NITROGEN FIXATION

1. Introduction

Although $\sim 80\%$ of the Earth's atmosphere is made up of nitrogen gas (dinitrogen or N_2) and so seemingly in plentiful supply, the availability of nitrogen is most often the limiting nutrient for agricultural productivity. Plants and animals cannot use N_2 directly, it has to be converted to a usable "fixed" form, like ammonium (NH_4^+) or nitrate (NO_3^-), before they can assimilate it. In Nature, only certain prokaryotes, the simplest forms of life, can convert atmospheric N_2 gas into a "fixed" usable form. Using the enzyme called nitrogenase, N_2 is converted into ammonium by a processs known as biological nitrogen fixation to provide the basic building block of both proteins and nucleic acids. At any time, $< 0.001\%$ ($\sim 10^{11}$ metric tons) of the global inventory of all forms of nitrogen on Earth ($> 10^{17}$ metric tons) is present as "fixed" nitrogen, which occurs in several forms; in living plants and animals, as dead organic matter, and as soluble inorganic forms (like NO_3^-) in the sea (1-5). The remaining 99.9% is N_2 that is either trapped in primary and sedimentary rocks (2×10^{17} and 4×10^{14} metric tons, respectively, and amounting to $> 97\%$) or free in the atmosphere ($\sim 4 \times 10^{15}$ metric tons or $\sim 2\%$).

Various processes in the nitrogen cycle (Fig. 1) allow transformations among the various forms of nitrogen, thus allowing movement between the inert atmospheric pool and the fixed, usable terrestrial and marine pools. Nitrogen fixation is a pivotal process in global nitrogen cycling. It drives the atmosphere-to-land–sea portion of the cycle. Two other biological processes, called nitrification and denitrification, convert ammonium to nitrate and then, via nitrogen oxides, to N_2, which is returned to the atmosphere. Physical processes, like the leaching and erosion of soils, result in the movement of fixed-nitrogen forms between land and sea. The biological world stays just ahead of a fixed-nitrogen deficiency because the fixation rate slightly exceeds the denitrification rate (6). This slight positive balance could be enhanced if more of the available fixed nitrogen from both biological and chemical (see below) sources could be assimilated by plants. It is estimated that only about one-third of the available fixed nitrogen is assimilated by plants, whereas about one-third is lost by leaching, and about one-third is nitrified–denitrified and lost to the atmosphere.

In addition to the biological process, N_2 is fixed either by natural abiological processes or by industrial ammonia (qv) production (1,2). The total annual biological contribution is estimated at $> 100 \times 10^6$ t, whereas commercial nitrogen fertilizer production contributes $\sim 70 \times 10^6$ t/year (see Fertilizers). Other natural processes, eg, lightning, combustion, and volcanic eruptions, contribute $\sim 30 \times 10^6$ t/year. Thus, the biological process represents the majority ($\sim 60\%$) of the total annual fixation rate and contributes about twice as much as commercial fertilizer production (1,2,5,7,8).

Although plants, and subsequently animals, depend for growth on the availability of fixed-nitrogenous compounds produced ultimately from atmospheric N_2, only a relatively few microbial species are capable of "fixing" N_2 into ammonium. However, these microbes usually do so for their own benefit and, only in certain crops, eg, the legumes (peas, beans, alfalfa), has Nature provided a mechanism for a direct biological interaction between the plant and

nitrogen fixing bacteria. Both partners receive benefit from this mutually beneficial symbiotic association; the plant receives fixed nitrogen directly from the bacteria, which, in turn, are sheltered in nodules on its roots (usually) and are nourished by energy-rich compounds produced by the plant. The most important food crops, eg, the cereal grains (rice, wheat, and corn) and root and tuber crops, do not harbor symbiotic partners. Hence, for crop productivity to reach commercially acceptable levels, extensive augmentation by commercial nitrogen fetilizer is necessary.

Considerable progress in understanding biological nitrogen fixation has been made since the 1970s. For example, the crystal structures of both component proteins of nitrogenase have been solved (9,10) and these provide essential information for the continuing, so far unsuccessful, efforts of chemists to synthesize close representations of the nitrogen-reducing site of nitrogenase. Furthermore, although the mechanism of biological nitrogen reduction is still unknown, a useful numerical model has been developed to assist in the interpretation of experimental data (11). Moreover, purely chemical processes have been devised that bind N_2 and, in some cases, activate the nitrogen sufficiently so that reduced nitrogen compounds (ammonia and/or hydrazine) are produced.

2. Industrial Processes

Until the early nineteenth century, the fixed usable nitrogen stockpiled over millions of years by various natural processes was enough to sustain the needs of the Earth's population. Then, the dramatic growth of cities and populations led to the beginnings of the nitrogen fertilizer industry. Guano, hardened bird droppings, was imported into Europe from Peru, as was saltpeter (sodium nitrate) from Chile. These fertilizer forms were supplemented in the industrialized nations by the ammoniacal by-products from coal-gas production. Further increases in demand led to the invention of several nitrogen fixation processes, some of which were exploited commercially.

