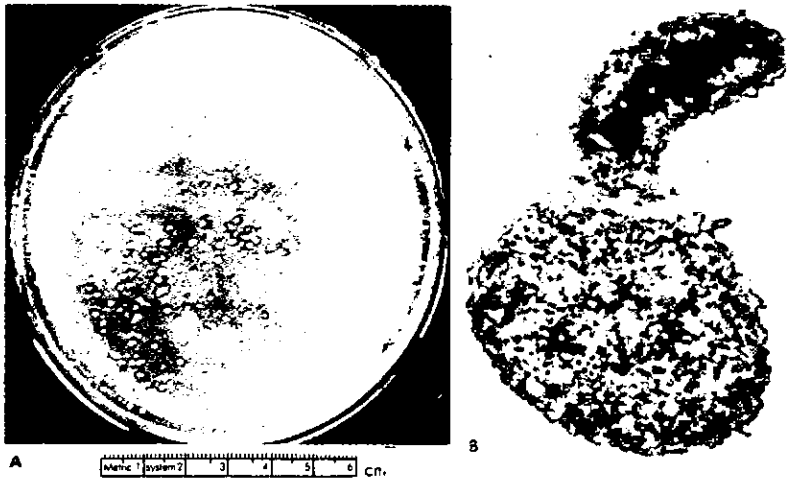

Figure 25-6. Bacteriolysis produced by *Bdellovibrio bacteriovorus*. (A) Plate culture of *B. bacteriovorus* on a lawn of *E. coli* showing whitish-gray colony surrounded by circular plaque-like clearing zone. Central colony consists of bdellovibrios and the clear zone contains a few intact *E. coli* cells and spheroplasts of the host cells (*E. coli*). (B) Electron micrograph thin section showing *B. bacteriovorus* penetration into *E. coli* cell (X48,000). (*Courtesy of J. C. Burnham, T. Hashimoto, and S. F. Conti, J Bacteriol*, 96:1366, 1968.)

to a host cell at a special region and eventually causes the lysis of that cell (see Chap. 13). As a consequence, plaquelike areas of lysis (Fig. 25-6) appear when these parasites are plated along with their host bacteria. There are also many strains of fungi which are parasitic on algae and other fungi by penetration into the host.

Viruses which attack bacteria, fungi, and algae are strict intracellular parasites since they cannot be cultivated as free-living forms. The phenomenon of lysogeny is quite important because of the possibility for genetic recombination in natural populations and the subsequent expression of new characteristics.

BIOGEOCHEMICAL ROLE OF SOIL MICROORGANISMS

Soil microorganisms serve as biogeochemical agents for the conversion of complex organic compounds into simple inorganic compounds or into their constituent elements. The overall process is called **mineralization**. This conversion of complex organic compounds into inorganic compounds or elements provides for the continuity of elements (or their compounds) as nutrients for plants and animals including people.

It is possible to construct a sequence of reactions to illustrate that microorganisms perform an essential role in maintaining a cyclic process for the reutilization of elements under natural conditions. In this respect we can view the planet earth as a closed system dependent upon the process of recycling for maintenance of life as we know it.

In the following paragraphs we shall discuss the role of soil microorganisms with respect to the transformations they bring about on nitrogen, carbon, sulfur, phosphorus, and their compounds.

BIOCHEMICAL TRANSFORMATIONS OF NITROGEN AND NITROGEN COMPOUNDS: THE NITROGEN CYCLE

Because of the importance of nitrogen for plant nutrition, the biochemical events that make up the nitrogen cycle have been studied in considerable detail.

