Microorganisms play a major role in the nitrogen cycle. A unique group of bacteria fix atmospheric nitrogen (dinitrogen) into ammonia and assimilate the ammonia into amino acids. Certain nitrogen-fixing bacteria have established a symbiotic relationship with plants where they fix atmospheric nitrogen into forms that can be utilized by the host plants. Degradation of nitrogenous compounds by microorganisms is also an important aspect of the nitrogen cycle. Without this degradation and subsequent return of nitrogen from a wide variety of complex natural and artificial compounds to the nitrogen cycle, higher forms of life could not exist. An in-depth knowledge of these processes may aid materially in overcoming the imbalances created in the nitrogen cycle by overloading it with waste and excretory materials and improper use of agricultural lands by overabundant applications of fertilizers, herbicides, and pesticides.

In this chapter, we consider the contributions of microorganisms to the nitrogen cycle and the underlying mechanisms of the processes of nitrogen fixation, metabolism of inorganic nitrogen compounds, and assimilation of inorganic nitrogen into amino acids. While urea is not an inorganic compound, it is widely used as a fertilizer because it is readily broken down into carbon dioxide and ammonia. For this reason, urea metabolism is also described in this chapter. Reactions involved in the interconversion of the amino acids, especially amino group transfer (transamination), and other important reactions of amino acids are discussed. The biosynthesis of amino acids, purines, pyrimidines, and other nitrogen-containing compounds is covered in Chapters 15 and 16.

**BIOLOGICAL NITROGEN FIXATION**

Dutch farmers associated the establishment of rich stands of clover with the improvement of subsequent crops grown in the same fields. Although they were
unaware of the precise nature of the relationship, they noted the development of nodules on the root system of clover. These observations provided the foundations for systematic crop rotation implemented by Townsend in England during the agricultural revolution of the eighteenth century. The photograph in Figure 14-1 shows extensive nodule development on the root system of a soybean plant, which is a legume. A legume is a plant that bears seed pods, such as pea or soybean. These plants often form a symbiotic association with bacteria that form prominent nodules on the roots. These nodules in the root system harbor millions of bacteria that convert atmospheric nitrogen (dinitrogen) to ammonia (NH₃).

The works of Atwater and of Hellriegel and Wilfarth, published in the 1880s, were among the first to provide scientific data supporting the importance of the root nodules and the bacteria within them in the process of nitrogen fixation by clover and other leguminous plants. Despite criticisms by scientists of the time, their reports initiated a concerted effort to determine the nature of the symbiotic organisms associated with the

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Fig. 14-1. Extensive root nodule development in the root system of soybean (Glycine max), a leguminous plant. (Photo courtesy of R. S. Smith, Milwaukee, WI.)
root nodules of legumes (and other plants), as well as characterization of the numerous free-living microorganisms capable of nitrogen fixation.

For many years, it has been considered that the ammonia produced by the bacteria in the nodule was transferred to the plant where it was then assimilated into amino acids and other nitrogenous compounds. More recently, it has been determined that under certain conditions the bacteria assimilate the ammonia into the amino acid alanine, which is then transported into the plant system. A number of examples of nitrogen-fixing organisms and their associated plant partners are listed in Table 14-1.

Symbiotic nitrogen-fixing organisms and the photosynthetic nitrogen fixers appear to account for most of the atmospheric nitrogen assimilated into organic forms in nature. Under conditions in which fixed nitrogen is low, root-nodulated angiosperms and gymnosperms and the cyanobacteria may be especially valuable in improving soil fertility. The estimated amount of nitrogen fixed by symbiotic bacterial-inoculated legumes in the United States approximately equals the amount of nitrogen supplied by farmers as nitrogen fertilizer. In the past, the contribution of free-living forms was probably underestimated. With the introduction of the acetylene reduction technique* for assessing the nitrogen-fixing potential of organisms in natural environments, it has been possible to show the presence of nitrogen-fixing organisms in a number of settings, including the intestinal tract of nonruminant mammals. Although free-living organisms, in general, appear less efficient in their ability to fix nitrogen, their number, variety, and ubiquitous distribution suggest that they are of major ecological importance. The cyanobacteria have a distinct advantage in that the energy (as ATP) and the reducing power (as NADH) required for nitrogen fixation can be supplied by photosynthesis, a process that makes it possible for them to become established in environments unfavorable for the development of other nitrogen-fixing organisms.

Rapidly rising energy and labor costs have made it less economical to increase plant growth by the use of ammonia fertilizer. Commercial production of ammonia by the Haber-Bosch process (catalyst-mediated reduction of hydrogen with nitrogen under high pressure and temperature) is expensive. Furthermore, continued application of chemical fertilizers at a high rate has threatened water supplies and the ecological balance of rivers and streams. Thus, attention has been focused on the improvement of plant yields through the development of new associations between nitrogen-fixing bacteria and plants. In the pursuit of this goal, intensive efforts have been directed toward studies of the genetics, biochemistry, and ecology of both free-living and symbiotic nitrogen-fixing organisms. It has already become apparent that the photosynthetic capacity of plants is one limiting factor in nitrogen fixation. Work on the selection and breeding of plant strains with greater photosynthetic efficiency has also been intensified. Another long-range goal has been to attempt development of an association between nitrogen-fixing bacteria and the root system of highly efficient photosynthetic plants such as corn.

* Originally, mass spectrometry was used to measure the amount of 15N-labeled nitrogen gas reduced to ammonia by nitrogenase. The discovery that nitrogenase can reduce acetylene to ethylene proved very useful, since this reaction can be measured by the somewhat simpler technique of gas chromatography.
TABLE 14-1. Examples of Nitrogen-Fixing Organisms

*Symbiotic Association of Various Genera with Leguminous Plants*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azorhizobium caulindans</td>
<td>tropical legume (Sesbania rostrata)</td>
</tr>
<tr>
<td>Allorhizobium undicola</td>
<td>lotus (Lotus albus)</td>
</tr>
<tr>
<td>Bradyrhizobium japonicum</td>
<td>soybean (Glycine max)</td>
</tr>
<tr>
<td>Mesorhizobium amorphae</td>
<td>false indigo (Amorpha fruticosa)</td>
</tr>
<tr>
<td>Rhizobium trifolii</td>
<td>clover (Trifolium, Crotalaria)</td>
</tr>
<tr>
<td>Sinorhizobium meliloti</td>
<td>alfalfa (Medicago sativa)</td>
</tr>
</tbody>
</table>

*Symbiotic Association of Actinomycetes with Angiosperms*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frankia sp.</td>
<td>alder (Alnus)</td>
</tr>
<tr>
<td>Frankia sp.</td>
<td>bog myrtle or sweet gale (Myrica)</td>
</tr>
<tr>
<td>Frankia sp.</td>
<td>oleasters (Shepherdia, Eleagnus, Hippophae)</td>
</tr>
<tr>
<td>Frankia sp.</td>
<td>New Jersey tea (Ceanothus)</td>
</tr>
</tbody>
</table>

*Symbiotic Association with Leaf-Nodulating Plants*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella aerogenes</td>
<td></td>
</tr>
</tbody>
</table>

*Symbiotic Association of Marine Bacteria with Bivalves*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic, chemoheterotrophic sp.</td>
<td>bivalves (Teredinidae)</td>
</tr>
</tbody>
</table>

*Symbiotic Association with Marine Diatoms*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Richelia intracellularis</td>
<td>(cyanobacterium), Rhizoselena</td>
</tr>
</tbody>
</table>

*Associative Interaction with Grasses*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azospirillum brasiliense</td>
<td>tropical grasses</td>
</tr>
<tr>
<td>Azospirillum lipoferum</td>
<td>tropical grasses, maize</td>
</tr>
<tr>
<td>Azotobacter paspali</td>
<td>tropical grass (Paspalum notatum)</td>
</tr>
</tbody>
</table>

*Free-Living Bacteria and Cyanobacteria*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic, heterotrophic</td>
<td>Azotobacter, Derxia, Azomonas, Biejerinka, Nocardia, Pseudomonas</td>
</tr>
<tr>
<td>Aerobic, phototrophic</td>
<td>Anabaena, Calothrix, Nostoc, Gleotheca, Cylindropermum, Aphanocapsa</td>
</tr>
<tr>
<td>Faculative, heterotrophic</td>
<td>Enterobacter cloacae, Klebsiella pneumoniae, Bacillus polymyxa, Desulfovibrio desulfuricans, D. gigas, Achromobacter</td>
</tr>
<tr>
<td>Anaerobic, heterotrophic</td>
<td>Clostridium pasteurianum, C. butyricum, Propionispira arboris</td>
</tr>
<tr>
<td>Anaerobic, phototrophic</td>
<td>Chromatium vinosum, Rhodospirillum rubrum, Rhodopseudomonas sphaeroides, R. capsulata, Rhodococcus verniellii, Rhodocyclus, Chlorobium limicola</td>
</tr>
<tr>
<td>Nonphotosynthetic, autotrophic</td>
<td>Methanobacterium, Methylloccus, Methylosinus, Methanococcus, Methanococcus, Methanosarcina</td>
</tr>
</tbody>
</table>
THE NITROGEN FIXATION PROCESS

Fixation of atmospheric dinitrogen (N₂ or N≡N) is accomplished by a variety of bacteria and cyanobacteria utilizing a multicomponent nitrogenase system. Despite the variety of organisms capable of fixing nitrogen, the nitrogenase complex appears to be remarkably similar in most organisms (Fig. 14-2). Nitrogenase consists of two oxygen-sensitive proteins. Component I (dinitrogenase) is a molybdenum-iron protein containing two subunits. Component II (dinitrogenase reductase) is an iron-sulfur protein that transfers electrons to dinitrogenase. These proteins, together with ATP, Mg²⁺, and a source of electrons, are essential for nitrogen-fixing activity.