It was clear at the beginning of the twentieth century that several approaches were available for "fixing" N_2. It was also just as clear that many of them would requre high temperature–high pressure conditions, probably a catalyst, and that, as exothermic equilibrium reactions, rapid cooling of the product. The first process to be exploited commercially was the Birkeland-Eyde (or Norwegian Arc) process for N_2 oxidation, which was implemented in 1905 (7,8,12). In this process, air is passed through an electric arc, which is stabilized by a magnetic field, at temperatures $> 3000^{\circ}C$ to generate nitric oxide [10102-43-9], NO. Effectively, N_2 is burned in an atmosphere of O_2. On cooling the air stream, further oxidation gives nitrogen dioxide [10102-44-0], NO_2, which on absorption into water gives a mixture of nitric, HNO_3, and nitrous, HNO_2, acids (see Nitric Acid). The low ($\sim 2\%$) yield of NO, the tendency to revert to N_2 and O_2 if the product stream is not quenched rapidly, the consumption of large ($\sim 60{,}000$ kWh/t N_2 fixed) amounts of electricity, and the concomitant expense to sustain the arc all led to the demise of this process. The related Wisconsin process for oxidizing N_2 at high temperatures in a pebble-bed furnace was developed in the 1950s (13). Although a plant that produced over 40 t/day of

nitric acid was built, the product recovery costs were not economically competitive.

$$N_2 + O_2 \longrightarrow 2\,NO$$
$$2\,NO + O_2 \longrightarrow 2\,NO_2$$
$$2\,NO_2 + H_2O \longrightarrow HNO_2 + HNO_3$$

At about the same time that the Birkeland-Eyde process was developed, the Frank-Caro cyanamide process was commercialized (14). This process presented a series of different problems to those of the Norwegian Arc and other (see below) processes in that it involved solids as product and reactant. In this process, limestone is heated to produce lime, which is then reacted with carbon in a highly energy-demanding reaction to give calcium carbide. Subsequent reaction with N_2 is exothermic at $\sim 1000^\circ C$ and produces calcium cyanamide [150-62-7], which was applied directly as a solid fertilizer. On contact with ground water, it hydrolyzes to ammonia and calcium carbonate (see Cyanamides).

$$CaCO_3 \longrightarrow CaO + CO_2$$
$$CaO + 3\,C \longrightarrow CaC_2 + CO$$
$$CaC_2 + N_2 \longrightarrow CaCN_2 + C$$
$$CaCN_2 + 3\,H_2O \longrightarrow CaCO_3 + 2\,NH_3$$

Even though the overall energy requirement of this process is only ~ 20–25% of the Arc process, the Haber-Bosch process, also developed in the early 1900s, proved to be much more economical. The low fixed-nitrogen content of calcium cyanamide, the lime (often inappropriate for continuous application to soils) produced on its hydrolysis, and the decreasing use of cyanamide as a weed killer, all contributed to its decline. A fourth process, the Serpak process for the catalytic nitriding of aluminum, was never commercially exploited to any significant degree, again because of the large energy requirement (12).

The synthetic ammonia industry of the latter part of the twentieth century employs only the Haber-Bosch process (5,7,12–16), which was developed in Germany just before World War I. Development of this process was aided by the concurrent development of a simple catalyzed process for the oxidation of ammonia to nitrate, needed at that time for the explosives industry. Haber was the academic who researched the necessary conditions and catalyst requirement. Although his early results were criticized by Nernst, he perservered and found that, at 200-atm pressure and $\sim 1000^\circ C$ over a suitable catalyst, up to 80 g NH_3 could be produced in 1 h from a 3:1 mixture of H_2 and N_2. For the simple reason that H_2 was expensive at that time, he developed a system to freeze out the NH_3 after each pass and this allowed him to recirculate the gases through the reactor.

$$N_2 + 3\,H_2 \longrightarrow 2\,NH_3$$

Bosch's role was, in many ways, harder. His job was to scale-up and commercialize Haber's system. He had to find a stable and commercially viable catalyst (together with Mittasch), a suitable source of H_2, and to determine the

minimal commercially acceptable operating conditons. His labors resulted in the methodology described below. Although ammonia itself is commonly used as a fertilizer in the United States, elsewhere it is often converted into solid or liquid fertilizers, such as urea (qv), ammonium nitrate or sulfate, and various solutions (see Ammonium Compounds).

2.1. The Modern Haber-Bosch Process. A modern ammonia plant performs two distinct functions. The more energy demanding and complex function is the preparation and purification, from various feedstocks, of synthesis gas, known as syngas, which contains N_2 and H_2 in a 1:3 ratio. The second function is the catalytic conversion of syngas to ammonia (Fig. 2). In the years since its commercial introduction in 1913, many process changes have been made in syngas production to lower costs and to give greater efficiencies.