The sequence of changes from free atmospheric nitrogen to fixed inorganic nitrogen, to simple organic compounds, to complex organic compounds in the tissues of plants, animals, and microorganisms, and the eventual release of this

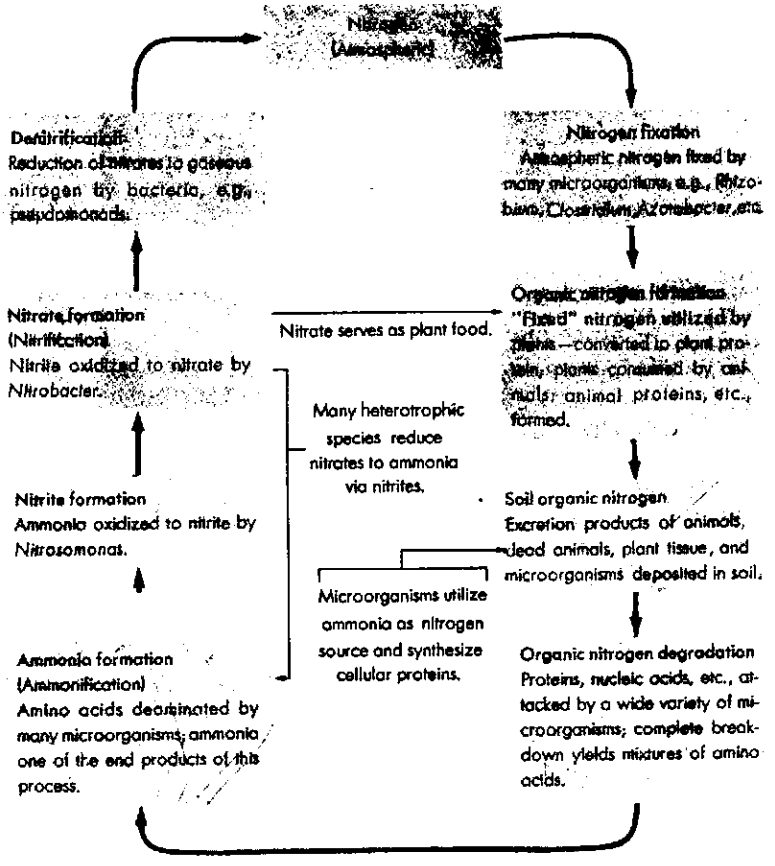

Figure 25-7. Nitrogen cycle in nature (schematic), showing the role of microorganisms.

nitrogen back to atmospheric nitrogen is summarized in Fig. 25-7, the nitrogen cycle.

Proteins, nucleic acids, purine and pyrimidine bases, and amino sugars (glucosamine and galactosamine) represent the complex organic nitrogenous substances which are deposited in soil in the form of animal and plant wastes or their tissues. Synthetic processes of microorganisms also contribute some amount of complex organic nitrogen compounds.

The simplest form of nitrogen involved in biological transformations is gaseous elementary nitrogen. The overall transformations in which microorganisms are involved range from nitrogen gas to protein. A great many intermediate products and a corresponding large number of intricate enzymatic reactions are involved in bringing about these changes.

Some of the biochemical events in the nitrogen cycle are summarized below.

Proteolysis

The nitrogen in proteins (as well as in nucleic acids) may be regarded as the end of the line as far as synthesis of nitrogenous compounds is concerned. The nitrogen in proteins is "locked" and is not available as a nutrient to plants. In

order to set this organically bound nitrogen free for reuse, the first process that must take place is the enzymatic hydrolysis of proteins (proteolysis). This is accomplished by microorganisms capable of elaborating extracellular proteinases that convert the protein to smaller units (peptides). The peptides are then attacked by peptidases, resulting ultimately in the release of individual amino acids. The overall reactions may be summarized:

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

Some bacterial species elaborate large amounts of proteolytic enzymes. Among the most active in this respect are some of the clostridia, e.g., *Clostridium histolyticum* and *C. sporogenes*; a lesser degree of activity is found in species of the genera *Proteus*, *Pseudomonas*, and *Bacillus*. Many fungi and soil actinomycetes are extremely proteolytic. Peptidases, however, occur widely in microorganisms as demonstrated by the fact that peptones (partially hydrolyzed proteins) are a common constituent of bacteriological media and provide a readily available source of nitrogen.

