound which loses a hydrogen atom has essentially lost an electron and therefore has been oxidized.

An oxidizing agent (oxidant) will absorb electrons and will therefore become reduced, as illustrated by the following examples.

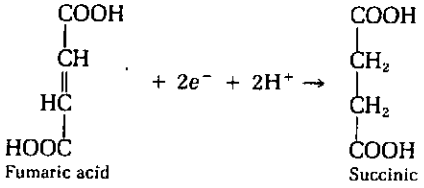
The ferric ion is an oxidizing agent; it absorbs electrons and becomes reduced to ferrous ion:

$$\underset{\text{Ferric ion}}{Fe^{3+}} + \underset{\text{Electron}}{e^-} \rightarrow \underset{\text{Ferrous ion}}{Fe^{2+}}$$

The hydrogen ion is an oxidizing agent; it absorbs electrons and becomes reduced to atomic hydrogen:

$$\underset{\text{Hydrogen ion: proton}}{2H^+} + \underset{\text{Electron}}{2e^-} \rightarrow \underset{\text{Hydrogen atom: proton plus electron}}{2H}$$

Fumaric acid is an oxidizing agent; it absorbs hydrogen atoms (which contain electrons) and becomes reduced to succinic acid:



Fumaric acid — Succinic acid

A reducing agent (reductant) donates electrons, becoming oxidized in the process. The ferrous ion is a reducing agent, it donates electrons and becomes oxidized to ferric ion:

$$Fe^{2+} \rightarrow Fe^{3+} + e^-$$

From this example, one can see that the reverse of each oxidation reaction is a reduction and the reverse of a reduction reaction is an oxidation. Moreover, in each reaction, a *pair* of substances is involved: one is the *reduced form*, the other the *oxidized form*, e.g., ferrous ion and ferric ion, succinic acid and fumaric acid. Each such pair of substances is referred to as an oxidation-reduction (O/R) system.

One O/R system may tend to absorb electrons from another O/R system; i.e., the first system will oxidize the second. On the other hand, the tendency of the

first system to absorb electrons may be so low that the second system may oxidize the first. This power (the tendency to absorb electrons) is expressed by the **standard oxidation-reduction potential** or the **electromotive potential** (E_0') of an O/R system, which is measured electrically under standardized conditions of comparison (electron donor and its conjugate at 1.0 *M* concentration, 25°C, and pH 7.0) and expressed in volts. The more positive the E_0', the greater the oxidizing ability of the system. Consequently, any system listed in Table 10-2 can oxidize any other system listed above it, but not below it, under the standard conditions. Such relationships are very important in understanding the orderly sequence in which biological oxidations occur.

As discussed previously, when one O/R system oxidizes another, energy is released. It is important to know the values of E_0' for each system, because the $\Delta G^{0'}$ of the overall reaction is directly proportional to the difference in E_0' values. If the voltage difference is large, an amount of free energy sufficient to drive the synthesis of ATP may be liberated.

In respiration, an oxidizable substrate is the primary electron donor. In aerobic respiration the terminal electron acceptor is oxygen; in anaerobic respiration the final electron acceptor is a compound like fumarate, NO_3^-, SO_4^{2-}, or CO_3^{2-}. In fermentation, an organic compound is the final electron acceptor; an oxidizable substrate is the electron donor. In photosynthesis carried out by bacteria, bacteriochlorophylls serve as both electron donors and acceptors. In photosynthesis by green plants, algae, and cyanobacteria, water serves as a primary electron donor and $NADP^+$ (nicotinamide adenine dinucleotide phosphate) as a terminal electron acceptor. The paths through which these electrons flow in the various processes are called electron-transport chains.

Electron-transport chains are sequences of oxidation-reduction reactions that occur in cells. These reactions are mediated by a number of electron carriers and electron-carrier enzymes (discussed later). As the electrons flow through the chains, much of their free energy is conserved in the form of ATP; this process is called **oxidative phosphorylation**.

The multicomponent electron-transport chains are always associated with membranes. In eucaryotes, they are in mitochondrial or chloroplast membranes; in procaryotes, they are in the cytoplasmic membrane.

THE RESPIRATORY CHAIN

A respiratory chain is an electron-transport chain. When a pair of electrons or hydrogen atoms (which contain electrons) from an oxidizable substrate is coupled with the reduction of an ultimate electron acceptor, such as oxygen, there is a large free-energy change (ΔG°). The flow of electrons through the transport chain allows a stepwise release of this energy, some of which is conserved in the form of ATP at several steps in the chain. At these specific steps the difference in E_0' values is great enough to permit sufficient energy to be liberated for oxidative phosphorylation to occur.

The component O/R systems of a common type of respiratory chain are shown in Table 10-2 and are illustrated in Fig. 10-2.

A respiratory chain consists of enzymes having prosthetic groups or coenzymes. These can be regarded as the working parts of the enzymes, and in the case of the respiratory chain each is in fact an O/R system. The oxidized form of each prosthetic group or coenzyme has an absorption spectrum different from

Table 10-2. Component O/R Systems of a Respiratory Chain, with Their Corresponding E_0' Values

O/R System	E_0', V
$NAD^+/NADH + H^+$*	−0.32
Flavoprotein/flavoprotein-H_2	−0.03
CoQ/CoQ-H_2	+0.04
Cyt b–Fe^{3+}/cyt b–Fe^{2+}	+0.07
Cyt c_1–Fe^{3+}/cyt c_1–Fe^{2+}	+0.21
Cyt c–Fe^{3+}/cyt c–Fe^{2+}	+0.23
Cyt a–Fe^{3+}/cyt a–Fe^{2+}	+0.29
Cyt a_3–Fe^{3+}/cyt a_3–Fe^{2+}	+0.53
Oxygen/water	+0.82

* $NADH + H^+$ may also be designated as $NADH_2$ or simply as NADH since the other hydrogen atom appears as a free H^+ ion.

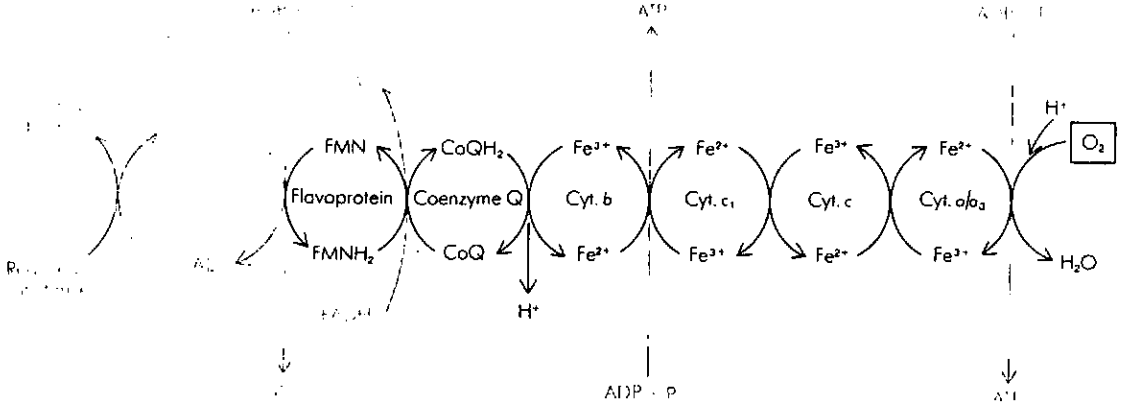


Figure 10-2. A respiratory chain, showing sequential oxidation steps and points where sufficient energy is liberated to permit synthesis of ATP. Electron transfer is accompanied by a flow of protons (H^+) from $NADH_2$ through coenzyme Q but not in later steps involving cytochromes. Note that three ATP are formed per molecule of $NADH_2$ reoxidized but only two ATP per molecule of $FADH_2$ reoxidized. (Note that this is an abbreviated representation. Actually, there are more than 15 chemical substances in the chain.)

that of the reduced form, so that the two states can be distinguished by spectrophotometry. A summary of these coenzymes follows.