Synthesis Gas Production. Through World War II, coal (qv) was the primary raw material for ammonia synthesis, either directly as in the original water–gas plant or indirectly via coke-oven gas. As of the 1990s, petroleum-based products are preferred and represent $\sim$90% of all feedstocks. Hydrogen for synthesis gas is produced either by steam reforming of natural gas and other lighter hydrocarbons (qv), eg, naphtha, or by the partial oxidation of heavy oils and coal. Other processes include coal carbonization, oil refining, and the electrolysis of water (14) (see Coal Conversion Processes; Gas, Natural; Hydrogen).

In the catalytic steam reforming of natural gas (see Fig. 2), the hydrocarbon stream, principally methane, is desulfurized and, through the use of superheated steam (qv), contacts a nickel catalyst in the primary reformer at $\sim$30-atm pressure and 800°C to convert methane to H_2. Reforming is completed in a secondary reformer, where air is added both to elevate the temperature by partial combustion of the gas stream and to produce the 3:1 ratio of H_2 to N_2 downstream of the shift converter as is required for ammonia synthesis. The water-gas shift converter then produces more H_2 from carbon monoxide and water. A low temperature shift process that uses a zinc–chromium–copper oxide catalyst has replaced the earlier iron oxide-catalyzed high temperature system. The majority of the CO_2 is then removed.

$$CH_4 + H_2O \longrightarrow 3\,H_2 + CO$$

$$CO + H_2O \longrightarrow H_2 + CO_2$$

The partial-oxidation process differs only in the initial stages before the water-gas shift converter. Because it is a noncatalyzed process, desulfurization can be carried out further downstream. The proportions of a mixture of heavy oil or coal, etc, O_2, and steam, at very high temperature, are so adjusted that the exit gases contain a substantial proportion of H_2 and carbon monoxide. These gases are then fed to the water-gas converter as in the steam-reforming process, after which they are compressed to $\sim$200 atm for processing in the catalytic ammonia converter.

$$3\,C + H_2O + O_2 \longrightarrow H_2 + 3\,CO$$

A breakthrough in this area was the development of centrifugal turbine compressors, which gave a significant energy savings compared to the reciprocating compressors, but only at ammonia outputs of >600 t/day. This economy means that all newer plants operate at 1000–2000 t/day. Energy can also be saved by increasing the operating pressure for the reformers in synthesis-gas production because this lowers compression costs at the catalytic ammonia converter and improves heat recovery from the unreacted steam. The general trend is for ammonia plants to become independent of external electricity sources. Many recovery systems are employed in such a way that waste heat is recaptured in a reusable form.

Catalytic Conversion to Ammonia. A large number of catalysts have been tested in the ammonia synthesis reaction. Haber originally proposed osmium as the catalyst and achieved an equilibrium yield of ammonia of 8% at 550°C and 150 atm. However, on an industrial scale, only iron, cobalt, molybdenum, and tungsten are practical. The most economical catalyst is metallic iron, using alumina and potassium oxide as promoters. The alumina acts as a structural promoter to prevent catalyst sintering and K_2O provides an electronic promoter, acting to increase the synthesis rate per unit area of the catalyst (17,18) and also aiding in desorption of ammonia from the surface. Of the three known iron oxides, only magnetite, Fe_3O_4, yields an efficient catalyst after reduction with H_2 to spongy iron (14) (see Catalysis). A cobalt–molybdenum catalyst actually has better ammonia-synthesis activity at low ammonia concentrations.

Because the ammonia synthesis reaction is an equilibrium, the quantity of ammonia formed depends on temperature, pressure, and the H_2/N_2 ratio. At 500°C and 200-atm pressure, the equilibrium mixture contains 17.6% ammonia. The ammonia formed is removed from the exit gases by condensation at -20°C and the gases are recirculated with fresh synthesis gas into the reactor. The ammonia must be removed continually as its presence decreases both the equilibrium yield and the reaction rate by reducing the partial pressure of the N_2–H_2 mixture.

The mechanism of the synthesis reaction remains unclear. Both a molecular mechanism and an atomic mechanism have been proposed. Strong support has been gathered for the atomic mechanism through measurements of adsorbed nitrogen atom concentrations on the surface of model working catalysts, where dissociative N_2 chemisorption is the rate-determining step (17,18). The likely mechanism, where (ad) indicates surface-adsorbed species, is as described below.

$$\begin{aligned} H_2 &\leftrightarrow 2\,H\,(ad) \\ N_2 &\leftrightarrow 2\,N\,(ad) \\ N\,(ad) + 3\,H\,(ad) &\leftrightarrow NH_3\,(ad) \\ NH_3\,(ad) &\leftrightarrow NH_3 \end{aligned}$$

Density functional theory (DFT) calculations have been applied to this industrial process and show a linear relationship between the activation energy for N_2 dissociation and the binding energy of atomic nitrogen on various transition-metal surfaces, plus a dependence on the structure of the active site. This relationship led to a volcano-type dependence of the catalytic activity on nitrogen

heat of adsorption. Together, these results show that DFT calculations can predict the relative activity of different potential catalysts. Such calculations have been extended to show the feasibility of industrial ammonia synthesis at decreased temperatures and pressures (18–21).