Amino Acid Degradation: Ammonification

The end products of proteolysis are amino acids. Their fate in the soil may be utilization as nutrients by microorganisms or degradation by microbial attack. Amino acids are subject to a variety of pathways for microbial decomposition. We are concerned here with the liberation of nitrogen from these compounds, which is accomplished by deamination, i.e., removal of the amino group. Although several variations of deamination reactions are exhibited by microorganisms, one of the end products is always ammonia, NH_3. An example of a specific deamination reaction is

$$\underset{\text{Alanine}}{CH_3CHNH_2COOH} + \tfrac{1}{2}O_2 \xrightarrow[\text{deaminase}]{\text{alanine}} \underset{\text{Pyruvic acid}}{CH_3COCOOH} + \underset{\text{Ammonia}}{NH_3}$$

This reaction is classified as an oxidative deamination. Many microorganisms can deaminate amino acids. The production of ammonia is referred to as ammonification. The fate of the ammonia thus produced varies, depending upon conditions in the soil. Ammonia is volatile and, as such, leaves the soil; however, if solubilized, NH_4^+ is formed. Some of the subsequent possibilities include accumulation and utilization by plants and microorganisms and, under favorable conditions, oxidation to nitrates.

Nitrification

Microorganisms convert ammonia to nitrate, and the process is called nitrification. The process occurs in two steps, each step performed by a different group of bacteria.

1 Oxidation of ammonia to nitrite by ammonia-oxidizing bacteria

$$2NH_3 + 3O_2 \rightarrow 2HNO_2 + 2H_2O$$

2 Oxidation of nitrite to nitrate by nitrite-oxidizing bacteria

$$HNO_2 + \tfrac{1}{2}O_2 \rightarrow HNO_3$$

Bacteria of both physiological groups, ammonia oxidizers and nitrite oxidiz-

Table 25-3. Composition of Medium for Isolation of Nitrifying Bacteria Using Enrichment Culture Technique

Ingredients	g/L
$(NH_4)_2SO_4$	2.0
K_2HPO_4	1.0
$MgSO_4$	0.5
$FeSO_4$	0.4
NaCl	0.4
$CaCO_3$	1.0
$MgCO_3$	1.0

ers, are Gram-negative chemolithotrophs. Their main source of carbon is obtained through carbon dioxide fixation; energy is derived by the oxidation of NH_3 or NO_2^- depending upon the group. Nitrifying bacteria occur widely in nature in a variety of habitats, including soil, sewage, and aquatic environments.

Nitrifying bacteria cannot be isolated directly by the usual techniques employed to isolate heterotrophic bacteria. Some of the reasons for this are: they are slow-growing compared with heterotrophs; and they may be present in very small numbers compared with other physiological types. Accordingly, enrichment cultures are used for their isolation. An example of a medium for this purpose is shown in Table 25-3. A relatively large inoculum is used, and incubation is in the dark at 25 to 30°C for a period of 1 to 4 months.

Species of ammonia-oxidizing bacteria vary in morphology (rod, spherical, spiral, or lobular) and usually have an extensive membrane system within their cytoplasm. They frequently form cysts and zooglea. See Fig. 25-8. The following species have been recognized as ammonia oxidizers:

Nitrosomonas europaea
Nitrosovibrio tenuis
Nitrosococcus nitrosus
Nitrosococcus oceanus

Species of nitrite-oxidizing bacteria exhibit some of the same morphological characteristics as the ammonia oxidizers. Only a few species have been isolated and described. These include *Nitrobacter winogradskyi* and *Nitrospina gracilis*.