Nicotinamide Adenine Dinucleotide (NAD) and Nicotinamide Adenine Dinucleotide Phosphate (NADP)

Certain enzymes which remove electrons and hydrogen ions from reduced substrates (referred to as dehydrogenases) have NAD^+ or $NADP^+$ as their coenzyme. NAD^+ can exist in a reduced form, $NADH + H^+$, to form an O/R system:

$$NAD^+ + 2H^+ + 2e^- \rightleftharpoons NADH + H^+$$

In the same way, $NADP^+$ can exist in a reduced state.

The vitamin niacin (nicotinic acid) forms part of the structure of NAD and NADP and is a precursor (building block) in their biosynthesis.

Flavin Adenine Dinucleotide (FAD) and Flavin Mononucleotide (FMN)

Another class of dehydrogenases known as flavoproteins exists and contains either FAD or FMN as prosthetic groups. One of the basic parts of their coenzyme structure is the vitamin riboflavin. Riboflavin can exist in either an oxidized or

reduced form: $\text{Riboflavin} + 2H \rightleftharpoons \text{riboflavin-}H_2$

The reduced forms of the coenzymes are $FADH_2$ and $FMNH_2$.

Coenzyme Q

Coenzyme Q is also called **ubiquinone** because it is a quinone and is present in all cells. Coenzyme Q is a fat-soluble coenzyme. It functions as an acceptor of reducing power from the flavin-linked dehydrogenases:

$$\text{Flavoprotein-}H_2 + \text{ubiquinone} \rightleftharpoons \text{ubiquinone-}H_2 + \text{flavoprotein}$$

NAD^+, $NADP^+$, flavoproteins, and ubiquinones carry $2H^+$ and $2e^-$ but the cytochromes (discussed below) transfer only electrons, the protons being associated with an $—NH_2$ group or a $—COO^-$ group and eventually transferred to O_2 (see Fig. 10-2).

Cytochromes

Another major class of oxidative enzymes in the respiratory chain is the cytochromes. The prosthetic group of a cytochrome is a derivative of heme and contains a single iron atom, which is responsible for the oxidative or reductive properties of the enzyme. On the basis of differences in absorption spectra, cytochromes can be divided into three main categories: cytochromes *a*, cytochromes *b*, and cytochromes *c*. Each of these groups has a different function in the respiratory chain and can be further subdivided on the basis of minor differences in absorption spectra, e.g., cytochromes *c* and c_1 or cytochromes *a* and a_3. Each cytochrome type can exist in either an oxidized or reduced form, depending on the state of the iron atom contained in their structure:

$$\underset{\text{Ferric}}{\text{Cyt } b\text{–}Fe^{3+}} + e^- \rightleftharpoons \underset{\text{Ferrous}}{\text{cyt } b\text{–}Fe^{2+}}$$

The cytochromes act sequentially to transport electrons from coenzyme Q to O_2. Cytochromes *a* and a_3 together are called cytochrome oxidase. Both of them also contain copper. But only cytochrome a_3 can react directly with oxygen.

Sequence of Oxidation

The arrangement of O/R systems in Table 10-2 according to E_0' values is based on the experimentally determined sequence of oxidation reactions in the respiratory chain illustrated in Fig. 10-2. Sufficient energy for ATP synthesis is liberated at three points along the chain. The incremental release of energy in the respiratory chain results in a more efficient trapping of energy in ATP than would be true of direct oxidation of the reduced substrate by oxygen.

The respiratory chain of bacteria is associated with the *cytoplasmic membrane*; that of eucaryotes is present in mitochondrial membranes. Much of the electron transfer in membranes is accomplished within highly integrated particles or complexes.

ENERGY PRODUCTION BY ANAEROBIC PROCESSES

Heterotrophic bacteria can use a variety of organic compounds as energy sources. These compounds include carbohydrates, organic and fatty acids, and amino acids. For many microorganisms the preferred compounds are carbohydrates, especially the 6-carbon sugar glucose.

Glycolysis

The most common pathway of glucose catabolism is the Embden-Meyerhof pathway of glycolysis ("splitting of sugar"). This process occurs very widely and is found in microorganisms as well as in animals and plants. Glycolysis does not require the presence of oxygen and therefore can occur in both aerobic and anaerobic cells. Aerobic cells degrade glucose by glycolysis, and this process constitutes the preparatory stage for the aerobic phase of glucose oxidation. Thus, under anaerobic conditions this situation prevails:

$$\text{Glucose} \xrightarrow{\text{fermentation}} \text{fermentation products}$$

whereas under aerobic conditions, the following occurs:

$$\text{Glucose} \xrightarrow{\text{fermentation}} \underset{\text{(pyruvate)}}{\text{intermediate}} \xrightarrow[O_2]{\text{respiration}} CO_2 + H_2O$$

In glycolysis, as shown in Fig. 10-3, fructose-1,6-diphosphate formed from glucose is split into two 3-carbon units (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate), and they are subsequently oxidized to pyruvic acid. At the step where glyceraldehyde-3-phosphate is oxidized, a pair of electrons (two hydrogen atoms) is removed. In the absence of oxygen, this pair of electrons may be used to reduce pyruvic acid to lactic acid or ethanol. In the presence of oxygen, this pair of electrons may enter the respiratory chain.

Many of the reactions of the glycolytic pathway are freely reversible and can be used for the synthesis of glucose as well as for its breakdown. Only three of the reactions are not reversible by common enzymes; but the presence of other enzymes can reverse them for glucose synthesis to occur. Thus phosphoenolpyruvate is synthesized from pyruvate by the action of phosphoenolpyruvate synthase and specific phosphatases hydrolyze fructose-1,6-diphosphate and glucose-6-phosphate in the biosynthetic direction. The enzymes at these steps in the degradative direction are kinases and require ATP. (See Fig. 10-3.) For each molecule of glucose metabolized, two molecules of ATP are used up and four molecules of ATP are formed. Therefore for each molecule of glucose metabolized by glycolysis, there is a net yield of two ATP molecules. This is shown in Fig. 10-3.

The overall reaction of glycolysis can be summarized as follows:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + 2NAD + 2ADP + \underset{\substack{\text{Inorganic}\\\text{phosphate}}}{2P_i} \rightarrow \underset{\text{Pyruvic acid}}{2CH_3COCOOH} + 2NADH_2 + 2ATP$$

The Pentose Phosphate Pathway

The pentose phosphate pathway, like the glycolytic one, is another catabolic reaction pathway that exists in both procaryotic and eucaryotic cells. Since it involves some reactions of the glycolytic pathway, it has been viewed as a "shunt" of glycolysis; hence it may also be called the hexose monophosphate shunt. Its other synonym is the phosphogluconate pathway.