The overall process of nitrogen fixation is accomplished at considerable expense of energy, requiring from 12 to 16 molecules of ATP and 6 to 8 electrons, depending on the manner in which the equation is viewed. In one form, the equation may be written

\[
N₂ + 6 H^+ + 6 e^- + 12 ATP + 12 H₂O \rightarrow 2 \text{NH}_3 + 12 \text{ADP} + 12 \text{Pi}
\]

This form of the equation does not take into account the fact that dihydrogen (H₂) is an obligate product of the nitrogenase reaction. If H₂ is considered, then the equation

\[
8 \text{NAD}^+ + 16 \text{MgATP} + 8 \text{Fd} \rightarrow 8 \text{NADH} + 8 \text{Fd} + 8 \text{H}^+ + 16 \text{ATP} + 16 \text{Pi} + 8 \text{H}_2
\]

\[
8 \text{NADH} + 8 \text{Fd} \rightarrow 8 \text{NAD}^+ + 8 \text{Fd} + 8 \text{H}^+ + 8 \text{e}^-
\]

\[
8 \text{Fd} + 8 \text{H}^+ + 8 \text{e}^- \rightarrow 8 \text{Fd} + 8 \text{H}^+ + 8 \text{e}^-
\]

\[
8 \text{H}^+ + 8 \text{e}^- \rightarrow 8 \text{H}_2
\]

\[
2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{H}^+ + 2 \text{e}^- + 2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^- \rightarrow 2 \text{NH}_3 + 2 \text{ATP} + 2 \text{Pi}
\]

\[
16 \text{MgATP} + 16 \text{Pi} \rightarrow 16 \text{MgADP} + 16 \text{Pi}
\]

\[
2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
\text{N}_2 + 6 \text{H}^+ + 6 \text{e}^- + 12 \text{ATP} + 12 \text{H}_2O \rightarrow 2 \text{NH}_3 + 12 \text{ADP} + 12 \text{Pi}
\]

This form of the equation does not take into account the fact that dihydrogen (H₂) is an obligate product of the nitrogenase reaction. If H₂ is considered, then the equation

\[
16 \text{MgATP} + 16 \text{Pi} \rightarrow 16 \text{MgADP} + 16 \text{Pi}
\]

\[
2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{H}^+ + 2 \text{e}^- + 2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^- \rightarrow 2 \text{NH}_3 + 2 \text{ATP} + 2 \text{Pi}
\]

\[
16 \text{MgATP} + 16 \text{Pi} \rightarrow 16 \text{MgADP} + 16 \text{Pi}
\]

\[
2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{H}^+ + 2 \text{e}^- + 2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^- \rightarrow 2 \text{NH}_3 + 2 \text{ATP} + 2 \text{Pi}
\]

This form of the equation does not take into account the fact that dihydrogen (H₂) is an obligate product of the nitrogenase reaction. If H₂ is considered, then the equation

\[
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\]

\[
2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{H}^+ + 2 \text{e}^- + 2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^- \rightarrow 2 \text{NH}_3 + 2 \text{ATP} + 2 \text{Pi}
\]

This form of the equation does not take into account the fact that dihydrogen (H₂) is an obligate product of the nitrogenase reaction. If H₂ is considered, then the equation

\[
16 \text{MgATP} + 16 \text{Pi} \rightarrow 16 \text{MgADP} + 16 \text{Pi}
\]

\[
2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{H}^+ + 2 \text{e}^- + 2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^- \rightarrow 2 \text{NH}_3 + 2 \text{ATP} + 2 \text{Pi}
\]

This form of the equation does not take into account the fact that dihydrogen (H₂) is an obligate product of the nitrogenase reaction. If H₂ is considered, then the equation

\[
16 \text{MgATP} + 16 \text{Pi} \rightarrow 16 \text{MgADP} + 16 \text{Pi}
\]

\[
2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{H}^+ + 2 \text{e}^- + 2 \text{H}_2 \rightarrow 2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^-
\]

\[
2 \text{N}_2 + 4 \text{H}^+ + 4 \text{e}^- \rightarrow 2 \text{NH}_3 + 2 \text{ATP} + 2 \text{Pi}
\]
can be shown as

\[ \text{N}_2 + 8 \text{H}^+ + 8 \text{e}^- + 16 \text{ATP} + 12 \text{H}_2\text{O} \rightarrow 2 \text{NH}_3 + \text{H}_2 + 16 \text{ADP} + 16 \text{P}_i \]

Thus, the theoretical stoichiometric relationship between \( \text{N}_2 \) fixation and \( \text{H}_2 \) production is related by the equation

\[ \text{N}_2 + 8 \text{H}^+ + 8 \text{e}^- \rightarrow 2 \text{NH}_3 + \text{H}_2 \]

The production of hydrogen during nitrogen fixation is an energy-expensive process. In actuality, most aerobic nitrogen fixers rarely evolve \( \text{H}_2 \) because a membrane-bound uptake hydrogenase recycles the \( \text{H}_2 \) and produces ATP through respiration to help support the ATP requirements of the system.

An anaerobic environment is essential for nitrogenase activity because of the oxygen lability of both proteins in the complex. An uptake hydrogenase coupled to a dioxygen-consuming pathway helps to maintain an anaerobic environment. Anaerobiosis is essential because oxygen represses the hydrogen uptake system. In the cyanobacteria, the problem of conducting oxygen-evolving photosynthesis and nitrogen fixation simultaneously is circumvented by sequestration of the nitrogenase system in specialized cells called heterocysts. Otherwise, they can only fix \( \text{N}_2 \) in the dark under anoxic conditions.

Components of the Nitrogenase System

The nitrogen fixation (\( \text{nif} \)) genes in \( \text{K. pneumoniae} \) are listed in Table 14-2 along with their known product or function. The nitrogenase complex of this organism contains two separable proteins. Component I (dinitrogenase) is a \( \alpha_2\beta_2 \) tetramer of 240 kDa encoded by \( \text{nifK} \) and \( \text{nifD} \). The iron-molybdenum cofactor (FeMo-co) of the dinitrogenase is synthesized under the direction of six \( \text{nif} \) genes (\( \text{nifQ,B,V,N,E,H} \)). Mutants of \( \text{nifB} \) and \( \text{nifNE} \) accumulate an inactive apo-component I (ApoI). ApoI is an oligomer containing an additional protein, the product of \( \text{nifY} \), which disassociates from the complex upon activation by the addition of FeMo-co with restoration of the ability to

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Gene</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q</td>
<td>FeMoCo, Mo uptake</td>
<td>U</td>
<td>Protein associated with FeS center</td>
</tr>
<tr>
<td>B</td>
<td>FeMoCo synthesis</td>
<td>X</td>
<td>Regulatory protein</td>
</tr>
<tr>
<td>A</td>
<td>Positive regulator</td>
<td>N</td>
<td>FeMoCo</td>
</tr>
<tr>
<td>L</td>
<td>Negative regulator</td>
<td>E</td>
<td>FeMoCo synthesis</td>
</tr>
<tr>
<td>F</td>
<td>Flavodoxin, electron acceptor</td>
<td>Y</td>
<td>Unknown function</td>
</tr>
<tr>
<td>M</td>
<td>Fe protein activation</td>
<td>T</td>
<td>Unknown function</td>
</tr>
<tr>
<td>Z</td>
<td>Insertion of FeMoCo protein</td>
<td>K</td>
<td>MoFe protein ( \beta )-subunits</td>
</tr>
<tr>
<td>W</td>
<td>Activation of dinitrogenase</td>
<td>D</td>
<td>MoFe protein ( \alpha )-subunits</td>
</tr>
<tr>
<td>V</td>
<td>Homocitrate synthesis</td>
<td>H</td>
<td>Dinitrogenase reductase</td>
</tr>
<tr>
<td>S</td>
<td>Protein associated with FeS center</td>
<td>J</td>
<td>Pyruvate oxidoreductase</td>
</tr>
</tbody>
</table>

*The genes are listed in the order in which they appear on the detailed chromosome map of \( \text{Klebsiella pneumoniae} \).*
fix N₂. Component II (dinitrogenase reductase, encoded by nifH) is an α₂ protein (ca. 60 kDa) containing a single four-iron four-sulfur (Fe₄S₄) center. This protein binds and hydrolyzes Mg ATP when an electron is transferred from reduced ferredoxin to dinitrogenase. Ferredoxin and/or flavodoxin can serve as electron donors.