An interesting historical event involving nitrification and production of gunpowder may be cited. During the Napoleonic wars, France was unable to import nitrate, which was needed for the manufacture of gunpowder. To solve this

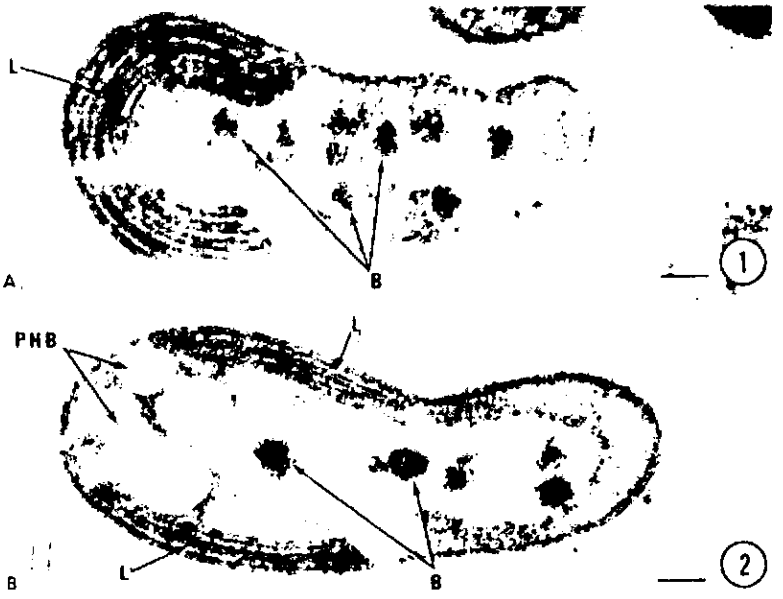

Figure 25-8. Ultrastructure of *Nitrobacter winogradskyi*. (A) Thin section from cell grown chemoautotrophically and harvested during exponential phase of growth, showing lamellar membrane system (L) at the swollen end of the cell and electron-dense polyhedral bodies (B). (B) Thin section from cell grown on nitrite mineral-salts medium supplemented with 5 mmol sodium acetate and harvested during exponential phase of growth. Section shows lamellas (L), polyhedral bodies (B), and electron-transparent bodies believed to be poly-β-hydroxybutyrate (PHB) reserve material. (*Courtesy of L. M. Pope, D. S. Hoare, and A. J. Smith, J Bacteriol, 97:936, 1969.*)

dilemma, artificial niter beds were made, consisting of soil mixed with animal waste and vegetable materials, ashes, etc. Aeration was performed by turning the heap over from time to time. After a long period of incubation, crude saltpeter (mineral nitrates) was extracted with hot water. This occurred, of course, long before the specific activities of microorganisms were known. Nitrification was discovered to be a biological process by Schloesing and Muntz in 1877; Winogradsky isolated the bacteria responsible for the process in 1890.

Reduction of Nitrate to Ammonia

Several heterotrophic bacteria are capable of converting nitrates into nitrites or ammonia. This normally occurs under anaerobic conditions, e.g., in waterlogged soil. The oxygen of the nitrate serves as an acceptor for electrons and hydrogen. The process involves several reactions, and the overall result is

$$HNO_3 + 4H_2 \rightarrow NH_3 + 3H_2O$$

This reaction is not of major significance in well-cultivated agricultural soil.

Denitrification

The transformation of nitrates to gaseous nitrogen is accomplished by microorganisms in a series of biochemical reactions. The process is known as **denitrification.** From the standpoint of agriculture, this is an undesirable process in that it results in loss of nitrogen from the soil and hence a decline in nutrients for plant growth.

Species of several genera of bacteria are capable of transforming NO_3^- to N_2, e.g., *Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium, Hyphomicrobium, Pseudomonas, Thiobacillus,* and *Vibrio.*

The overall biochemical reaction which expresses the process of denitrification is

$$\underset{\text{Nitrate}}{2NO_3^-} \rightarrow \underset{\text{Nitrite}}{2NO_2^-} \rightarrow \underset{\text{Nitric oxide}}{2NO} \rightarrow \underset{\text{Nitrous oxide}}{N_2O} \rightarrow \underset{\text{Nitrogen}}{N_2}$$

Experimental results which illustrate the order in which products rise and fall during denitrification are shown in Fig. 25-9.