Glucose can be oxidized by the pentose phosphate pathway with the liberation of electron pairs, which may enter the respiratory chain. However, this cycle is not generally considered a major energy-yielding pathway in most microorganisms. It provides reducing power in the form of $NADPH + H^+$, which is required in many biosynthetic reactions of the cell, and it provides pentose phosphates

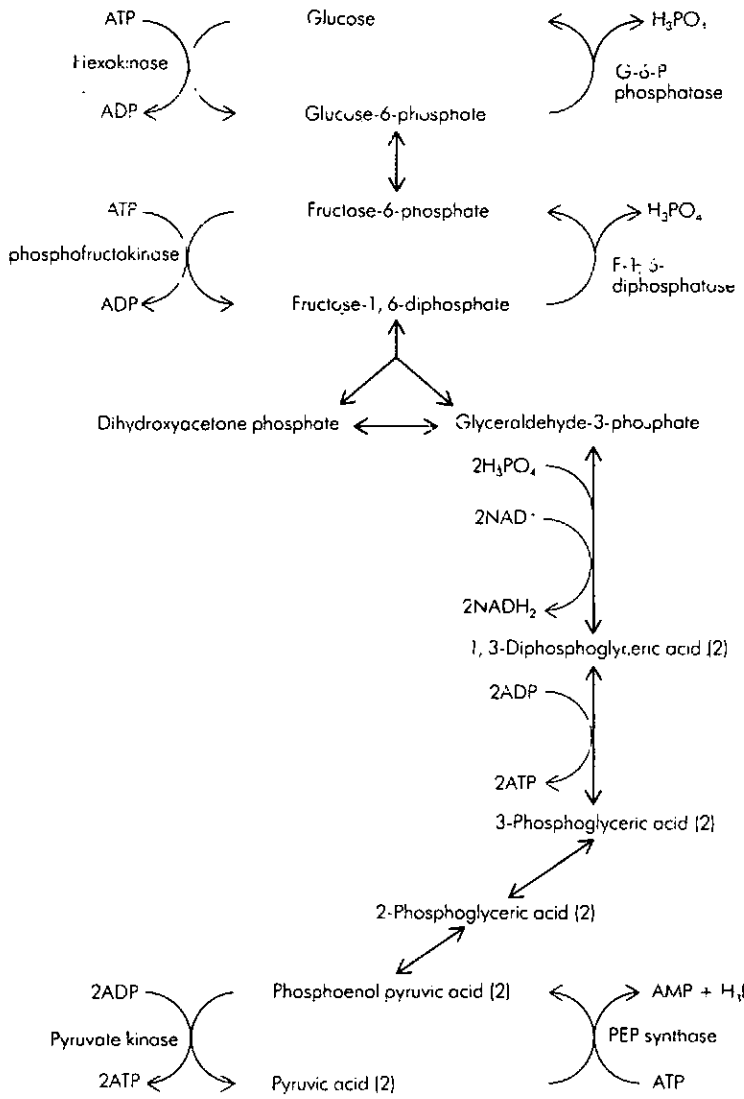


Figure 10-3. The Embden-Meyerhof (glycolytic) pathway of glucose catabolism. Enzymes shown are for those steps which are not freely reversible by a common enzyme.

for use in nucleotide synthesis. Although it can produce energy for the cell as an alternate pathway for the oxidation of glucose, it is also a mechanism for obtaining energy from 5-carbon sugars.

As seen in Fig. 10-4, the pentose phosphate pathway involves the initial phosphorylation of glucose to form glucose-6-phosphate; the latter is oxidized to 6-phosphogluconic acid with the simultaneous production of NADPH. Decarboxylation of 6-phosphogluconic acid, together with a yield of NADPH, produces ribulose-6-phosphate. Epimerization reactions yield xylulose-5-phosphate and ribose-5-phosphate. These two compounds are the starting point for

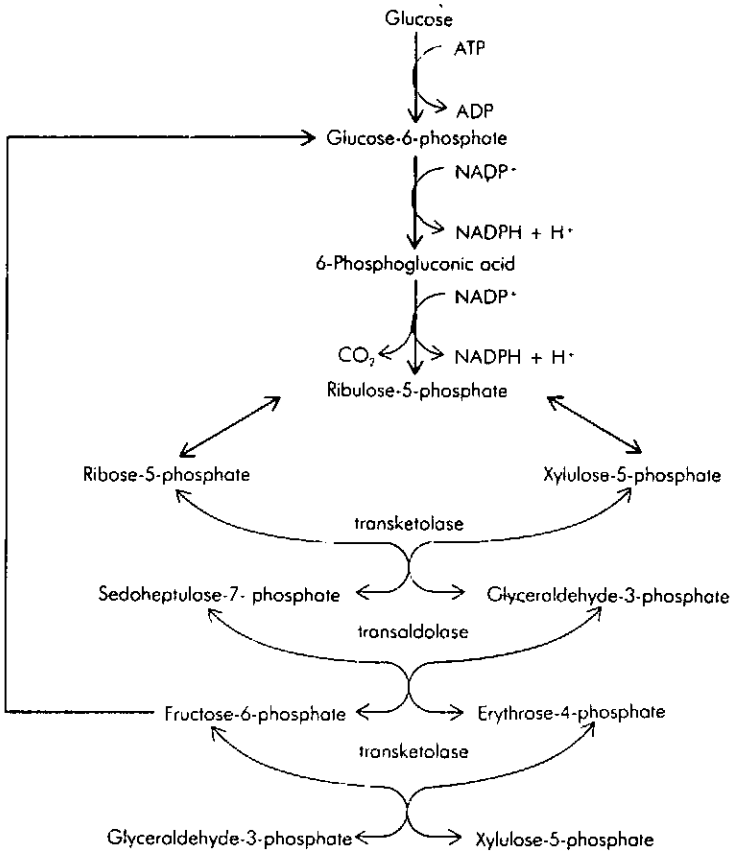


Figure 10-4. The pentose phosphate pathway of glucose catabolism yielding ribose-5-phosphate and NADPH + H^+.

a series of transketolase reactions and transaldolase reactions leading subsequently to the initial compound of the pathway, 6-phosphogluconic acid, thus completing the cycle. Note that two intermediates of glycolysis—fructose-6-phosphate and glyceraldehyde-3-phosphate—are generated. Theoretically, by means of this cycle, the cell can carry out the complete oxidation of glucose-6-phosphate to CO_2. Specifically, six molecules of glucose-6-phosphate are oxidized to six molecules each of ribulose-5-phosphate and CO_2; five molecules of glucose-6-phosphate are then regenerated from the six molecules of ribulose-5-phosphate. The overall equation is as follows:

$$6 \text{ Glucose-6-phosphate} + 12NADP^+ \rightarrow 5 \text{ glucose-6-phosphate} + 6CO_2 + 12NADPH + 12H^+ + P_i$$

The net equation is therefore:

$$\text{Glucose-6-phosphate} + 12NADP^+ \rightarrow 6CO_2 + 12NADPH + 12H^+ + P_i$$

In the real situation, it is more probable that the pentose phosphate pathway feeds into the glycolytic pathway by means of fructose-6-phosphate and glyceraldehyde-3-phosphate.

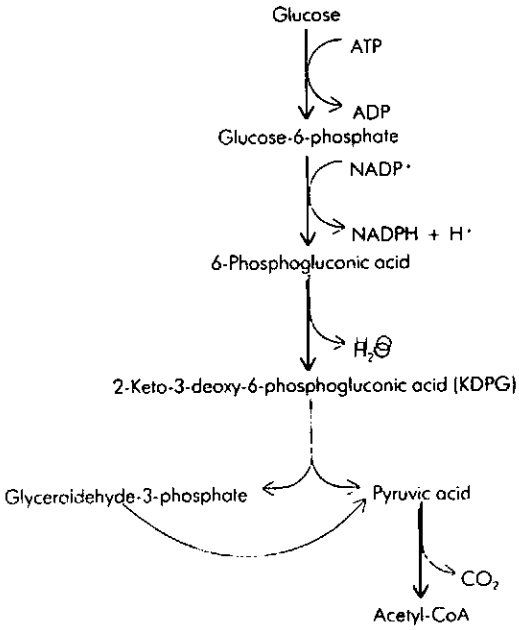


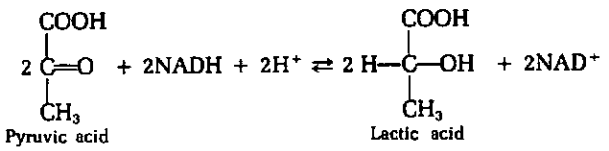
Figure 10-5. The Entner-Doudoroff pathway of glucose catabolism for some bacteria.