Regulation of the 17 nif genes in K. pneumoniae is under the direction of the nifLA operon. The NtrC protein activates the transcription of the nifLA operon under the conditions of nitrogen limitation. The NifA protein is a positive regulatory factor required for nif gene transcription (Table 14-2). The NifL gene product interacts with the NifA protein to prevent NifA activation in the presence of fixed nitrogen (ammonia or amino acids) or oxygen. The functions of nifZ, W, U, S, X, and T are still not well defined. However, there is evidence that the products of the nifW and nifZ genes may be involved in processing one of the structural components of nitrogenase and that the product of the nifX genes is a positive regulator of the nif regulon in response to ammonia and oxygen (Fig. 14-3).

The nitrogen-fixing systems of Azotobacter species have been well characterized. Three different nitrogenases found in A. vinelandii and A. chroococcum are regulated by the Mo or V content of the culture medium. Nitrogenase 1 is produced by both organisms in the presence of Mo. Dinitrogenase reductase is composed of two identical subunits encoded by nifH. Dinitrogenase 1 is a tetramer of two pairs of nonidentical subunits encoded by nifD and nifK. The three structural genes appear in the order nifHDK and comprise an operon. Nitrogenase 2 is produced by both A. vinelandii and A. chroococcum grown in a nitrogen-free medium in the presence of V. Dinitrogenase reductase 2 is a dimer encoded by vnfH. Dinitrogenase 2, encoded by vnfD and

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\( \text{vnfK} \), is a tetramer composed of two pairs of subunits containing Fe and V. A third nitrogenase, nitrogenase 3, is encoded by alternate nitrogen fixation (\( \text{anfHDK} \)) genes, which are expressed in \( A. \text{vinelandii} \) only in the absence of Mo and V. Dinitrogenase reductase 3 contains two identical subunits and dinitrogenase 3 is present in two active configurations, \( \alpha_2 \beta_2 \) and \( \alpha_1 \beta_2 \). The regulatory genes \( \text{nifA}, \text{vnfA}, \) and \( \text{anfA} \) are required for the expression of nitrogenases 1, 2, and 3, respectively. An additional regulatory gene, \( \text{nfrX} \), is required for growth on \( N_2 \) (diazotrophic growth) in the presence or absence of Mo.

The nitrogenase system from \( C. \text{pasteurianum} \) is similar to that found in \( K. \text{pneumoniae} \) and \( A. \text{vinelandii} \). Two proteins are required for nitrogen fixation and ATP-dependent \( H_2 \) evolution. One protein, component I, is an FeMo protein having dinitrogenase activity. A second protein, the Fe protein, or component II, has dinitrogenase reductase activity. Although there are structural similarities between the nitrogenase complexes of various organisms, the primary structure of those from \( C. \text{pasteurianum} \) is quite different from those of \( K. \text{pneumoniae} \) and \( A. \text{vinelandii} \). The nitrogenase components from a wide variety of bacteria can interact successfully with one another. The FeMo and Fe proteins from different organisms show a remarkably high degree of successful formation of active hybrid nitrogenases. However, the components of \( C. \text{pasteurianum} \) nitrogenase are much less effective in forming heterologous complexes than are mixtures of the components from gram-negative organisms.

A number of phototrophic bacteria including strict anaerobes such as \( C. \text{chromatium}, C. \text{chlorobium}, \) and \( R. \text{rubrum} \) can fix nitrogen anaerobically in the light because their photosynthetic systems do not evolve \( O_2 \). However, the most important phototrophs from an ecological viewpoint are the cyanobacteria. They are capable of performing oxygenic photosynthesis and fixing \( CO_2 \) through the reductive pentose phosphate pathway (see Chapter 12). Cyanobacteria lack \( \alpha \)-ketoglutarate dehydrogenase but utilize this intermediate as a substrate for nitrogen assimilation. Nitrate, ammonia, urea, and \( N_2 \) are used as nitrogen sources. In the absence of fixed nitrogen, most cyanobacteria are able to fix \( N_2 \) using a nitrogenase system similar to that described in other diazotrophic organisms. However, they face a dilemma in that nitrogenase is extremely sensitive to oxygen. \( A. \text{baena} \) and \( N. \text{ostoc} \) form heterocysts, which are specialized cells that contain most of the nitrogenase activity. In heterocysts, the photosynthetic system is effectively inactivated and any residual \( O_2 \) is eliminated by highly active respiration.

Some cyanobacteria such as \( A. \text{baena variabilis} \) produce two Mo-dependent nitrogenases: Nif1 functions in heterocysts while Nif2 functions under anoxic conditions in vegetative cells. A number of cyanobacteria that do not form heterocysts are also known. They apparently operate under microaerophilic conditions when fixing nitrogen. Among the free-living nitrogen-fixing bacteria, the cyanobacteria contribute much more significantly to the soil nitrogen because they obtain their energy from photosynthesis and therefore capable of colonizing soil or other habitats where conditions are sparse. They also function symbiotically with primitive plants such as lichens, liverworts, and water ferns.

Of the many genes required for heterocyst function, only \( \text{ntcA}, \text{hanA}, \) and \( \text{hetR} \) appear to be required for the initiation of heterocyst development. NtcA functions as a nitrogen-dependent global transcriptional regulator in all cyanobacteria. In \( A. \text{baena} \), ntcA mutants fail to grow on nitrate, do not form heterocysts, or undergo nitrogenase
SYMBIOTIC NITROGEN FIXATION

Fig. 14-4. Diagrammatic view of regulation of nitrogenase by ADP ribosylation of the Fe protein in *Rhodospirillum rubrum*. DRAG, dinitrogenase reductase–activating glycohydrolase; DRAT, dinitrogenase reductase ADP ribosyltransferase; Fe$_2$S$_4$, the iron-sulfur in the Fe protein; arg, arginine 101 position in the Fe protein. (Simplified version of diagram in C. M., Halbleib et al., *J. Biol. Chem.* 275:3493–3500, 2000.)

Nitrogenase synthesis in a variety of organisms is generally subject to the close regulatory controls at the level of *nif* gene transcription. However, many of these organisms also display a posttranslational regulation of nitrogenase activity effected by small extracellular concentrations of ammonia. In some organisms this rapid and reversible inhibition of nitrogenase, termed ammonia switch-off, is the result of a covalent modification of the Fe protein (dinitrogenase reductase) in response to the addition of ammonia. Formation of an inactive form of nitrogenase in the photosynthetic bacterium *R. rubrum* results from ADP ribosylation of an arginine residue in the Fe-protein component (Fig. 14–4). This activity is catalyzed by dinitrogenase reductase ADP ribosyltransferase (DRAT). Activation of the modified protein by removal of the ADP-ribose moiety is catalyzed by dinitrogenase reductase activating glycohydrolase (DRAG). Other systems in which covalent modification of the Fe protein occurs include *A. brasiliense*, *A. lipoferrum*, and *A. vinelandii*. In *A. amazonense* a different, noncovalent inhibitory mechanism results in only a partial inhibition of nitrogenase activity by ammonia. In *Rhodospirillum rubrum* nitrogen fixation is also tightly regulated by transcriptional regulation of *nif* gene expression.
formation of nodules that conduct active nitrogen fixation, is a complex process that occurs in several stages: attachment of the bacteria to root hairs, root hair curling, formation of a “shepherd’s crook,” development of infection threads within root hairs, growth of the threads toward the inner cortex of the root, and formation of a nodule meristem in the inner root cortex (Fig. 14-5). These cytological changes in the root hair provide a means of passage of the organism into the internal root system where it infects a root cell. This infection causes the cell to swell and divide, forming a thick mass of cells called the root nodule. In the root nodule a differentiated form of the bacterium, termed a **bacteroid**, is capable of nitrogen fixation.

A portion of the genomes of both the plant and the bacteria are expressed only in the symbiotic state. Considering the complexity of the interaction between the bacterium and the host plant in the development of effective symbiosis, it is not surprising that attempts to develop strains that are more efficient in their nitrogen-fixing capability have been hampered by the fact that indigenous strains in the soil are more competitive than laboratory-developed strains in their ability to initiate nodule development. Nevertheless, continued efforts to engineer more effective strains of nitrogen-fixing bacteria are important because of the increase in the cost of nitrogen fertilizer and the undesirable contamination of rivers and streams that occurs through the heavy application of it.