Operational Constraints and Problems. Synthetic ammonia manufacture is a mature technology and all fundamental technical problems have been solved. However, extensive know-how in both the construction and operation of the facilities is an absolute requirement and severe problems can arise when industrial expertise is lacking. Although apparently simple in concept, these facilities are complex in practice. Some of the myriad operational parameters, eg, feedstock source or quality, change frequently and the plant operator has to adjust accordingly. Most modern facilities rely on computers to monitor and optimize performance on a continual basis.

3. Biological Systems

In contrast to the large industrial facilities required to produce ammonia economically, some microorganisms are capable of diazotrophy, which is the ability to use N_2 gas as the sole source of nitrogen for growth. Only prokaryotes, which are those living things without an organized nucleus (eubacteria, cyanobacteria, archebacteria, and actinomycetes) can perform biological nitrogen fixation, the result of which is the reduction of N_2 to ammonia. These microbes can be either free-living, eg, *Azotobacter* and *Clostridium*, or symbiotic, like the rhizobia. The latter group, which form tight associations with higher leguminous plants, are much more important agriculturally. The energy supplied by the legume to the rhizobia in exchange for the supply of fixed nitrogen is generated by photosynthesis. Thus, renewable solar energy (qv) powers this biological fertilizer-production system, which operates at ambient temperature and pressure in marked contrast to commercial ammonia fertilizer production. However, industrial nitrogen-fertilizer production is almost at the theoretical energy-efficiency limit and accounts for only 5% of the annual global fuel consumption. It has driven the enormous increase in food production over the second half of the twentieth century, particularly in tandem with the "green revolution" crop varieties. Today, food is more plentiful than ever before and ammonia is as cheap as it is ever likely to get (5). Unfortunately, this abundance has led to excessive application rates, which has resulted in eutrophication of surface waters and contamination of ground water. It has also led to the loss of soil tilth. If these problems continue to increase in developed countries and as food demands increase in developing countries, the exploitation of biological nitrogen fixation becomes more and more attractive as an alternative to commercial fertilizer production. Current research in this area ranges from selecting the most efficient and competitive microsymbionts for legume plants through the increased use of associative symbioses to the development of catalysts based on nitrogenase for N_2 reducing processes.

The ability to fix N_2 is widely spread among bacterial genera (22–24). It occurs in free-living obligate aerobes, microaerobes, facultative anaerobes, and strict anaerobes, with examples in each group capable of photosynthesis, as

well as in a variety of associations with higher plants, liverworts, and fungi. Although farmers recognized the benefits of crop rotations utilizing legumes centuries ago, the source of that benefit was unknown to them. The first report of nitrogen fixation, in 1838, compared the growth and nitrogen content of cereals and leguminous plants in both greenhouse and field experiments (25,26). Using clover in rotations with wheat and tuber crops, Boussingault clearly showed that the increased nitrogen content was associated with the presence of the clover plants. When he speculated that nitrogen entered plants directly, there was much skepticism. It was not until the 1880s that his work was convincingly confirmed. The perplexing question of the source of the newly fixed nitrogen was solved by Hellriegel and Wilfarth, who localized the activity to the bacteria-filled nodules on the roots of pea plants (25–29).

3.1. Plant–Bacterial Associations. Certain nitrogen-fixing bacteria enter into a variety of associations with their plant hosts (22,23,25,26,30,31). In addition to the *Rhizobium*-leguminous plant associations that are mentioned above and that were first recognized in the 1880s (25–30), there are associations that involve: (*1*) the nitrogen-fixing actinomycete *Frankia* harbored in root nodules on nonleguminous woody plants and trees, such as alder; (*2*) cyanobacteria intimately associated with fungi, ferns, cycads, and even the angiosperm, *Gunnera*; and (*3*) the less formalized associative symbioses of grasses with, eg., *Azotobacter*, *Azosprillum*, *Gluconacetobacter*, and *Azoarcus*.