The Entner-Doudoroff Pathway

Another pathway of glucose catabolism is called the Entner-Doudoroff pathway. It is found in both aerobic and anaerobic procaryotes but not in eucaryotes. It is fairly widespread, particularly among Gram-negative bacteria. As shown in Fig. 10-5, glucose is phosphorylated to glucose-6-phosphate. It is then oxidized to 6-phosphogluconic acid. A dehydration step follows to yield 2-keto-3-deoxy-6-phosphogluconic acid (KDPG); the latter is cleaved to pyruvic acid and glyceraldehyde-3-phosphate, which is metabolized via some Embden-Meyerhof pathway enzymes to produce a second molecule of pyruvic acid. In the aerobic pseudomonads the catabolism is completed via acetyl-CoA and the tricarboxylic acid cycle (see later in this chapter).

Fermentation

Anaerobes also produce energy by reactions called fermentations, which use organic compounds as electron donors and acceptors. Facultative anaerobic bacteria and obligately anaerobic bacteria employ many different kinds of fermentations to produce energy. The lactic fermentation is a typical example. *Streptococcus lactis*, the bacterium responsible for the normal souring of raw milk, dissimilates glucose to lactic acid, which accumulates in the medium as the sole fermentation product. How does this happen? By glycolysis (Fig. 10-3), one molecule of glucose is converted to two molecules of pyruvic acid with concomitant production of two NADH + H^+. The pyruvic acid is converted to lactic acid in the following reaction:



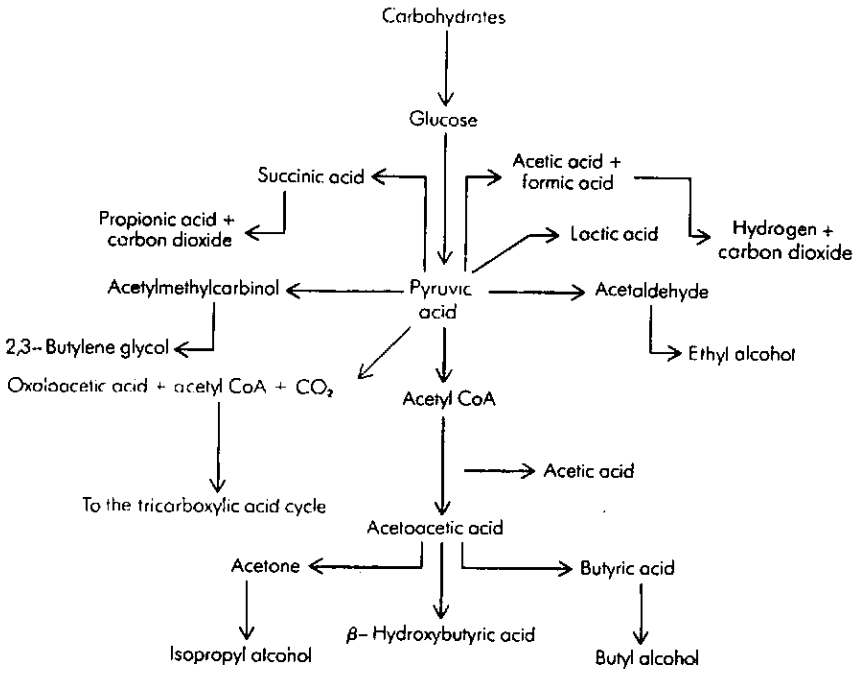


Figure 10-6. Pyruvic acid is regarded as the key compound in the dissimilation of glucose, as shown in this schematic illustration.

Insufficient energy for ATP synthesis results from this reaction; however, NAD^+ is regenerated for further use as an oxidant.

In other carbohydrate fermentations, the initial stages of glucose dissimilation frequently, but not always, follow the scheme of glycolysis. Differences in carbohydrate fermentations usually occur in the ways the resulting pyruvic acid is used. Thus pyruvic acid is the "hub" of carbohydrate fermentations. Figure 10-6 illustrates the variety of products resulting from the metabolism of pyruvic acid.

Most heterotrophic bacteria produce several end products of the types indicated in Fig. 10-6 from glucose dissimilation, but no single species produces all these end products. The types listed represent a summary of what can be expected when one takes an inventory of the end products of glucose dissimilation by all heterotrophs. Actually, it is possible to group microorganisms on the basis of their products of fermentation (the lactic acid group or the propionic acid group of bacteria, for example, as shown in Table 10-3). Such designations are established on the basis of the major end products of carbohydrate fermentation. From this it is evident that not all microorganisms metabolize the same substrate in exactly the same manner. For example, *Streptococcus lactis* and *Escherichia coli* both ferment glucose but by quite different pathways of fermentation, as shown in Fig. 10-7.

However, some anaerobes do not have a functional glycolytic system. They may have carbohydrate fermentation pathways that use the pentose phosphate pathway and the Entner-Doudoroff pathway. Fermentations of noncarbohydrate substrates, such as amino acids, involve highly specific pathways.

Table 10-3. Bacteria Grouped According to Major Products of Glucose Dissimilation

Groups with Examples of Some Genera	Representative Products
Lactic acid bacteria *Streptococcus* *Lactobacillus* *Leuconostoc*	Lactic acid only or lactic acid plus acetic acid, formic acid, and ethyl alcohol; species producing only lactic acid are homofermentative, and those producing lactic acid plus other compounds are heterofermentative
Propionic acid bacteria *Propionibacterium* *Veillonella*	Propionic acid plus acetic acid and carbon dioxide
Coli-aerogenes-typhoid bacteria *Escherichia* *Enterobacter* *Salmonella*	Formic acid, acetic acid, lactic acid, succinic acid, ethyl alcohol, carbon dioxide, hydrogen, 2,3-butylene glycol (produced in various combinations and amounts depending on genus and species)
Acetone, butyl alcohol bacteria *Clostridium* *Eubacterium* *Bacillus*	Butyric acid, butyl alcohol, acetone, isopropyl alcohol, acetic acid, formic acid, ethyl alcohol, hydrogen, and carbon dioxide (produced in various combinations and amounts depending on species)
Acetic acid bacteria *Acetobacter*	Acetic acid, gluconic acid, kojic acid

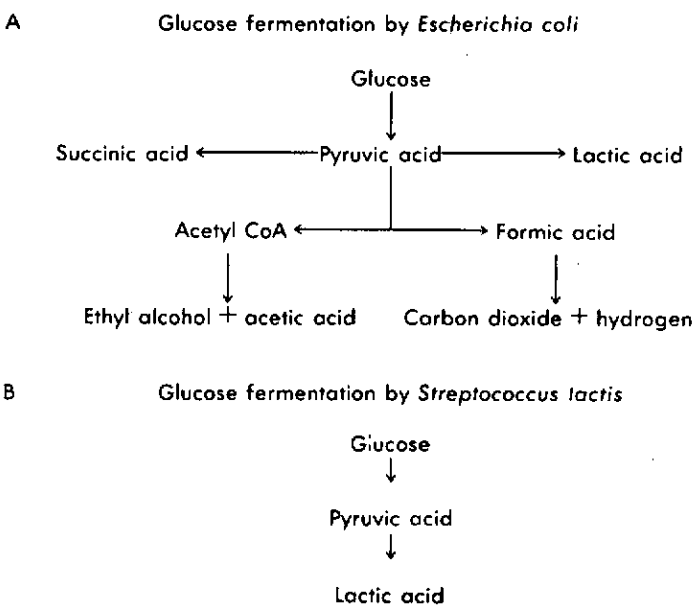
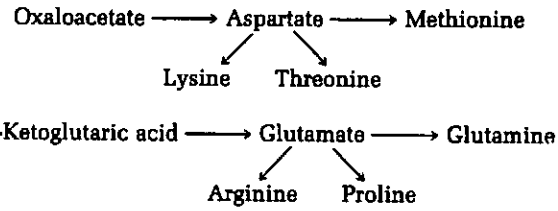


Figure 10-7. Glucose is fermented by many different bacteria and in many different ways. (A) *Escherichia coli* fermentation of glucose results in a mixture of products, whereas (B) *Streptococcus lactis* fermentation of glucose produces lactic acid almost exclusively.