The nodulation process requires the exchange of a series of signals between the plant and bacteria. Initially, plant genes coding for the production of flavonoids are expressed, and these compounds are excreted from the roots. Flavonoids activate NodD1 and start

---

**Fig. 14-5.** Root infection and modulation by *Sinorhizobium meliloti* in *Medicago sativa* (*alfalfa*). Assignment of development step control in common and specific *nod* loci of *S. meliloti*. Thick arrows represent development steps. Thin arrows represent genetic control. *(Source: From Debellé, F., et al., *J. Bacteriol* 168:1075–1086, 1986.)*
the sequence of Nod (nodulation factor) synthesis. Nod factors induce root hair curling and initiate nodule development (Fig. 14-6). Invasion of the root nodule by rhizobia is dependent on the production of at least one of several complex polysaccharides: succinoglycan, exopolysaccharide II, or a capsular polysaccharide (CPS or KPS) containing 3-deoxy-d-manno-2-octulosonic acid (see Fig. 14-7).

The early events in root nodule formation by rhizobia in leguminous plants are under the control of genes located on a large plasmid called symbiosis or Sym plasmid. Plant
TABLE 14-3. Genes Involved in Nodulation and Their Known Function or Activity

<table>
<thead>
<tr>
<th>Designation</th>
<th>Function or Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>nodD</em></td>
<td>Positive transcription activator; binds to the <em>nod</em> box upstream of all inducible operons</td>
</tr>
<tr>
<td><em>nod</em> box</td>
<td>Highly conserved DNA sequence; <em>cis</em>-acting regulatory element of <em>nod</em> gene expression</td>
</tr>
<tr>
<td><em>nodA</em></td>
<td>N-Acetyltransferase</td>
</tr>
<tr>
<td><em>nodB</em></td>
<td>Oligochitin deacetylase</td>
</tr>
<tr>
<td><em>nodC</em></td>
<td>Oligochitin synthase</td>
</tr>
<tr>
<td><em>nodFEL</em></td>
<td>Operon involved in addition of acyl substituents to core lipooligosaccharide</td>
</tr>
<tr>
<td><em>nodH</em></td>
<td>Addition of substituents to core lipooligosaccharide</td>
</tr>
<tr>
<td><em>nodI</em></td>
<td>Involved in efficiency of nodulation and development of infectious threads</td>
</tr>
<tr>
<td><em>nodJ</em></td>
<td>Involved in efficiency of nodulation and development of infectious threads</td>
</tr>
<tr>
<td><em>nodM</em></td>
<td>Fructose-6-phosphate:glutamine amidotransferase</td>
</tr>
<tr>
<td><em>nodP</em></td>
<td>Sulfate + APS (adenylyl sulfate) → PAPS (3′-phosphoadenylyl sulfate)</td>
</tr>
<tr>
<td><em>nodQ</em></td>
<td>Addition of substituents to core lipooligosaccharide</td>
</tr>
<tr>
<td><em>nodS</em></td>
<td>N-methyltransferase</td>
</tr>
<tr>
<td><em>nodU</em></td>
<td>6-O-Carbamoyltransferase</td>
</tr>
<tr>
<td><em>nodX</em></td>
<td>Addition of acetyl substituent to core lipooligosaccharide</td>
</tr>
<tr>
<td><em>nodZ</em></td>
<td>6-O-Fucosyltransferase</td>
</tr>
<tr>
<td><em>noeA</em></td>
<td>Sulfuryltransferase</td>
</tr>
<tr>
<td><em>noeC</em></td>
<td>Addition of substituent to core lipooligosaccharide</td>
</tr>
<tr>
<td><em>noeI</em></td>
<td>2-O-Methyltransferase</td>
</tr>
<tr>
<td><em>noeJ</em></td>
<td>Mannose-1-phosphate guanyltransferase</td>
</tr>
<tr>
<td><em>noeL</em></td>
<td>GDP-Mannose-4,6-dehydratase</td>
</tr>
<tr>
<td><em>noeK</em></td>
<td>Phosphomannose mutase</td>
</tr>
<tr>
<td><em>nolL</em></td>
<td>O-Acetyltransferase</td>
</tr>
<tr>
<td><em>nolO</em></td>
<td>Carbamoyltransferase</td>
</tr>
<tr>
<td><em>nolK</em></td>
<td>Fucose synthase</td>
</tr>
</tbody>
</table>

Flavonoids, in concert with NodD, induce the expression of *nod* genes. The NodD protein serves as a positive transcription activator. It binds to the *nod* box, located upstream of all inducible *nod* genes and operons. The *nod* box, a highly conserved DNA sequence, appears to function as a *cis*-acting regulatory element of *nod* gene expression. The *nodABC* genes common to all rhizobia are required for the synthesis of a lipooligosaccharide that triggers root nodule formation. Addition of various substituents to the core compound imparts host specificity to the lipooligosaccharide. Addition of these components occurs under the influence of *nodH* and *nodQ* and the *nodFEL* operon (see Table 14-3). The NodI and NodJ proteins are involved in efficiency of nodulation and play a role in the normal development of infection threads. Once bacteroid development is complete, the inducible *nod* genes are no longer transcribed. This transcription switch-off prior to the release of the bacteria from the infection thread is a general phenomenon observed in all rhizobia and is the result of a negative regulatory control mechanism.
Under the conditions of symbiosis, the host plant provides the bacteroid with reduced carbon in the form of C\textsubscript{4}-dicarboxylic acids (succinate, malate, and fumarate). These compounds serve as energy sources for the fixation of nitrogen to ammonia. Dicarboxylic acids are present in high concentration in the nodule and are the most effective substrates for respiration and subsequent ATP-utilizing nitrogen fixation in the bacteroid. The dicarboxylic acid transport genes (\textit{dct}) are located on a megaplasmid. Mutants defective in dicarboxylate transport form ineffective nodules. The products of \textit{dctB} and \textit{dctD} regulate the expression of \textit{dctA}, which encodes a transport protein. \textit{DctB} is a sensor protein that activates \textit{DctD} by phosphorylation. The \textit{DctD} protein activates transcription at the \(\sigma^{54}\)-dependent \textit{dctA} promoter. In addition to activating \textit{dctA} transcription, \textit{DctD} can repress the expression of \textit{dctA}. In uninfected cells, inactive \textit{DctD} binds to the \textit{dctA} promoter and prevents its activation by NtrC.

For many years, the paradigm of symbiotic nitrogen fixation has been the release of ammonia directly from the bacteroid to the plant. Active ammonia transport systems have been described in some symbiotic microorganisms. However, recent studies reveal that alanine, rather than ammonia, is the form of nitrogen transported to the plant. It has been shown that rhizobia can assimilate ammonia into pyruvate, forming alanine via alanine dehydrogenase. The equilibrium of the alanine dehydrogenase reaction favors ammonia assimilation.

Labeling experiments with \(^{15}\text{N}_2\) using highly purified nodule bacteroids of \textit{B. japonicum} show that recently formed ammonia from nitrogenase was incorporated into alanine and then transported to the plant from the bacteroid. Secretion of labeled alanine was dependent on nitrogenase activity as bacteroids exposed to inactivating levels of oxygen or to the energy uncoupling agent CCCP (carbonyl cyanide \(m\)-chlorophenylhydrazone) did not excrete alanine. The peribacteroid membrane is inverted with respect to the bacteroid, so alanine secretion by the bacteroid to the plant cytoplasm can occur quickly, but it cannot be readily reabsorbed. It is considered that alanine serves primarily as a transport mechanism for fixed nitrogen, since most organisms use glutamate as the central compound in nitrogen metabolism (see “General Reactions of Amino Acids”).

**INORGANIC NITROGEN METABOLISM**

The assimilation of inorganic nitrogen ends with the incorporation of ammonia into organic compounds. Since ammonia is the only form of inorganic nitrogen that can be directly assimilated into amino acids, the ability of an organism to utilize other forms of inorganic nitrogen depends on the presence of enzymes or enzyme systems that are able to convert these compounds to ammonia. This process is referred to as \textbf{denitrification}. The reverse process, converting ammonia to nitrate and nitrite, is called \textbf{nitrification} (Fig. 14-8). Table 14-4 provides examples of organisms known to carry out reactions of inorganic nitrogen. Although it is not an inorganic compound, the metabolism of urea is discussed in this section because it is so readily degraded to ammonia and carbon dioxide by ureases found in many genera of bacteria and fungi.