Rhizobium-Legume Associations. The legumes (family Leguminoseae) include temperate and tropical flowering plants, ranging from small plants, like clover, to bushes and large trees, eg, acacia. Only $\sim$3500 of the known 17,000 species of legumes have been tested for root nodulation. About 90% of the species tested in the subfamilies, Papilionoideae and Mimosoideae, are nodulated by rhizobia, but $<25\%$ of the Caesalpinioideae species are nodulated (22,23,32). Two legume tree genera have nodules on their stems rather than on their roots. All nodules on legumes are caused by colonization by rhizobia bacteria, which are grouped into several genera. These are *Rhizobium*, which are usually fast-growing, unable to fix N_2 *ex planta*, and have plasmid-borne nitrogen fixation (*nif*) genes and a narrow host range; *Bradyrhizobium*, which are usually slow growers, some of which can fix nitrogen *ex planta*, and have chromosomal *nif* genes and often a broad host range; *Azorhizobium* and *Allorhizobium*, each of which consists of only one species that is a fast grower capable of fixing nitrogen *ex planta*; *Sinorhizobium*, which appear closely related to *Rhizobium* and were isolated originally in China; *Mesorhizobium*, which was originally isolated in Mexico and Central America and has species with *nif* genes either on the chromosome or on symbiotic plasmids, suggesting that it is intermediate between *Rhizobium* and *Bradyrhizobium*; and *Photorhizobium*, which has been proposed, but not yet generally accepted (33,34). There is only one known example of rhizobia nodulating a nonlegume; *Parasponia*, which is a woody member of the elm family that grows in southeast Asia and the Pacific islands, has nodules (35).

The best studied associations are those for important crops, eg, the pulses (peas and beans, including soybeans) (see Soybeans and Other Oilseeds), clovers, and alfalfa. These associations show a degree of specificity. Certain rhizobia only infect certain host plants. However, some legumes may be nodulated simulta-

neously by several rhizobial species, and a single rhizobial species may nodulate more than one legume host. The rhizobia are divided into species and biovars, based on the range of acceptable hosts. This situation gives the appearance of promiscuity, however, recognition among plant and microbe is a highly refined, but still incompletely understood, process (36–40).

The first visibly discernible stage of the developing symbiosis is root hair deformation, branching, and curling. The rhizobia promote invagination of the external surface of the root at these deformed root hairs to form an infection thread through which they enter the plant. As the infection thread elongates, and in some symbioses even before it is formed, root inner-cortical cells are induced to divide. These cells become the nodule primordium. The thread enters these cells and releases the rhizobia, which remain confined within vesicles bound by the plant-derived peribacteroid membrane. Within the infected plant cells, the bacteria cease growing and enlarge, and often take on unusual shapes to become bacteroids. Infection occurs as early as the appearance of the first leaves and fixation continues usually until pod filling.

Certain strains of rhizobia are relatively ineffective as N_2 fixers. Thus, seeds are coated with inoculum, often in the form of a dried effective rhizobial culture in peat, before planting so that indigenous strains of rhizobia in the soil are out-competed and good fixation rates ensured. This practice is important because, once a plant is infected by one strain, the ability of other strains to invade the plant is greatly decreased (41). Infection is also inhibited if the soil contains significant amounts of fixed nitrogen (22,23).

During the early rhizobial root-colonization stage, the root exudes various compounds, especially flavonoids and isoflavonoids, which are effective in rhizobial chemotaxis and stimulate the expression of the rhizobial nodulation (*nod*) genes (42–44). These inducing compounds are active at low concentrations and appear to be exuded specifically in the area of the root most responsive to nodulation (45). The first of the compounds to be identified was luteolin [491-70-3] (42), which, because it is also produced by seeds and even by nonleguminous plants, cannot be host specific as expected. It appears to be the range of compounds produced by the plant that determines host specificity (44). Every stage in nodule formation is accompanied by the expression of nodule-specific plant genes, each encoding a plant protein called a nodulin (40,46). In these early stages of nodulation, the rhizobial *nod* genes (40,47–49) are stimulated by the flavonoids, which, together with the product of the rhizobial regulatory *nodD* gene, induce the expression of the other *nod* genes. This concerted action results in the synthesis of the bacterial signal molecules called Nod factors.

Nod factors consist of three to five β1-4-linked *N*-acetylglucosamine residues. The nonreducing sugar is acylated with a variety of either saturated or unsaturated fatty acids and may also be modified by either *O*-acetylation or carbamylation. Substitution at the reducing sugar, eg, either with sulfate in *Rhizobium meliloti* (38) or with 2-*O*-methylfucose in *Bradyrhizobium japonicum* (50), is essential for biological activity. The common *nodABC* genes are required for the core oligosaccharide, whereas the other host-specific *nod* genes are responsible for the appended side chains, which likely confer host specificity on the individual Nod factors (49).

Rhizobia were once thought to fix N_2 only after becoming bacteroids within the plant nodule. It is now known, however, that some of them can fix *ex planta*, but only when the O_2 concentration is $< 0.5\%$. This low O_2 requirement mimics the role in nodules of leghemoglobin, which is to supply a high flux of O_2 at a low concentration to the bacteroids for metabolism without causing damage to the oxygen-sensitive nitrogenase (51). Leghemoglobin, however, is not restricted to legume nodules because the genes encoding leghemoglobin are widespread in plants and are even expressed at low levels in some in the absence of nodulation (52). Leghemoglobin may act generally to signal an O_2 deficit and, thus, the need for the plant to shift from oxidative to fermentative metabolic processing (53). Leghemoglobin is the protein that gives cut nodules their red color and is plant produced (54).