ENERGY PRODUCTION BY AEROBIC PROCESSES

The Tricarboxylic Acid Cycle

The tricarboxylic acid (TCA) cycle is a sequence of reactions that generate energy in the form of ATP and reduced coenzyme molecules ($NADH_2$ and $FADH_2$). It also performs other functions. Many intermediates in the cycle are precursors in the biosynthesis of amino acids, purines, pyrimidines, etc. For example, oxaloacetic acid and α-ketoglutaric acid are amino acid precursors as shown in the following:



Thus the TCA cycle is an amphibolic cycle, which means that it functions not only in catabolic (breakdown) but also in anabolic (synthesis) reactions. The cycle is shown in Fig. 10-8.

The overall reaction of the TCA cycle can be summarized as follows:

$$\text{Acetyl-CoA} + 3H_2O + 3NAD^+ + FAD + ADP + P_i \rightarrow 2CO_2 + CoA + 3NADH_2 + FADH_2 + ATP$$

Since the breakdown of glucose by glycolysis yields two acetyl-CoA molecules

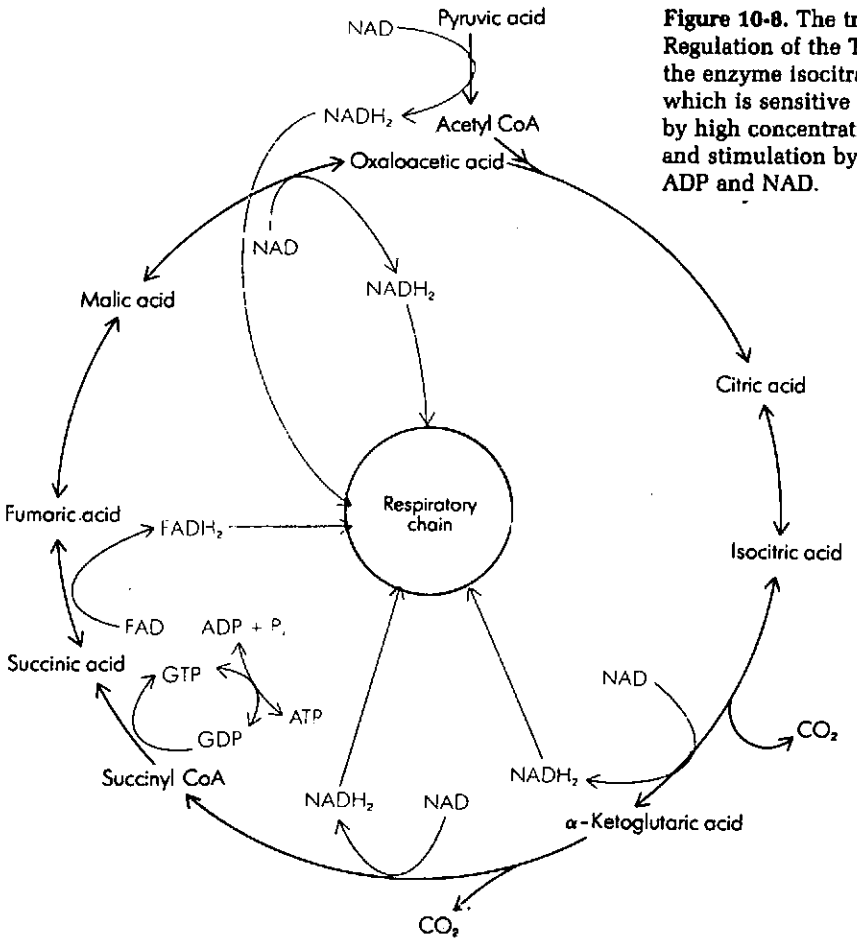


Figure 10-8. The tricarboxylic acid cycle. Regulation of the TCA cycle focuses on the enzyme isocitrate dehydrogenase, which is sensitive to feedback inhibition by high concentrations of ATP and $NADH_2$ and stimulation by high concentrations of ADP and NAD.

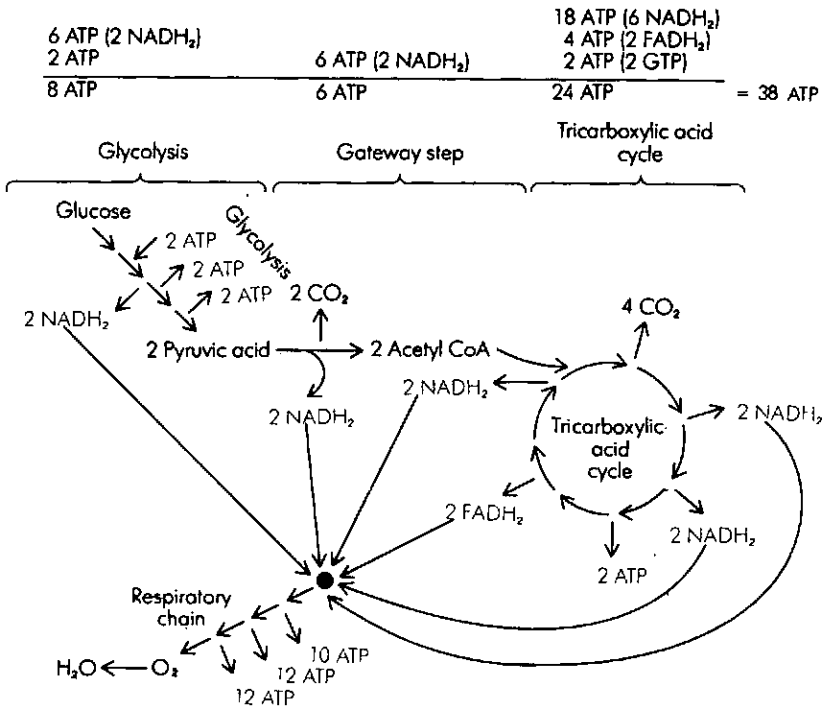


Figure 10-9. ATP yield per glucose molecule broken down in aerobic respiration.

which can enter this cycle, the overall equation for the cycle, per glucose molecule broken down, is twice the above.

Energy Yield in Aerobic Respiration

We may now look at the energy yield from the aerobic breakdown of one molecule of glucose when the electrons stored in the reduced coenzyme molecules are fed into the electron-transport chain. As shown previously, the electrons are transferred stepwise from the coenzyme carriers to molecular oxygen, and this transfer is coupled to the generation of ATP by oxidative phosphorylation.

For each glucose molecule broken down, there are 12 reduced coenzymes to be oxidized: 2 $FADH_2$ (1 from each turn of the TCA cycle) and 10 $NADH_2$ (2 from glycolysis; 2 from the gateway step between glycolysis and the TCA cycle, i.e., pyruvic acid to acetyl-CoA; and 6 from two turns of the TCA cycle). Since 3 ATP are produced from each $NADH_2$ and 2 ATP from each $FADH_2$, there are 34 ATP generated from the reduced coenzymes via oxidative phosphorylation through the respiratory chain. But the total yield of ATP from the aerobic respiration of 1 glucose molecule is 38: 34 from the oxidation of reduced coenzymes, 2 from glycolysis, and 2 from the side reaction of the TCA cycle, that is, from 2 GTP. The total ATP yield per glucose molecule from aerobic respiration is summarized in Fig. 10-9.