Environmental conditions in a soil have a significant effect on the level of denitrification. For example, the process is enhanced in soils (1) by an abundance of organic matter, (2) by elevated temperatures (25 to 60°C), and (3) by neutral or alkaline pH. Availability of oxygen has a dual effect. Denitrification proceeds only when the oxygen supply is limited. However, oxygen is necessary for nitrite and nitrate formation.

Nitrogen Fixation

A number of microorganisms are able to use molecular nitrogen in the atmosphere as their source of nitrogen. The conversion of molecular nitrogen into ammonia is known as **nitrogen fixation.** Two groups of microorganisms are involved in this process: (1) **nonsymbiotic** microorganisms, those living freely and independently in the soil; and (2) **symbiotic** microorganisms, those living in roots of plants. Several types of experiments are used to detect nitrogen fixation by microorganisms. One approach is to demonstrate growth in a nitrogen-free medium. More specific evidence of fixation can be obtained by cultivating the microorganism in the presence of nitrogen labeled with isotopic nitrogen. $^{15}N_2$ can be measured by using a mass spectrometer. In essence, after

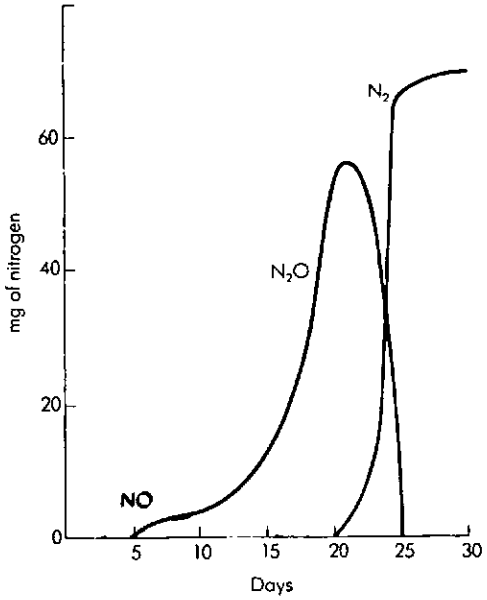

Figure 25-9. Sequence of products during denitrification in Norfolk sandy loam. (*Courtesy of F. B. Cady and W. V. Bartholomew, Soil Sci Soc Am Proc,* **24**:*477, 1960.*)

Table 25-4. Some Examples of Nitrogen-Fixing Bacteria

Cyanobacteria
- *Anabaena* spp.
- *Nostoc* spp.
- *Gloeotrichia* spp.
- *Synechococcus* spp.
- *Plectonema* spp.
- *Oscillatoria* spp

Phototrophic bacteria
- *Rhodospirillum rubrum*
- *Rhodopseudomonas palustris*
- *Rhodomicrobium vannielii*
- *Chromatium vinosum*
- *Chlorobium thiosulfatophilum*

Chemotrophic bacteria
- *Azospirillum lipoferum*
- *Azotobacter chroococcum*
- *Beijerinckia indica*
- *Rhizobium leguminosarum*
- *Methylomonas methanitrificans*
- *Escherichia coli*
- *Enterobacter aerogenes*
- *Bacillus macerans*
- *Clostridium butyricum*
- *Xanthobacter autotrophicus*

the organism is grown in the mixture of atmospheric nitrogen and $^{15}N_2$, the culture is examined for evidence of $^{15}N_2$ incorporated in any compounds. Its presence is positive proof that nitrogen has been fixed. Under suitable conditions an increase of as little as 0.001 μg of nitrogen can be detected by this technique.