The nitrogen -reducing enzyme, nitrogenase (see the section Nitrogenase), is of bacterial origin and the rhizobial nitrogenase is very similar to the enzyme from the free-living nitrogen fixing organisms (55,56). In some symbioses, nitrogenase-produced H_2 is evolved directly from the nodule (57), whereas other symbioses recapture this H_2 through a bacterial uptake hydrogenase and so recycle this otherwise lost energy. This type of recapture has been suggested as both an index of efficiency and a criterion for the selection of rhizobia for agricultural use (58).

Although root nodules are the most common sites of N_2-fixing symbioses, some tropical legumes, like *Sesbania*, produce stem nodules in association with *Azorhizobium caulinodans* (59). In contrast to root nodules, some stem nodules are photosynthetic and contain, in the case of *Aeschynomene indica*, rhizobia themselves capable of photosynthesis (60). This close relationship of photosynthesis to fixation may ease the energy supply demand of nodules.

Nodulated Nonleguminous Angiosperms. Actinomycetes nodulate a wide variety of host plants that appear to be unrelated taxonomically, but almost all are woody trees and shrubs. The best studied association involves *Alnus* (alder), which develops root nodules called actinorhizae, that harbor actinomycetes of the genus *Frankia* (22,23,61,62). The plants have a wide geographical distribution and often are the first plant types to colonize poor or devastated soils. They, therefore, play an important ecological role, and some may be of great significance in biomass production. Alder is increasing in importance in timber production in the northwestern United States because the growth of Douglas fir is apparently stimulated by intercropping with alder (63).

Distinct *Frankia* species are apparent from DNA-hybridization studies and several *Frankia* host-specificity groups have been described based on the ability to infect the same group of plants. Unlike the rhizobia and other N_2-fixing microsymbionts (see below), this organism is multicellular and differentiated. Even so, infection occurs similarly to that with legumes. The hyphae-like filaments penetrate the plant tissue and end in club-shaped vesicles, which are the site of N_2 fixation (64). Cell-free, partially purified preparations of nitrogenase from these vesicles have properties similar to those of the purified enzyme from free-living bacteria (65). Most actinorhizal nodules (64) have little or none of a hemoglobin-like protein present and the only barrier to O_2 diffusion, and for nitrogenase protection, appears to be the vesicle envelope. Thus, most actinorhizae show maximum rates of nitrogen fixation at atmospheric O_2 levels. There

are, however, some actinorhizae that produce a hemoglobin-like protein, with about 50% amino acid sequence similarity to that in soybean, and also have a physical barrier. In this second group, the hemoglobin-like protein probably plays a similar role in O_2 diffusion to the one it has in legume root nodules.

Cyanobacterial Associations. Symbiotic associations of cyanobacteria range from lichens, involving a fungus, through liverworts and ferns, to gymnosperms and an angiosperm (22,23,66). Most are less formalized than the legume-*Rhizobium* symbiosis. Almost all the involved cyanobacteria are capable of growth and nitrogen fixation without the host. In the symbiosis, the cyanobacterium's primary function is to provide fixed nitrogen for both partners. This role is clearly shown by the increased number of heterocysts, the specialized nitrogen-fixing cells, that develop. No specialized structure, like the nodule, is developed to accommodate the symbiosis. The microsymbiont usually invades normal host structures, like the leaf cavity in *Azolla*, although modifications of these structures may subsequently occur.

Lichens are exceptional in that the symbiotic state is classified as a separate organism. All nitrogen-fixing lichens have a fungal and cyanobacterial symbiont (most often a *Nostoc* species), but some may have a green alga as a third partner. Each lichen genus accommodates only one cyanobacterial genus, either in layers just below the surface or restricted to spherical bodies called cephalodia. In bryophytes (mosses and liverworts), the cyanobacteria, again often a *Nostoc* species, are enclosed in cavities in the ventral side of the thallus. Bryophytes also form casual epiphytic associations with cyanobacteria.

The water fern *Azolla* is unique in being the only known fern genus to associate symbiotically with a nitrogen-fixing cyanobacterium, *Anabaena azollae*. It is globally distributed and is variously considered as either a waterway-blocking weed or as an important contributor of fixed nitrogen to rice culture. Either as a green manure or grown in dual culture, estimates indicate that *Azolla* can supply fixed nitrogen to rice in amounts comparable to those supplied by the rhizobial symbiont to the legume (67). In Nature, the fern is always associated with the cyanobacterium, but it can be freed of the microsymbiont and, when provided with a fixed-nitrogen source, grown alone. The alga from this association is difficult to grow alone and the evidence for reinfection of an algal-free plant is controversial. The microsymbiont lives in a cavity in the dorsal leaf lobe of the fern. Transmission from fern to fern is ensured by retention of some *Anabaena* filaments within the sexual reproductive organs, so that as the young fern develops through these filaments, its leaves become rapidly infected (67).