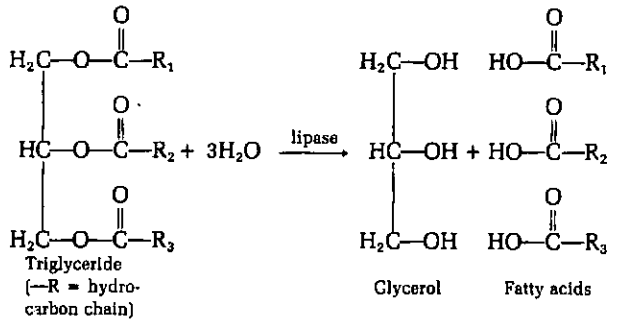
The complete oxidation of glucose via glycolysis, the TCA cycle, and the respiratory chain is summarized in this overall reaction:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

Catabolism of Lipids

Glucose is the single most important source of energy for most cells. However, for many microorganisms, other substances, such as lipids and proteins, may be used as alternate sources of energy. There is a general rule that governs their utilization: they are converted as quickly and efficiently as possible into intermediates of the glycolytic and TCA pathways so that a minimum number of additional enzymes is required to effect complete breakdown. This rule highlights the fact that the glycolytic pathway and the TCA cycle serve as a common center around which other catabolic pathways are built.

The breakdown of lipids or fats begins with the cleavage of triglycerides by the addition of water to form glycerol and fatty acids by means of enzymes called lipases:



Glycerol as a component of fats can be converted into an intermediate of the glycolytic pathway (dihydroxyacetone phosphate) by the following reactions:

$$\text{Glycerol} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{glycerol kinase}} \text{ADP} + \text{glycerol-3-phosphate}$$

$$\text{Glycerol-3-phosphate} + \text{NAD}^+ \xrightarrow{\text{glycerol phosphate dehydrogenase}} \text{dihydroxyacetone phosphate} + \text{NADH}_2$$

The dihydroxyacetone phosphate formed would be broken down by the mechanisms shown in Fig. 10-3. Fatty acids are oxidized by the successive removal of 2-carbon fragments in the form of acetyl-CoA, a process known as β-oxidation. The acetyl-CoA formed can then enter the TCA cycle, and the hydrogen atoms and their electrons enter the respiratory transport chain, leading to oxidative phosphorylation.

There is more energy yield per gram of fat than per gram of carbohydrate. However, relatively few microbial species are effective in breaking down lipids of either simple or complex types, partly because of the limited solubility of lipids.

Catabolism of Proteins

Many heterotrophic microorganisms can degrade exogenous proteins, using the products as carbon and nitrogen energy sources. Since protein molecules are too large to pass into the cell, bacteria secrete exoenzymes called proteases that hydrolyze exogenous proteins to peptides, which are then transported into the cell cytoplasm.

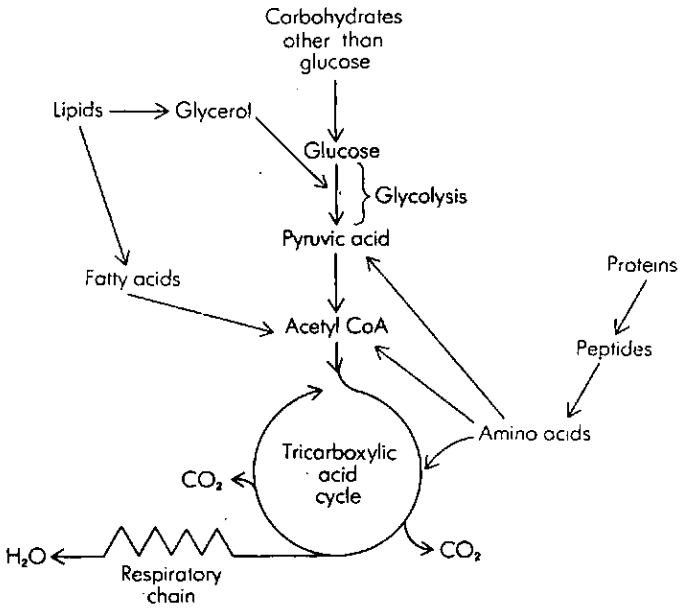


Figure 10-10. Metabolism of carbohydrates, lipids, and amino acids. As can be seen from the diagram, acetyl-CoA is a common intermediate of carbohydrate and lipid metabolism, and the TCA cycle is the common pathway for oxidation of carbohydrates, lipids, and amino acids.

Bacteria produce peptidases that break down peptides to the individual amino acids, which are then broken down according to the specific amino acid and the species or strain of bacteria breaking it down. This process may be shown as follows:

$$\text{Proteins} \xrightarrow{\text{proteases}} \text{peptides} \xrightarrow{\text{peptidases}} \text{amino acids}$$

Where amino acids are broken down, the carbon skeletons of the amino acids undergo oxidation to compounds that may enter the TCA cycle for further oxidation. Entry into the TCA cycle can be via acetyl-CoA, α-ketoglutaric acid, succinic acid, fumaric acid, or oxaloacetic acid.

An overall view of the dissimilation of carbohydrates, lipids, and proteins is shown in Fig. 10-10.

Respiration without Oxygen in Some Bacteria

Some bacteria which are ordinarily aerobic can grow anaerobically if nitrate is present. For example, *Aquaspirillum itersonii*, an aquatic bacterium, is dependent on oxygen unless potassium nitrate is added to the medium. In such cases nitrate essentially substitutes for oxygen as the final electron acceptor in the respiratory chain. This process is termed **anaerobic respiration**. The pathways for the dissimilation of the carbon and energy sources are identical with those in aerobic respiration, and electron transport occurs via a respiratory chain similar to that in aerobic cells. Oxygen is replaced as the terminal electron acceptor by nitrate. However, in some strict anaerobes, other compounds, such as carbon dioxide, or ions, such as sulfate ion, can be the terminal electron acceptors.

Heterotrophic CO_2 Fixation

This phenomenon (unrelated to autotrophic CO_2 fixation) is important because it provides a mechanism for synthesis of compounds of the TCA cycle from the

products of carbohydrate metabolism. Two types of CO_2-fixing reactions occur in heterotrophic bacteria.

1 This first type of reaction is essentially irreversible and occurs in many bacteria:

$$\text{Phosphoenolpyruvate (PEP)} + CO_2 \xrightarrow[\text{acetyl-CoA}]{\text{PEP carboxylase}} \text{oxaloacetate} + P \text{ (inorganic phosphate)}$$

A variation of this reaction requires a nucleoside diphosphate:

$$PEP + ADP + CO_2 \xrightleftharpoons[Mg^{2+}]{\text{PEP carboxykinase}} \text{oxaloacetate} + ATP$$

2 The second type requires the vitamin biotin for activity:

$$ATP + \text{pyruvate} + CO_2 \xrightleftharpoons[\text{biotin, } Mg^{2+}, \text{ acetyl-CoA}]{\text{pyruvate carboxylase}} \text{oxaloacetate} + ADP + P_i$$

The Glyoxylate Cycle

The glyoxylate cycle is used by some microorganisms when acetate is the sole carbon source or during oxidation of primary substrates (such as higher fatty acids) that are cleaved to acetyl-CoA without the intermediate formation of pyruvic acid. This pathway does not occur in higher organisms because they are never forced to feed on 2-carbon molecules alone.