Many of the organisms that conduct the reactions shown in Table 14-4 do not actually assimilate the nitrogen. In some organisms, nitrate may be used as a terminal electron acceptor in place of oxygen. The end product is nitrite or \(\text{N}_2\). Nitrate respiration yields biologically useful energy under anaerobic conditions. Nitrate assimilation occurs by sequential reduction to nitrite, hydroxylamine, and, finally, ammonia. Organisms that
Fig. 14-8. Pathways of inorganic nitrogen metabolism. Denitrification and nitrification. The superscript numbers on the nitrogen indicate the valence state or oxidation level of the nitrogen in the compounds.

conduct both nitrate respiration and nitrate assimilation contain two nitrate reductases. In *E. coli* and *Neurospora* the assimilatory enzyme is a soluble cytoplasmic enzyme, whereas the respiratory enzyme is membrane-bound and sensitive to oxygen. Both enzymes contain flavin as the prosthetic group. In most organisms the assimilatory enzyme is repressed by ammonia and induced in the presence of either nitrate or nitrite. Algae readily utilize nitrate as a source of inorganic nitrogen, but the route of assimilation is less well characterized than in bacteria and fungi. Ammonia is considered to be the final product of nitrate reduction by algae.

Denitrification by members of the obligately chemolithotrophic *Nitrobacteriaceae* is considered to be the major source of assimilable nitrogen (ammonia) in soils. The ammonia oxidizer, *Nitrosomas europaea*, and the nitrite oxidizer, *Nitrobacter winogradskyi*, have been widely studied. *Nitrosomonas europaea* obtains all of its energy for growth from the oxidation of ammonia to nitrite. The oxidation of ammonia to hydroxylamine is an oxygen-dependent reaction catalyzed by ammonia monooxygenases:

\[
\text{NH}_3 + \text{O}_2 + 2 \text{e}^- + 2 \text{H}^+ \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O}
\]

Hydroxylamine is further oxidized to nitrite by hydroxylamine oxidoreductase:

\[
\text{NH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + 5 \text{H}^+ + 4 \text{e}^-
\]

Two of the four electrons generated from hydroxylamine oxidation are used to support the oxidation of additional ammonia molecules, while the other two electrons enter the electron transfer chain and are used to support ATP synthesis via oxidative phosphorylation and CO₂ reduction. The oxidative chain from NO₂⁻ to O₂ in *N. winogradskyi* consists of membrane-bound cytochrome c oxidase. *Nitrobacter* is obligately dependent on nitrite as the sole source of nitrogen and energy and requires carbon dioxide for growth. *Nitrobacter* is, therefore, dependent on *Nitrosomonas* or other organisms in the natural environment for its nitrogen supply.
<table>
<thead>
<tr>
<th>Reaction</th>
<th>Electrons Required</th>
<th>Representative Organisms Performing Reaction</th>
<th>Requirements (comments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{NO}_3^- \rightarrow \text{NO}_2^-$</td>
<td>2</td>
<td><em>Escherichia coli</em>, <em>Micrococcus denitrificans</em>, <em>Bacillus subtilis</em>, <em>Haemophilus influenzae</em>, <em>Pseudomonas aeruginosa</em>, <em>Neurospora crassa</em>, <em>Achromobacter fischeri</em></td>
<td>Varies among organisms NAD, NADP, FAD, FMN, Mo, cytochromes c, b</td>
</tr>
<tr>
<td>$\text{NO}_2^- \rightarrow \text{NH}_4^+$</td>
<td>6</td>
<td><em>Bacillus pyocyaneus</em>, <em>Neurospora crassa</em>, <em>Bacillus pumilis</em>, <em>Clostridium pasteurianum</em>, <em>Desulfovibrio desulfuricans</em></td>
<td>NADPH, NADH, FAD, Cu, Fe</td>
</tr>
<tr>
<td>$\text{NO}_3^-$ or $\text{NO}_2^-$ → NO, N$_2$O, and N$_2$ Denitrification</td>
<td>Varies from 1 to 10</td>
<td><em>Micrococcus denitrificans</em>, <em>Denitrobacillus</em>, <em>Pseudomonas</em>, <em>Spirillum itersonii</em>, <em>Bacillus licheniformis</em>, <em>Achromobacter</em>, <em>T. thioparus</em>, <em>Thiobacillus denitrificans</em></td>
<td>See requirements for individual reactions</td>
</tr>
<tr>
<td>$\text{NO}_3^- \rightarrow \text{NO}$ Nitrite reduction</td>
<td>1</td>
<td><em>Pseudomonas stutzeri</em>, <em>E. coli</em>, <em>Bacillus subtilis</em>, <em>P. aeruginosa</em></td>
<td>NAD, NADP, FMN, FAD, Fe$^{3+}$</td>
</tr>
<tr>
<td>NO → N$_2$ or N$_2$O Nitric oxide reductase</td>
<td>2 or 4</td>
<td><em>Pseudomonas stutzeri</em>, <em>E. coli</em>, <em>Bacillus subtilis</em>, <em>Pseudomonas aeruginosa</em>, <em>Clostridium pasteurianum</em>, <em>Pseudomonas aeruginosa</em></td>
<td>NAD, NADP, FAD, FMN, Fe$^{3+}$</td>
</tr>
<tr>
<td>N$_2$O → N$_2$ Nitrous oxide reductase</td>
<td>2</td>
<td><em>Pseudomonas stutzeri</em></td>
<td>Cu</td>
</tr>
<tr>
<td>$\text{H}_2\text{N}_2\text{O}_2 \rightarrow 2\text{NH}_4^+$ Hyponitrite assimilation</td>
<td>8</td>
<td><em>Neurospora crassa</em>, <em>E. coli</em></td>
<td>NADPH, Fe$^{3+}$</td>
</tr>
</tbody>
</table>

(continued overleaf)
<table>
<thead>
<tr>
<th>Reaction</th>
<th>Electrons required</th>
<th>Representative Organisms Performing Reaction</th>
<th>Requirements (comments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{N}_2 \rightarrow 2\text{NH}_4^+$</td>
<td>6</td>
<td>See Table 14-1 for representative organisms</td>
<td>Mo, Fe, V</td>
</tr>
<tr>
<td>Nitrogen fixation</td>
<td></td>
<td><em>Neurospora crassa, Azotobacter vinelandii, Desulfovibrio desulfuricans, Bacillus pumilis, Clostridium pasteurianum, Pseudomonas aeruginosa</em></td>
<td>Varies—NADH, FAD, Mn$^{2+}$</td>
</tr>
<tr>
<td>$\text{NH}_2\text{OH} \rightarrow \text{NH}_4^+$</td>
<td>2</td>
<td><em>Neurospora crassa, Azotobacter vinelandii, Desulfovibrio desulfuricans, Bacillus pumilis, Clostridium pasteurianum, Pseudomonas aeruginosa</em></td>
<td>Varies—NADH, FAD, Mn$^{2+}$</td>
</tr>
<tr>
<td>Hydroxylamine reductase</td>
<td></td>
<td><em>Neurospora crassa, Azotobacter vinelandii, Desulfovibrio desulfuricans, Bacillus pumilis, Clostridium pasteurianum, Pseudomonas aeruginosa</em></td>
<td>Varies—NADH, FAD, Mn$^{2+}$</td>
</tr>
<tr>
<td>$\text{NH}_2\text{NH}_2 \rightarrow 2\text{NH}_4^+$</td>
<td>2</td>
<td><em>Micrococcus lactilyticus</em></td>
<td>Measured hydrogen uptake with hydrazine added</td>
</tr>
<tr>
<td>Hydrazine reductase</td>
<td></td>
<td><em>Micrococcus lactilyticus</em></td>
<td>Measured hydrogen uptake with hydrazine added</td>
</tr>
<tr>
<td>$\text{NH}_4^+ \rightarrow$ organic compounds</td>
<td>In some cases, 2</td>
<td>Large group</td>
<td>Varies</td>
</tr>
<tr>
<td>$\text{NH}_4^+ \rightarrow \text{NO}_2^-$ or $\text{NH}_2\text{OH} \rightarrow \text{NO}_2^-$</td>
<td>$-6$ or $-4$</td>
<td><em>Nitrosomonas spp.</em></td>
<td>Acceptor—cytochrome c or phenazine methosulfate</td>
</tr>
<tr>
<td>Nitrification</td>
<td></td>
<td><em>Nitrobacter spp.</em></td>
<td>Cytochrome, Fe$^{3+}$</td>
</tr>
</tbody>
</table>
Nitrite formation by heterotrophic soil organisms appears to be rather limited. Nitrate formation, on the other hand, is accomplished by a significant number and variety of heterotrophic species present in the soil microflora. *Aspergillus flavus* is particularly active and must be considered as a major source of nitrate in soils.