Only the Cycads, which commonly grow in Africa, South America, and Australia, among the gymnosperms form nitrogen fixing associations with cyanobacteria. These plants produce modified lateral roots called coralloid roots, which often extend above the ground. Infection of these roots is common and almost always involves *Nostoc* (22,23,66). The site of entry of the microsymbiont is unknown, but they are usually located intercellularly. In the angiosperms, only *Gunnera* forms an association with *Nostoc*, through invasion of its secretory glands just behind the shoot apex (66). Although located above the ground and within the stem, these sites of nitrogen fixation are still often referred to as nodules. Unusual aspects of this cyanobacterial association and indications of

its advanced nature are the intracellular location of the bacteria, the well-developed vascular system surrounding the microsymbiont, the high rates of nitrogen fixation, and the efficient transport of fixed nitrogen such that all the needs of the host are met.

Associative Symbioses. There are some rather informal associations in which a measure of interdependence exists among some grasses (family Gramineae) and bacteria, but where no specialized structure is developed. The earliest discovered examples are the association of the tropical grass *Paspalum* with the bacterium *Azotobacter paspali*, and that of the grass *Digitaria* with *Azospirillum brasilense* (68). In the former, a mucilaginous sheath forms around the root, within which the bacteria live and fix nitrogen. The bacteria do not invade the plant tissue. In the *Digitaria–Azospirillum* asssociation, however, the roots are invaded, but no nodule develops. The extent to which the plants benefit from the association remains uncertain. *Azospirillum lipoferum*, which occurs in temperate zones, associates with certain corn and sorghum cultivars, but the effect on the plants appears to be due to increased phytohormone production rather than nitrogen fixation (69,70). More recently, formalized endophytic associations have been discovered. The best characterized examples involve *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. with sugar cane (71), and *Azoarcus* spp. with Kallar grass and possibly rice (72). It appears that some of these associations can supply up to 60% of the fixed nitrogen required for the host plant's growth, indicating a significant agronomic and economic potential.

Other Associations. It is often difficult to separate nitrogen fixation arising from free-living species on the surface of a plant from that which is intimately associated with plant organs. Furthermore, whether bacterial invasion is directed or adventitious is not always clear. For example, *Pseudomonas* spp. and *Azospirillum* spp. are often found inside the roots of grasses, but how they entered and what function they perform is often unclear. Other associations occur with leaf surfaces, and the nitrogen-fixing bacteria that multiply in the phyllosphere could upgrade the local soil conditions after rain washes their products onto the ground. A more formal association, involving the leaves of certain tropical angiosperms, like *Psychotria*, and *Klebsiella* bacteria exists. Here, the bacteria occupy nodules on the leaf surface but, even though they are known to fix N_2 as a free-living organism, the *Klebsiella* cease to do so in the nodule. Although some benefit is likely to be derived by each of the partners, it is not from nitrogen fixation (73). Similarly, mycorrhizae, which are symbiotic fungi, were once thought to fix N_2 in association with the roots of the gymnosperm *Podocarpus*. In fact, they only create an environment conducive to nitrogen fixation by soil bacteria (74).

Finally, associations with animals exist (75). For higher animals, eg, humans with *Klebsiella* and ruminants with *Clostridium*, it is unlikely that much is contributed to the nitrogen status of the animal because sufficient fixed nitrogen likely exists in the diet to repress any nitrogen-fixing activity by the bacteria. However, for termites and shipworms, the associations are significant. *Citrobacter* infects the intestinal tract of termites and can, if necessary, fix nitrogen there. The amount of nitrogen fixed and the benefit gained by the insect appears to depend on its diet (76,77). The nitrogen-fixing, cellulose-decomposing bacterium that inhabits the Deshayes gland in wood-boring shipworms has yet to

be identified. However, it forms a significant association, which contributes significantly to the mollusc's well being by providing up to 35% of its fixed-nitrogen requirement (78). This whole area of animal symbioses has been only sparsely researched.

3.2. Free-Living Microorganisms. Except for the cyanobacteria, free-living bacteria are generally not agriculturally important. They contribute only ~0.1% of the fixed nitrogen of a leguminous association. The free-living cyanobacteria, by comparison, contribute ~2–5% as much as a leguminous association. The difference probably lies in the cyanobacteria's ability to photosynthesize, which relieves their dependence on the often limiting carbon, and therefore energy-yielding, substrates in the soil.

Free-living bacteria are, however, used as the source of the enzyme nitrogenase, which is responsible for nitrogen fixation (8,28,31,79,80), for research purposes because these are easier to culture. The enzyme is virtually identical to that from the agriculturally important rhizobia. These free-living nitrogen fixers can be simply classified into aerobes, anaerobes, facultative anaerobes, photosynthetic bacteria, and cyanobacteria.