The capability of the nitrogen-fixing enzyme to act upon acetylene, discovered in the mid-1960s, has led to the development of a simple, rapid, relatively inexpensive technique now widely used to measure nitrogen fixation. The test is based on the observation that the nitrogen-fixing enzyme (*nitrogenase*) interacts with triple-bonded compounds, e.g., acetylene, to form ethylene as follows:

$$\underset{\text{Acetylene}}{HC{\equiv}CH} \xrightarrow[\text{enzyme nitrogenase}]{2H} \underset{\text{Ethylene}}{H_2C{=}CH_2}$$

The comparable reaction with nitrogen is

$$\underset{\text{Nitrogen}}{N{\equiv}N} \xrightarrow[\text{enzyme nitrogenase}]{6H} \underset{\text{Ammonia}}{2NH_3}$$

The technique involves exposing the specimen being assayed for nitrogenase activity to acetylene in a suitable vessel and, after a period of incubation, analyzing the gas phase for ethylene by gas-liquid chromatography. The amount of ethylene produced is a measure of nitrogenase activity.

The essential reactants in the bacterial nitrogen fixation process are:

1 **The nitrogenase enzyme complex.** This has been characterized as two components, and neither is active without the other. Component I is nitrogenase and

component II is nitrogenase reductase. Component I is known as the MoFe protein (Mo for molybdenum, Fe for iron). Component II, which is a smaller molecule is designated the Fe protein. Both molecules contain sulfur.

2 A strong reducing agent such as ferredoxin or flavodoxin
3 ATP
4 A regulating system for NH_3 production and utilization
5 A system that protects the nitrogen-fixing system from inhibition by molecular oxygen

The overall biochemical reaction for nitrogen fixation can be expressed as:

$$N_2 + 6e^- + 12ATP + 12H_2O \xrightarrow[\text{complex}]{\text{nitrogenase}} 2NH_4^+ + 12ADP + 12P_i + 4H^+$$

Nonsymbiotic Nitrogen Fixation

Nonsymbiotic nitrogen fixation has been studied extensively with *Clostridium pasteurianum* and species of *Azotobacter*. For many years, these bacteria were the only ones known to be capable of this activity. The former is an anaerobic bacillus, and the latter are aerobic oval to spherical cells; both are widely distributed in soils. The nitrogen-fixing capacity of the *Azotobacter* species is greater than that of *Cl. pasteurianum*. In recent years many other microorganisms have been found to fix nitrogen (see Table 25-4).

It has been estimated that the amount of nitrogen fixed by the nonsymbiotic process ranges between 20 and 50 lb/acre annually. This estimate is no doubt subject to much variation depending upon the conditions peculiar to a particular soil.

Symbiotic Nitrogen Fixation

Symbiotic nitrogen fixation is accomplished by bacteria of the genus *Rhizobium* in association with legumes (plants that bear seeds in pods, e.g., soybeans, clover, and peas). Before these bacteria can fix nitrogen, they must establish themselves in the cells of root tissue of the host plant. Infection of the root system by the rhizobia bacteria is closely associated with the formation of an "infection thread" that develops in certain root hairs (see Fig. 25-10). The nitrogen-fixing bacteria invade the host plant cells via this infection thread. Some of the cells of the plant are thus infected, causing cell enlargement and an increased rate of cell division, leading to the formation of abnormal growths (nodules) on the root system. Several types of nodulation are illustrated in Fig. 25-11.

The legume, the bacteria, and the nodule constitute the system for this type of nitrogen fixation. It is a process where both the bacteria and the plant benefit by the association. The bacteria convert atmospheric nitrogen to fixed nitrogen which is available to the plant, and in turn, the bacteria derive nutrients from the tissues of the plant.

Not all species of *Rhizobium* produce nodulation and nitrogen fixation with any legume. There is a degree of specificity between the bacteria and legumes. For purposes of inoculation with commercial preparations of these bacteria, legumes are divided into seven major categories as follows: alfalfa, clover, peas and vetch, cowpeas, beans, lupines, and soybeans. *Rhizobium* species or strains effective for one group are less effective or ineffective for other groups. Even within a species, certain strains are more effective than others with a given host plant. Evidence of this specificity is demonstrated in Fig. 25-12.