The specific enzymes of the glyoxylate cycle are isocitrate lyase and malate synthase. Figure 10-11 shows how these two enzymes fit together with other

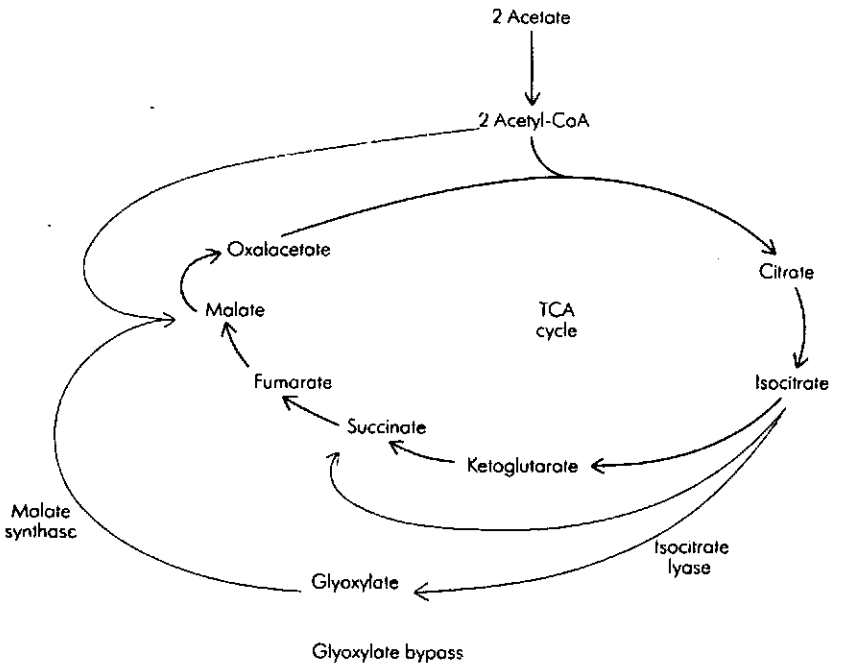


Figure 10-11. The glyoxylic acid cycle or bypass. Its reactions permit the replenishment of the pool of intermediates of the TCA cycle. The specific enzymes are isocitrate lyase and malate synthase.

reactions of the tricarboxylic acid cycle to provide a bypass around some of the TCA-cycle reactions. The overall reaction of the glyoxylate cycle is

$$2 \text{ Acetyl-CoA} \rightarrow \text{succinate} + 2H + 2CoA$$

As seen in Fig. 10-11, acetyl-CoA enters the cycle at two places. It condenses with oxalacetate to give citrate, which is the entry point for the TCA cycle, and the further reaction leads to the formation of isocitrate. Isocitrate lyase is a splitting enzyme that produces succinate and glyoxylate. The second acetyl-CoA molecule condenses with glyoxylate to give malate by the action of malate synthase. Enzymes which carry out replenishment reactions such as this are known as anaplerotic enzymes; their function is to maintain the pool of essential intermediates for biosynthesis.

ENERGY PRODUCTION BY PHOTOSYNTHESIS

Plants, algae, and cyanobacteria are photoautotrophs. They use light as their source of energy and carbon dioxide as their sole source of carbon. In order for carbon dioxide to be useful for metabolism, it must first be reduced to carbohydrate. This process, by which light is used to convert carbon dioxide to carbohydrate, is called photosynthesis. The overall reaction can be written as

$$\underset{\substack{\text{In the presence of light}\\ \text{(radiant energy)}\\ \text{and the green pigment}\\ \text{chlorophyll}}}{2H_2O + CO_2} \rightarrow \underset{\substack{\text{Carbo-}\\ \text{hydrate}}}{(CH_2O)_x} + O_2 + H_2O$$

Here $(CH_2O)_x$ is a formula representing any carbohydrate.

Photosynthesis has two important requirements: (1) a large amount of energy in the form of ATP, and (2) a large quantity of a chemical reductant, in this case water.

Several groups of bacteria—the photoautotrophic green and purple bacteria—are also characterized by their ability to perform photosynthesis. But unlike plants, algae, and cyanobacteria, they do not use water as their chemical reductant, nor do they produce oxygen as one of their end products of photosynthesis. The general equation for bacterial photosynthesis is:

$$\underset{\substack{\text{In the presence of}\\ \text{light (radiant energy)}\\ \text{and the green pigment}\\ \text{bacteriochlorophyll}}}{2H_2A + CO_2} \rightarrow \underset{\substack{\text{Carbo-}\\ \text{hydrate}}}{(CH_2O)_x} + 2A + H_2O$$

Here H_2A represents the chemical reductant, such as the inorganic compounds H_2, H_2S, or $H_2S_2O_3$, or the organic compounds lactate or succinate. If H_2A in this equation stood for H_2S, then A would stand for S.

Both of the preceding equations represent the overall results of photosynthesis. A great deal has been learned about the specific chemical reactions involved in bacterial and plant photosynthesis. What follows is a look at the light-dependent energy-yielding processes involving bacteriochlorophyll in bacteria and chlorophyll in plants, algae, and cyanobacteria. What is presented is

in accord with the latest results of many investigators but may require modification as further evidence is accumulated.

Cyclic and Noncyclic Photophosphorylation

Anoxygenic photosynthetic bacteria possess chlorophylls, called bacteriochlorophylls, that differ from the chlorophylls of plants in structure and in light-absorbing properties. Bacteriochlorophylls absorb light in the infrared region (725 to 1,035 nm). They are not contained in chloroplasts but are found in extensive membrane systems throughout the bacterial cell.

When a molecule of bacteriochlorophyll absorbs a quantum of light, the energy of the light raises the molecule to an excited state. In this excited state an electron is given off by bacteriochlorophyll. Bacteriochlorophyll thus becomes positively charged. It then serves as an electron trap or strong oxidizing agent.

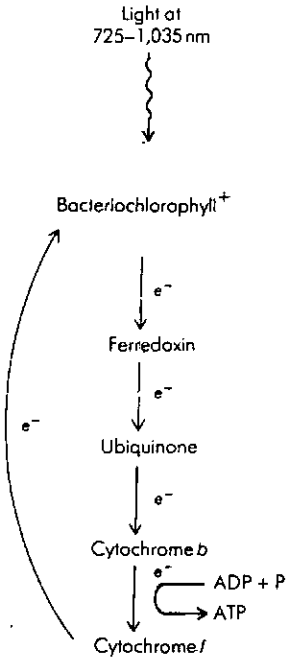


Figure 10-12. Cyclic photophosphorylation as it occurs in anoxygenic photosynthetic bacteria. The electron returns, at a lower energy state, to the bacteriochlorophyll, which had become positively charged after the initial ejection of the electron. No NADP is reduced and no external donor is necessary for this process.

The electron, carrying some of the energy absorbed from light, is transferred to an iron-containing heme protein known as **ferredoxin**. From there it is passed successively to ubiquinone, to cytochrome *b*, and to cytochrome *f*, and finally back to the positively charged bacteriochlorophyll. Essentially, the electron has gone around in a cycle, beginning with, and returning to, bacteriochlorophyll. This relatively simple process is illustrated in Fig. 10-12.

The energy released in the step between cytochrome *b* and cytochrome *f* is used for **photophosphorylation**—the generation of ATP from ADP and inorganic phosphate.

Note that no $NADP^+$ has been reduced in these reactions. The reduction of $NADP^+$ in photosynthetic bacteria is accomplished not by photosynthesis but by using reducing power from constituents of the environment, such as H_2S and other inorganic and organic compounds. Such reduced compounds usually abound in the anaerobic environment of photosynthetic bacteria.

It may be added that light of higher energy than that absorbed by bacteriochlorophylls can contribute to bacterial photosynthesis since there are carotenoids and other accessory pigments in the bacterial cells which absorb light at shorter wavelengths and transfer the energy to the bacteriochlorophylls.