Denitrifying bacteria, such as *Pseudomonas stutzeri*, use the reduction of nitrous oxide (N\textsubscript{2}O) to dinitrogen (N\textsubscript{2}) for the generation of energy. Reduction of N\textsubscript{2}O is usually the terminal step of bacterial denitrification, proceeding from nitrate to nitrite to nitric oxide to nitrous oxide and the final reduction of nitrous oxide to N\textsubscript{2} by N\textsubscript{2}O reductase. A gene cluster containing the genes necessary for the reduction of nitrite (*nir*), nitric oxide (*nor*), and nitrous oxide (*nos*) has been identified in *P. stutzeri*. There are at least 15 genes in this cluster arranged in the order *nos-nir-nor*. The structural gene *nosZ* codes for the copper-containing enzyme N\textsubscript{2}O reductase. Three other genes, *nosDFY*, are involved in the formation of the copper chromophore of the enzyme. A regulatory gene, *nosR*, is present within the *nos* region of the denitrification gene cluster of *P. stutzeri*. It is essential for the expression of the *nosZ* gene, and there is indirect evidence that the transcriptional regulator Fnr may also be involved in *nosZ* expression (see Chapter 5 for a discussion of regulatory mechanisms).

Urea is a simple organic compound with the structure H\textsubscript{2}NCONH\textsubscript{2}. It is widely used as an inexpensive nitrogen fertilizer because it is readily degraded to ammonia and carbamate by the enzyme urease:

\[
\text{H}_2\text{NCONH}_2 + \text{H}_2\text{O} \rightarrow \text{NH}_3 + \text{H}_2\text{NCOOH}
\]

Carbamate spontaneously decomposes to yield ammonia and carbonic acid:

\[
\text{H}_2\text{NCOOH} + \text{H}_2\text{O} \rightarrow \text{NH}_3 + \text{H}_2\text{CO}_3
\]

Complete urease gene clusters have been found in *Bacillus* spp, *Helicobacter pylori*, *Klebsiella aerogenes*, *Proteus mirabilis*, and *Yersinia enterocolitica*. In addition, a number of other bacteria, including soil bacteria such as *Sinorhizobium meliloti*, have been studied in some detail with regard to the genetics of urease production and its regulation. The enzyme has been identified as a virulence factor in *H. pylori* and other organisms that cause infections in humans and animals. Urease activity can serve as a diagnostic test for infection by *H. pylori*. Urease is notable because it was the first enzyme to be crystallized—a feat accomplished by Dr. James B. Sumner of Cornell University in 1926 and for which he later received the Nobel Prize.

In enteric bacteria urease expression is positively regulated and transcription is activated either in the absence of an assimilable nitrogen source or in the presence of urea. On the other hand, in *Streptococcus salivarius*, urease expression is derepressed at low pH and is enhanced in the presence of excess carbohydrate. Ureolysis by *S. salivarius* appears to provide protection of the organisms against acid damage and may also enable the organisms to acquire nitrogen when carbohydrates are present in excess. Wild-type strains of *S. salivarius* are protected against acid killing through physiologically relevant concentrations of urea, whereas a urease-deficient mutant is not. This organism can use urea as a source of nitrogen for growth exclusively through a urease-dependent pathway.

The bacterial urease from *K. pneumoniae* is composed of three basic subunits, UreA, UreB, and UreC, which appear as a trimer in the intact enzyme. Each UreABC
unit contains two coordinated nickel ions, separated from one another by 3.6 Å and bridged by a carbamylated lysine residue. Within this center are three metal-coordinated water molecules, four histidine ligands, one aspartate ligand, and two noncoordinated histidines that function in the conversion of urea into ammonia and carbon dioxide.

ASSIMILATION OF INORGANIC NITROGEN

Many microorganisms synthesize all of their amino acids and other nitrogenous compounds using ammonia and carbon chains derived from carbohydrate metabolism. The available pathways for ammonia assimilation are quite limited, however. Of all the amino acids found in proteins and other cellular constituents, ammonia can be directly assimilated into only a few. These amino acids then serve as donors of their amino nitrogen via transamination to keto acid precursors to form all of the other amino acids. The potential routes of ammonia assimilation are through the synthesis of glutamate, alanine, or aspartate. The major enzymes involved in ammonia assimilation are the glutamate dehydrogenases (GDH) and two enzymes that operate in tandem, glutamine synthetase (GS) and glutamate synthase, as follows:

\[
\text{Glutamate dehydrogenases (GDH)}
\]

\[
\begin{align*}
\alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{NADH} + \text{H}^+ & \longrightarrow \text{l-glutamate} + \text{NAD}^+ \\
\alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{NADPH} + \text{H}^+ & \longrightarrow \text{l-glutamate} + \text{NADP}^+
\end{align*}
\]

\[
\text{Glutamine synthase (GS)–glutamate synthase (GOGAT)}
\]

\[
\begin{align*}
\text{l-glutamate} + \text{NH}_4^+ + \text{ATP} & \longrightarrow \text{l-glutamine} + \text{ADP} + \text{Pi} \\
\alpha\text{-ketoglutarate} + \text{l-glutamine} + \text{NADPH} + \text{H}^+ & \longrightarrow 2 \text{l-glutamate} + \text{NADP}^+
\end{align*}
\]

\[
\text{Net: } \alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{ATP} + \text{NADPH} + \text{H}^+ \longrightarrow \text{l-glutamate} + \text{ADP} + \text{Pi} + \text{NADP}^+
\]

The abbreviation GOGAT frequently used for this enzyme system is derived from its previous trivial name, glutamine amide-2-oxoglutarate amino transferase.

Other enzymes that may play a role in ammonia assimilation in some organisms are alanine dehydrogenases and aspartase:

\[
\text{Alanine dehydrogenases}
\]

\[
\begin{align*}
\text{pyruvate} + \text{NH}_4^+ + \text{NADH} + \text{H}^+ & \longrightarrow \text{l-alanine} + \text{NAD}^+ \\
\text{pyruvate} + \text{NH}_4^+ + \text{NADPH} + \text{H}^+ & \longrightarrow \text{l-alanine} + \text{NADP}^+
\end{align*}
\]

\[
\text{Aspartase}
\]

\[
\text{fumarate} + \text{NH}_4^+ \longrightarrow \text{l-aspartate}
\]
The route(s) of ammonia assimilation varies from one organism to another depending on the ammonia assimilation enzymes present. In the majority of organisms that have been studied, glutamate is the most widely used route for ammonia assimilation. In organisms capable of synthesizing both the NADP-linked glutamate dehydrogenase and the GS–GOGAT system, the NADP–GDH is functional at high concentrations of ammonia while the GS–GOGAT system is most active at concentrations of ammonia below 1 mM. These pathways are highly regulated as the concentration of ammonia, glutamate, and glutamine are key sensors that relate the nitrogen status of the organism. The yeast, Saccharomyces cerevisiae, assimilates at least two-thirds of its amino nitrogen requirement via glutamate, and, when the ammonia concentration in the culture medium is high, utilizes the NADP–GDH for glutamate synthesis. At low concentrations of ammonia, the GS–GOGAT system is operative. In nitrogen-fixing species of Bacillus (B. polymyxa, B. macerans), NADP–GDH activity is several-fold higher than that of GS and is the predominant pathway for ammonia assimilation.

A few organisms appear to be incapable of forming the GS–GOGAT system, and, therefore utilize one or more of the alternative routes for ammonia assimilation. Rhodospirillum purpureum, which does not fix N₂, uses exogenously supplied ammonia via the NADP-linked GDH. The only ammonia assimilation pathway in Streptococcus sanguis, S. bovis, S. mutans, and S. salivarius is NADP–GDH regardless of the external ammonia concentration. In some Bacillus species, the NADP–ADH and NADP–GDH enzymes are highest in activity when ammonia is in high concentration. Under N₂-fixing conditions rhizobia appear to form alanine via ADH and export alanine rather than ammonia to the plant host.

Aspartase is sufficiently active in Klebsiella aerogenes to function as an important assimilatory enzyme, particularly after growth on C₄-dicarboxylic acids. Under most conditions of growth, however, aspartase appears to serve in a dissimilatory (ammonia-releasing) role.