Figure 25-10. Nodule formation by *Rhizobium* on legume plants. The first stage in the establishment of the *Rhizobium*-legume N_2-fixing symbiosis is the infection of the host legume by the appropriate *Rhizobium* species. Root hairs are the site of infection. The first microscopically visible indication of the bacteria-plant interaction is deformation and curling of the normally straight root hairs. Aseptically cultured clover seedling with undeformed root hairs is shown (A), × 40. (B) A clover seedling (× 40) inoculated with *R. trifolii*. The bacteria are in clumps (flocs) in the rhizosphere. Note the change in appearance of the root hairs. A characteristic deformation is curling at the root hair tip to produce a "shepherd's crook" (C). The bacteria enter the root hair and are enclosed in a tubular structure, the infection thread (C and D), which is the first microscopically visible sign of a successful infection. The bacteria appear to enter the root hair by a process of invagination. Root hair cell-wall growth is redirected at a localized point resulting in the wall growing back into the root hair to form the tubular infection thread. There is no direct penetration through the root hair cell wall, and the bacteria remain extracellular within the infection thread. (E, F, G, H) A serial section sequence through a root hair which had a shepherd's crook at the origin of the infection thread. (E) A diagrammatic illustration of a serial sectioned root hair showing the infection thread (IT), nucleus (N), and the initiation of sectioning (top arrow). (F) A section before the invagination showing the infection thread (IT) which contained bacteria (B). The arrows indicate the region of the root hair cell wall where the invagination process has begun (× 4,500). (G) A section through the middle of the invagination showing the infection thread wall (arrows) of the pore, bacteria (B) within the infection thread, and the root hair nucleus (× 4,500). (H) A section past the pore; the arrows point out where the wall of the pore is grazed by the knife (× 4,500). Bacteroids within a nodule (I) are surrounded by membrane which is believed to be derived from the plant. The bacteroids contain electron-dense, unidentified inclusions. (*Courtesy of C. A. Napoli and H.Hubbell, Appl Microbiol,* **30**:*1003, 1975.*)

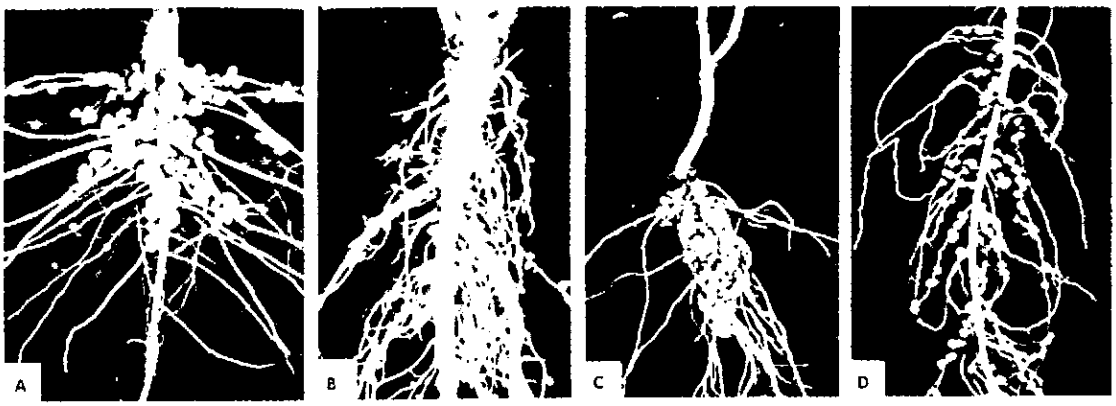

Figure 25-11. Root nodules are produced by effective strains of bacteria on several legumes: (A) soybean, (B) sweet clover, (C) pea, (D) Birdsfoot trefoil. (*Courtesy of the Nitragin Co., Inc.*)