In plants, algae, and cyanobacteria (oxygenic photosynthetic bacteria), noncyclic photophosphorylation occurs in photosynthesis. In this process, when a molecule in pigment system II (one of two systems of light reactions) absorbs light, this energy raises the molecule to an excited state and the molecule releases an electron. This electron is transferred to plastoquinone, to cytochrome *b*, to cytochrome *f*, and finally to pigment system I. Photophosphorylation occurs with generation of ATP from ADP and inorganic phosphate in the step between cytochrome *b* and cytochrome *f*. When pigment system I absorbs light, it releases an electron. This electron is transferred from ferredoxin, to flavoprotein, to $NADP^+$. Photophosphorylation occurs again between the release of the electron from pigment system I to ferredoxin. Also note that $NADP^+$ is reduced in this part of the process (see Fig. 10-13). This process differs from cyclic photophosphorylation because the electron lost by pigment system II is not cycled back to it. Instead, electrons are replaced in pigment system II by the light-generated breakdown of water, called **photolysis**. There is some evidence that this scheme of noncyclic photophosphorylation, shown in Fig. 10-13, may have to be modified. It appears that system II pigments alone can carry out the entire process

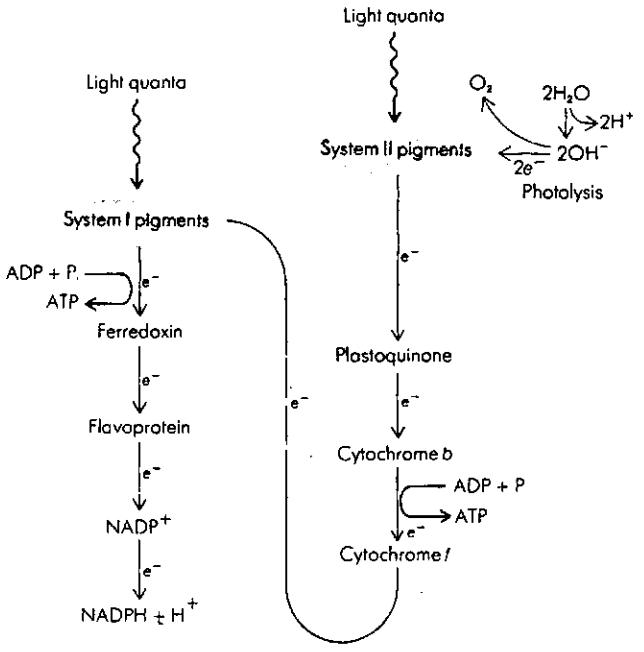


Figure 10-13. Noncyclic photophosphorylation as it occurs in green plants, algae, and cyanobacteria. In this process, electrons raised to a high energy state ultimately reduce $NADP^+$ and are not recycled to the light-pigment systems. The protons necessary for reduction come from the dissociation of water, which results in evolution of oxygen. Electrons are restored to the pigments of system II from the OH^- ion of H_2O. The OH^- ion is split to e^-, H^+, and $\frac{1}{2}$ O_2 by photolysis.

of noncyclic photophosphorylation. Thus the noncyclic reduction of ferredoxin need not involve system I pigments. Further, the most important role of plastoquinone is in the transport of protons originating from water. This modified process has been termed oxygenic photophosphorylation. Further evidence is needed for its confirmation.

THE MECHANISM OF ATP SYNTHESIS

The chemical reactions that lead to the synthesis of ATP are now well understood. But how the transfer of electrons through the respiratory transport chain is coupled to the synthesis of ATP is not very clear. Several alternate hypotheses have been proposed to explain how energy released during electron transport is conserved in the form of ATP. The prevailing theory is the chemiosmotic hypothesis advanced in 1961 by Peter Mitchell, a British biochemist. Mitchell was awarded the Nobel prize for his work in this field in 1978. According to this theory, the flow of electrons through the system of carrier molecules releases energy which drives positively charged hydrogen ions (H^+), or protons, across the membranes of chloroplasts, mitochondria, and bacterial cells (Fig. 10-14). This movement of hydrogen ions results in the acidification of the surrounding medium and the generation of a pH gradient (a difference in pH) across the organelle or cell membrane. In addition, such hydrogen-ion movements lead to the formation of an electric potential gradient (a difference in charge) across the membrane (since an electric charge is carried by the proton). In this way, energy released during the transfer of electrons through the respiratory chain is conserved as a "protonmotive force"; the electric potential gradients are produced by pumping hydrogen ions across the membrane.

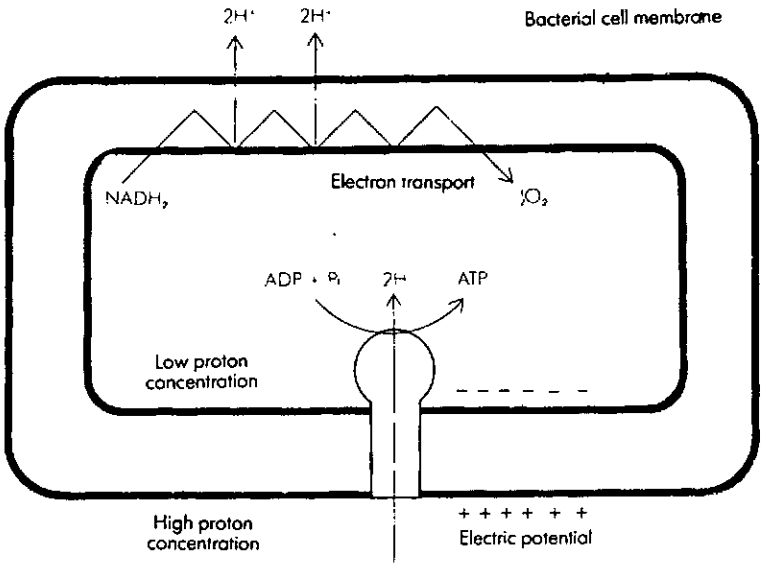


Figure 10-14. Mechanism of ATP synthesis. Flow of electrons through the respiratory chain drives hydrogen ions across the membrane. This results in a high hydrogen-ion concentration outside the cell and a low concentration inside the cell. This produces a pH and electrochemical gradient. ATP synthesis at the site of the ATPase complex (a knobbed structure on the membrane) is driven by the release of energy when hydrogen reenters the bacterial cell.

Following this first energy-conservation step, when the hydrogen ions reenter the organelle or cell, they are transported by the membrane-bound enzyme adenosine triphosphatase. The energy released on reentry drives the synthesis of ATP, the second energy-conservation step. This process is shown in Fig. 10-14.

QUESTIONS

1 What is meant by the following?
   (a) Free-energy change
   (b) Exergonic reaction
   (c) Endergonic reaction
   (d) ΔG, ΔG°, $\Delta G^{\circ\prime}$
2 Explain the relationship between ΔG° and the equilibrium constant, and the relationship between ΔG° and an oxidation-reduction potential difference.
3 What role does ATP play in energy exchanges in cells?
4 In what way is the coupling of exergonic reactions with endergonic reactions important in living organisms?
5 Define the meaning of a high-energy-transfer compound. Name those that occur in the glycolytic pathway.
6 Explain what is meant by an oxidation-reduction system.
7 What is oxidative phosphorylation? Where does it occur in the respiratory chain?
8 Briefly explain how glycolysis fits into the metabolism of glucose in aerobic cells.
9 Compare the disposition of electrons (or hydrogen atoms) obtained from the oxidation of glyceraldehyde-3-phosphate in aerobic and anaerobic cells.
10 Identify the three reactions in the glycolytic pathway that are not freely reversible by the same specific enzymes.

11 Account for the ATP yield per glucose molecule in glycolysis.
12 Describe the various ways in which the pentose phosphate cycle is useful to a cell.
13 Is the Entner-Doudoroff pathway found in (*a*) both aerobes and anaerobes; (*b*) both eucaryotes and procaryotes?
14 Explain why fermentation is a less efficient process for obtaining energy than aerobic respiration.
15 Explain how fermentation products can be used for the identification of bacteria. Provide specific examples to support your answer.
16 Why is the TCA cycle called an amphibolic cycle?
17 What general rule governs the utilization of substances other than carbohydrates for the production of energy?
18 Name the specific enzymes of the glyoxylate cycle and describe the reactions they catalyze.
19 What are the essential differences between photosynthesis by bacteria and by algae?
20 Explain why photophosphorylation in procaryotes is termed *cyclic*.
21 Describe the prevailing model for the mechanism of ATP synthesis.

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