As described in Chapter 5, the GS–GOGAT system and related enzymes are highly regulated by the Ntr (nitrogen regulation) system, particularly in E. coli and K. aerogenes. On the other hand, in Bacillus spp. there is no evidence for a global regulatory system analogous to the Ntr system. Activity of the GS–GOGAT system in B. subtilis and B. licheniformis is regulated by the available nitrogen source (feedback inhibition). The GS regulatory protein GlnR negatively regulates transcription of the B. subtilis GS structural gene, while GltC stimulates the expression of GS. In B. licheniformis, the GOGAT enzyme (GltS) consists of two unequal subunits. The larger subunit catalyzes the glutaminase reaction:

\[
\text{glutamine} + H_2O \longrightarrow \text{glutamate} + NH_3
\]

An ammonia transfer reaction is catalyzed by the small subunit:

\[
NH_3 + \alpha\text{-ketoglutarate} + NADPH + H^+ \longrightarrow \text{glutamate} + NADP^+
\]

In B. subtilis, GS (GlnA) and a regulatory protein, GlnR, are encoded in an operon. Regulation of the glnRA operon involves the action of both GlnR and GS. Here GlnR is a repressor that interferes with transcription under conditions of nitrogen excess.
In the nitrogen-fixing anaerobe *Clostridium kluyveri*, the NADP–GDH pathway plays an important role in ammonia assimilation in ammonia-grown cells but plays only a minor role to that of the GS–GOGAT pathway in nitrogen-fixing cells — conditions in which the intracellular ammonia concentration is low. In *C. butyricum* the GS–GOGAT system is the predominant pathway for ammonia assimilation with either ammonia or N\textsubscript{2} as the source of nitrogen. In *C. acetobutylicum* there is no evidence for a global Ntr system and the GS enzyme is not regulated by adenylylation. Instead, a promoter, P3, which controls the transcription of an antisense RNA, is present in the downstream region of *glnA* DNA.

In phototrophic bacteria, such as the nonsulfur purple bacterium *Rhodobacter capsulatum*, the NADP–ADH aminating activity can function as an alternative route for ammonia assimilation when GS is inactive. The ADH is induced in cells grown on pyruvate plus nitrate, pyruvate plus ammonia, or L-alanine under both light-anaerobic and dark-heterotrophic conditions. Aminating activity is strictly NADPH dependent, whereas deaminating activity is strictly NAD dependent.

**GENERAL REACTIONS OF AMINO ACIDS**

**Amino Acid Decarboxylases**

Microorganisms exhibit decarboxylase activity for many amino acids including aspartate, glutamate, ornithine, lysine, arginine, tyrosine, phenylalanine, cysteic acid, diaminopimelic acid, hydroxyphenyl serine, histidine, tryptophan, 5-hydroxytryptophan, and possibly others. The general reaction for all of these enzymes is

$$R-\text{CHNH}_2\text{COOH} \rightarrow R-\text{CH}_2\text{NH}_2 + \text{CO}_2$$

As far as is known, pyridoxal phosphate is the coenzyme of all the amino acid decarboxylases, with the exception of histidine decarboxylase, for which the cofactor is pyruvate. All of the amino acid decarboxylases are essentially irreversible and therefore are not of importance in the biosynthesis of most amino acids. However, in the case of diaminopimelic acid (DAP) decarboxylase, lysine is the final product. Amino acid decarboxylases are produced at low pH and their range of optimal activity is pH 3–5. Thus, excess acidity resulting from the production of acid end products may be regulated by amino acid decarboxylase activity, particularly in anaerobic proteolytic organisms such as the clostridia.

The diamine putrescine, the decarboxylation product of ornithine, is an essential growth factor for several organisms and is a biosynthetic precursor of both spermidine and spermine, polyamines found in a wide variety of microorganisms (see Chapter 15). Spermine is present in eukaryotic organisms but is found in only a few bacteria (e.g., *Pseudomonas aeruginosa* and *Bacillus stearothermophilus*). Spermidine is more widely distributed, being found in bacteria and fungi as well as in higher organisms.

Germinating conidia of *N. crassa* produce an active glutamate decarboxylase. This is the first step in a pathway that leads to a rapid increase in aspartate in the amino acid pool of this organism.
Amino Acid Deaminases

Deamination of amino acids occurs via several quite different reactions:

1. Oxidative deamination
   a. NAD\(^+\) - or NADP\(^+\)-linked deamination
   b. FAD- or FMN-linked deamination
2. Nonoxidative deamination

The oxidative deamination of glutamic acid by the reversible glutamate dehydrogenases has been discussed earlier in relation to ammonia assimilation. In most organisms the NAD-specific enzyme operates catabolically, whereas the NADP-linked enzyme functions in glutamate synthesis.

**Alanine dehydrogenase (ADH)** is present in members of the genus *Bacillus*, the photosynthetic nonpurple sulfur bacteria *Rhodobacter*, and others. The reaction proceeds via an \(\alpha\)-imino intermediate:

\[
\begin{align*}
\text{COOH} & \quad \text{COOH} \\
\text{H} & \quad \text{C} \\
\text{CH}_3 & \quad \text{C} \quad \text{CH}_3 \\
\text{NH}_2 & \quad \text{O} \\
\text{NAD}\(^+\)/\text{NADP}\(^+\) & \quad \text{H}^+ + \text{NH}_4^+ \\
\end{align*}
\]

The ubiquitous distribution and high activity of ADH in the aerobic bacilli has been considered as evidence that the reverse reaction catalyzed by this enzyme may serve as a major route of ammonia assimilation in these organisms. This conclusion has also been drawn in the case of the symbiotic rhizobia.

**Amino acid oxidases**, sometimes called aerobic or oxidative deaminases, involve reactions catalyzed by enzymes containing FAD (flavin adenine dinucleotide) or FMN (flavin mononucleotide). The reaction proceeds in two stages:

\[
\begin{align*}
R\text{-CHNH}_2\text{COOH} + \text{Enz-FAD} & \rightarrow \alpha\text{-keto acid} + \text{NH}_3 + \text{CO}_2 + \text{Enz-FADH}_2 \\
\text{Enz-FADH}_2 + \text{O}_2 & \rightarrow \text{Enz-FAD} + \text{H}_2\text{O}_2
\end{align*}
\]

Reduced FAD (or reduced FMN) may react nonenzymatically with molecular oxygen as shown above or it may transfer the hydrogen to other hydrogen acceptors. The amino acid oxidases are nonspecific in that a single enzyme may catalyze the oxidation of a variety of amino acids, for example, methionine, phenylalanine, tyrosine, leucine, isoleucine, valine, norvaline, alanine, tryptophan, and cysteine in decreasing order of activity. Another may oxidize proline, hydroxyproline, citrulline, histidine, and arginine. The rate of oxidation may differ for each amino acid, and the order of activity may differ with the source of the enzyme. Both D- and L-amino acid oxidases are known, but a single enzyme is usually specific for one configuration. These reactions yield no useful energy if the reduced FAD is oxidized nonenzymatically by molecular oxygen. It is highly unlikely that these enzymes play any significant role in the assimilation of ammonia.
Nonoxidative deaminases, such as aspartic acid deaminase (aspartase), serine, and threonine deaminases (dehydratases), and cysteine desulphhydrase, are specific in their substrate requirements.

Serine and threonine deaminases (dehydratases) catalyze the following type of reaction:

\[
\begin{align*}
\text{R} & = \text{H} \text{ in serine; CH}_3 \text{ in threonine} \\
\text{R} & = \text{H} \text{ in serine; CH}_3 \text{ in threonine}
\end{align*}
\]

An intramolecular transfer of hydrogen atoms occurs and water is removed (dehydration) to produce an imino acid via a $\beta$-elimination reaction. In the second stage of the reaction, water reacts nonenzymatically with the imino acid to release ammonia. Pyridoxal-5'-phosphate is the cofactor in this reaction. The two deaminating enzymes have been shown to be distinct. The level of L-serine deaminase varies as a function of nitrogen nutrition, carbon source, and the supply of glycine and leucine. Glycine and leucine induce the formation of serine deaminase. The enzyme seems to play a role in a number of pathways in which serine is generated and further metabolized as part of the main carbon pathway. Both *E. coli* and *S. enterica* produce biodegradative as well as biosynthetic threonine deaminases. The latter provides $\alpha$-ketobutyrate as an intermediate in the formation of isoleucine. The biodegradative enzyme is induced under anaerobic conditions in amino acid–rich medium, requires cAMP for its synthesis, and is sensitive to catabolite repression by glucose.

Aspartase is present in a number of organisms. It converts aspartate to fumarate and ammonia:

\[
\text{HOOCCH}_2\text{CHNH}_2\text{COOH} \rightleftharpoons \text{HOOCCH=CHCOOH} + \text{NH}_3
\]

There is no hydrogen exchange in this reaction. Instead, an intramolecular transfer of hydrogen occurs, and pyridoxal-5-phosphate is not involved as a cofactor in the reaction. The reaction is reversible, providing a potential route for ammonia assimilation. Although the equilibrium of the reaction is such that it could serve as an ammonia assimilation pathway, it is considered to function primarily in a catabolic capacity.

Cysteine desulphhydrase (dehydratase) has a reaction mechanism similar to that of serine and threonine deaminases except that hydrogen sulfide, rather than water, is removed via a $\beta$-elimination reaction:
Dehydratases and desulphhydrases are essentially irreversible reactions and are not considered reactions that could readily participate in ammonia assimilation.