Figure 25-12. Different strains of rhizobia have different effects on the growth of clover. Tests are carried out on Crimson clover in the following manner: Seeds are planted in sterile sand contained in a jar. The sand is then inoculated with the bacteria. Each jar contains a solution of nutrients—except nitrogen—which diffuse through the sand. Thus the extent of growth is indicative of the amount of nitrogen being supplied by the bacteria. (A) was not inoculated; (B), (C), and (D) were inoculated with different strains of rhizobia. Note the difference in growth response. (*Courtesy of L. W. Erdman, USDA.*)

Inoculation of seeds before planting is a desirable practice, since not all agricultural soils contain the right kinds of bacteria for optimum symbiotic nitrogen fixation with legume crops. Most of the commercial preparations consist of selected strains of bacteria dispensed in moist humus. This material is mixed with water and sprinkled over the seeds prior to planting.

Recombinant DNA and Nitrogen Fixation (Genetic Engineering)

The vast amount of knowledge that has accumulated in the last decade about microbial genetics, including the development of highly sophisticated techniques for gene splicing and cloning, has led to some dramatic developments in the field commonly referred to as genetic engineering. Applied aspects of this development are discussed later in Chap. 29, Industrial Microbiology. Nevertheless, we wish to mention here that many laboratories and research scientists

are concentrating their efforts on the possibility of developing new systems for nitrogen fixation using recombinant DNA technology.

One area of research is directed toward introducing the "package" of nitrogen-fixing genes from bacteria into plant cells. If this were achieved, plants might be capable of directly fixing nitrogen from the atmosphere. This would be a tremendous advance not only for agriculture but for the world at large in terms of producing food more economically and abundantly. Obviously, considerably more research is necessary before this kind of genetic engineering can be attempted at a practical level. For instance, nitrogenase is easily destroyed by oxygen, and some means of protection of this enzyme complex from oxygen would have to be provided in order for a plant cell to be able to fix nitrogen.

Alternatively, it may be possible to modify certain bacteria in a manner so that they would develop a relationship with the root system of other plants, as the *Rhizobium* species grow with legumes. For example, a symbiotic bacterial nitrogen-fixing system with cereal grains would have a tremendous effect on grain production both in yield and cost.

BIOCHEMICAL TRANSFORMATIONS OF CARBON AND CARBON COMPOUNDS: THE CARBON CYCLE

The ultimate source of organic carbon compounds in nature is the carbon dioxide present in the atmosphere (or dissolved in water). The process, carbon dioxide fixation, was discussed in Chap. 11. Although green plants and algae are the most important agents of carbon dioxide fixation, bacteria are also capable of synthesizing organic matter from inorganic carbon. The occurrence of photosynthesis among microorganisms has already been described. Other examples of carbon dioxide transformation or incorporation into organic compounds by bacteria are:

Carbon Dioxide Fixation

1 Utilization of carbon dioxide by autotrophic bacteria; the carbon dioxide represents the sole source of carbon for these organisms and is transformed by a reduction reaction to carbohydrates. The general reaction is

$$CO_2 + 4H \rightarrow (CH_2O)_x + H_2O$$

2 Carbon dioxide fixation by heterotrophic microorganisms is common among bacteria. A specific example of this type of reaction is

$$\underset{\text{Pyruvic acid}}{CH_3COCOOH} + CO_2 \rightarrow \underset{\text{Oxalacetic acid}}{HOOCCH_2COCOOH}$$

Organic Carbon Compound Degradation

The organic carbon compounds that eventually are deposited in the soil are degraded by microbial activity. The end product, carbon dioxide, is released into the air and soil. Fresh air contains approximately 0.03 percent carbon dioxide by volume. Bacteria and fungi are the principal microorganisms that degrade organic carbon compounds.

Under most natural systems of vegetation, e.g., forests, the amount of organic material in the soil remains approximately the same from year to year. This results from a balance established between the annual litter fall and death of the plants and the capacity of microorganisms to degrade these tissues.

The most abundant organic material in plants is cellulose. It is readily attacked by many species of bacteria and fungi. The initial enzymatic attack is by cel-