Phenylalanine deaminase (ammonia lyase) is produced by yeasts, molds, and bacteria and catalyzes the nonoxidative deamination of L-phenylalanine to trans-cinnamic acid:

\[ \text{CH}_2\text{CHNH}_2\text{COOH} \rightarrow \text{CH=CHCOOH} + \text{NH}_3 \]

A dehydroalanine group serves as a cofactor in the reaction. The enzyme is of interest because of its potential for use in the treatment and diagnosis of phenylketonuria and has industrial applications in the synthesis of L-phenylalanine from trans-cinnamic acid. In *Rhodotorula glutinis* this enzyme serves as the initial step in a metabolic pathway, leading to the formation of benzoate and 4-hydroxybenzoate.

Amino Acid Transaminases (Aminotransferases)

In 1945, Lichstein and Cohen first demonstrated transaminase activity in bacteria. Transfer of the α-amino nitrogen between glutamate, aspartate, alanine, and their corresponding α-keto acids was shown to be similar to reactions observed in mammalian tissues. The two major transaminases demonstrated were aspartate amino transferase and alanine aminotransferase:

\[
\begin{align*}
\text{aspartate} + \alpha\text{-ketoglutarate} & \quad \leftrightarrow \quad \text{oxaloacetate} + \text{glutamate} \\
\text{alanine} + \alpha\text{-ketoglutarate} & \quad \leftrightarrow \quad \text{pyruvate} + \text{glutamate}
\end{align*}
\]

Pyridoxal phosphate is the coenzyme for all known transaminase reactions.

Transamination was first thought to be a relatively limited activity confined to these three amino acids and their keto analogs. However, in the early 1950s, Feldman and Gunsalus, and Rudman and Meister, showed that a number of amino acids would undergo transamination with α-ketoglutarate to form the corresponding amino acids. Transaminases are fully reversible reactions, so glutamate is a key donor for the synthesis of most other amino acids. This result led to a more generalized view of transamination in which any amino acid and any keto acid could exchange the amino group:

\[ \text{amino acid}_1 + \text{keto acid}_2 \quad \leftrightarrow \quad \text{keto acid}_1 + \text{amino acid}_2 \]

Transaminase activity is ubiquitous as evidenced by the fact that keto analogs can replace many of the amino acids for the growth of amino acid–requiring mutants. The reaction is not totally universal, however. Four major transaminases have been identified in *E. coli* as shown in Table 14-5. By these criteria, strains lacking transaminase C (avtA mutants) should have no nutritional requirement, whereas strains lacking transaminase B (ilvE mutants) should require isoleucine. In practice, most *ilvE* mutants require only isoleucine, but some show additional requirements for valine or leucine as a result of reduced expression of either *avtA* or other genes distal to *ilvE*. These transaminases have not, in general, been well identified in other organisms.
TABLE 14-5. Major Transaminases in *Escherichia coli*

<table>
<thead>
<tr>
<th>Transaminase</th>
<th>Interacts with</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic amino acids</td>
<td>Tyrosine, Phenylalanine, Glutamate, Leucine, Aspartate, Methionine</td>
<td><em>tyrB</em></td>
</tr>
<tr>
<td>Aspartate (transaminase A)</td>
<td>Aspartate, Glutamate, Tyrosine, Phenylalanine</td>
<td><em>aspC</em></td>
</tr>
<tr>
<td>Transaminase B</td>
<td>Glutamate, Leucine, Isoleucine, Valine, Phenylalanine, Methionine</td>
<td><em>ilvE</em></td>
</tr>
<tr>
<td>(branched-chain aminotransferase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transaminase C</td>
<td>Alanine, Valine, α-Aminobutyrate</td>
<td><em>avtA</em></td>
</tr>
<tr>
<td>(alanine-valine aminotransferase)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

However, most of these activities appear to be present in organisms that display a general ability to synthesize the common amino acids.

The aspartate aminotransferase (*L*-aspartate:*2-oxoglutarate aminotransferase) is ubiquitous. However, the enzyme has been studied in detail only in *E. coli*, *P. putida*, *S. cerevisiae*, and the archeon *Sulfolobus solfataricus*. A thermophilic *Bacillus* species produces an aspartate aminotransferase that shows some sequence similarity in the N-terminal region between the eubacterial and archaeal enzymes.

Other transaminases are known. For example, *S. enterica* displays a glutamine amidotransferase encoded within the *trpD* gene. It serves a dual role in transferring the amino group of glutamine to chorismic acid in the synthesis of anthranilic acid. Glutamine amidotransferase activity is also present in a tryptophan gene (*trpE*) in *Bacillus pumilis*.

**Amino Acid Racemases**

A number of microorganisms contain enzymes that catalyze the conversion of D-amino acids to L-amino acids via the general reaction

\[
\text{COOH} \rightarrow \text{COOH} \\
\text{H} - \text{C} - \text{NH}_2 \rightarrow \text{H}_2\text{N} - \text{C} - \text{H} \\
\text{R} \quad \text{R}
\]

Most biochemical compounds are asymmetric and there is a tendency for one form to predominate over the other. Amino acids in naturally occurring proteins are usually in
L-configuration. However, the cell walls and polypeptide capsules of many organisms contain D-amino acids. Since most biosynthetic reactions lead to the synthesis of the L-amino acids, racemases are necessary for the conversion of certain amino acids to the D configuration for the synthesis of these specialized cell structures. As an example, D-alanine is a structural component of the cell wall of *Enterococcus faecalis* as well as several other gram-positive organisms. When grown in a medium lacking pyridoxal, D-alanine becomes a specific growth requirement for *E. faecalis* because alanine racemase is inactive under the conditions of pyridoxal deficiency. D-Amino acids are found in the polypeptide capsules of members of the genus *Bacillus* and in peptide antibiotics, providing other indications of the importance of racemases in microbial metabolism.

**Role of Pyridoxal-5'-Phosphate in Enzymatic Reactions with Amino Acids**

In the foregoing discussion of the various reactions in which amino acids participate, it was mentioned that pyridoxal-5'-phosphate (PLP), the coenzyme form of vitamin B₆, functions in many of these reactions. PLP is the most versatile of all enzyme cofactors in that it can catalyze several distinct chemical reactions. Such versatility seems to be due, in part, to the PLP cofactor acting as an electron sink. For example, a proton may be removed from the α-carbon of the amino acid substrate, with the resultant stabilization of the carbanion at the C₆ or C'⁴ position; or the electrons may flow into the ring, neutralizing a positively charged pyridine nitrogen (i.e., a quinoid structure may be one of the intermediates). It is assumed that appropriate groups on the apoenzyme hold the PLP in the precise alignment necessary for rupture of the −C−X bond (Fig. 14-9).

Depending on the type of electron shifts that take place, nine main types of reactions are recognized: transamination, β decarboxylation, α decarboxylation, aldol cleavage,
cleavage, γ elimination, β elimination, γ displacement, or β displacement may occur. In addition, various PLP enzymes carry out unique reactions that do not fall into any of these categories — for example, dialkyl amino acid transaminase, tryptophan synthase, threonine synthase, and δ-amino-levulinate synthase. Three enzymes are necessary for the reduction of cytidine diphosphate (CDP) sugars to 1,3-dideoxy sugars. One of these enzymes contains pyridoxamine. Pyridoxamine, not pyridoxal, is a required growth factor for certain organisms. These are the only known cases where the cofactor requirement is for the amine rather than the pyridoxal form. The 1,3-dideoxy sugars are important components of certain bacterial cell walls.

THE STICKLAND REACTION

Some members of the genus Clostridium can utilize amino acids as a source of energy by means of coupled oxidation–reduction reactions involving certain amino acids as hydrogen donors and others as receptors:

\[
R_1\text{-CH}_2\text{NH}_2\text{-COOH} + R_2\text{-CH}_2\text{NH}_2\text{-COOH} + H_2O \rightarrow R_1\text{-CH}_2\text{-COOH} + R_2\text{-COCH}_2\text{-COOH} + 2NH_3
\]

A specific example of such a reaction involving glycine and alanine takes place according to the following reaction scheme:

\[
\text{CH}_2\text{NH}_2\text{COOH} + \text{CH}_3\text{CHNH}_2\text{COOH} + H_2O \rightarrow \text{CH}_3\text{COOH} + \text{CH}_3\text{COCOOH} + 2NH_3
\]

Fig. 14-10. Mechanism of the Stickland reaction.
This type of reaction leads to the formation of short-chain fatty acids and keto acids. The Stickland reaction involves several steps in which NAD first accepts a hydrogen atom from the amino acid donor and transfers it to the acceptor amino acids. The latter reaction is catalyzed by an amino acid reductase (Fig. 14-10). Certain amino acids serve preferentially as hydrogen donors and others as hydrogen acceptors. The rate of the reaction is quite rapid, and this system is used by proteolytic clostridia to gain energy via substrate-level phosphorylation.

**BIBLIOGRAPHY**

**Nitrogen Fixation**


**Inorganic Nitrogen**


**Urease**


**Assimilation of Inorganic Nitrogen**