Aerobes and Microaerophiles. The best studied examples of aerobes are the azotobacters. All are obligate aerobes, which efficiently fix N_2 only in air. Many other genera fix N_2, but are usually more sensitive to O_2, eg, *Corynebacterium* and *Azospirillum*, and require microaerophilic conditions for fixation. A similar situation exists for the remarkable nitrogen-fixer, *Thiobacillus ferrooxidans*, which grows at pH 2 by oxidizing ferrous iron to ferric. All these organisms have systems, some more efficient than others, to protect nitrogenase from damage by O_2 while they are growing aerobically. Similarly, *Rhizobium* fixes only when the microaerophilic conditions existing in the nodule are simulated in a plant-free culture. Many other genera oxidize gases, eg, hydrogen or methane, to derive the energy necessary for growth and fixation.

Anaerobes. These bacteria, typified by *Clostridium*, are found in soil and water and, as obligate anaerobes, cannot use O_2. The clostridia metabolize glucose and related compounds to butyrate, carbon dioxide, and hydrogen, and many species fix N_2. A second group of nitrogen fixing anaerobes are the sulfur bacteria, such as *Desulfovibrio*. Again, not all species fix N_2. They grow by reducing sulfate or other oxidized sulfur compounds to sulfide, which is in part responsible for the smell of polluted environments. *Desulfovibrio*, which occurs naturally in the sea, is ecologically important as the principal nonphotosynthetic nitrogen-fixing contributor to the formation of nitrogen-containing marine sediments. A third group of anaerobic nitrogen-fixers are the methanogens, which are members of the Archeae, the third Kingdom of living things.

Facultative Anaerobes. These bacteria can grow with or without O_2, but they fix N_2 only under anaerobic conditions. The well-studied genus *Klebsiella* occurs in soil, water, and animal intestines and contains a number of species that fix N_2. They are related to *Escherichia coli*, which, although not a naturally occurring nitrogen fixer itself, can be genetically modified to fix N_2. Other genera, including *Citrobacter* and *Enterobacter*, are also of this type. Another nitrogen fixer is *Bacillus*, which, like *Clostridium*, has the ability to form spores to survive unfavorable conditions.

Photosynthetic Bacteria and Cyanobacteria. The photosynthetic bacteria are anaerobes that do not perform oxygen-generating photosynthesis and fix N_2 only in the light. Like the cyanobacteria, they can use CO_2 as their sole carbon source for growth. The photosynthetic bacteria are usually colored and include both sulfur and nonsulfur bacteria. *Chromatium* is red and oxidizes sulfur or sulfides to sulfate during photosynthesis to produce the reductant, and possibly the energy, necessary for nitrogen fixation. *Rhodospirillum* is a purple, nonsulfur, nitrogen-fixing genus. The cyanobacteria differ from the photosynthetic bacteria because cyanobacteria grow aerobically and like plants produce O_2 from photosynthesis. To protect nitrogenase from the evolved O_2, some genera, like *Anabaena*, produce heterocysts, which are incapable of oxygen-evolving photosynthesis, where nitrogenase is located. Others, like the filamentous *Plectonema*, do not have heterocysts, but fix N_2 only under lower O_2 pressures and low light intensity. In contrast, the unicellular *Gloethece* can fix N_2 in air.

4. Nitrogenase

The biological catalyst that reduces atmospheric N_2 to ammonia is the metalloenzyme nitrogenase. It exists as four types, three of which are closely related, but genetically distinct. The first of the three related types is the conventional molybdenum-based system, often called Mo-nitrogenase, which was isolated in the 1960s. The other two related types are the so-called "alternative" nitrogenases, namely, the vanadium-based V-nitrogenase and the iron-based Fe-nitrogenase (or nitrogenase-3). As early as 1930 (81), molybdenum was believed to be absolutely essential for nitrogen fixation, even though vanadium was found to be almost as stimulatory to bacterial growth on N_2 (82). The requirement for Mo was solidified both by the isolation of the larger of the two component proteins of nitrogenase, the molybdenum–iron (or MoFe) protein (83) containing a FeMo-cofactor (84), and later by the discovery of nitrogen-fixation specific (*nif*) genes involved in Mo-specific functions.

Biochemical-genetic studies have since shown that biological nitrogen fixation can also occur in the absence of Mo (85–88). The V-nitrogenase was demonstrated first when added vanadium stimulated diazotrophic growth of strains of both *Azotobacter vinelandii* and *A. chroococcum* from which the Mo-nitrogenase structural genes had been deleted and later by the isolation of V-nitrogenase (89,90). Since then, *A. vinelandii*, *Rhodobacter capsulatus*, and *Clostridium pasteurianum* among others have been shown to also harbor the third related nitrogenase, Fe-nitrogenase (91–93). The use of either specific DNA probes or the characteristic ethane production from acetylene reduction has established the distribution of these "alternative" nitrogenases among bacterial species (94–96). Although all nitrogen-fixing species studied so far have Mo-nitrogenase, it is still unclear why some species have only this single nitrogenase, whereas others have either two or three. The biosynthesis of each enzyme is transcriptionally regulated by the availability of the appropriate metal ion in the medium. If Mo is present, only Mo-nitrogenase is synthesized. When Mo is absent and V is plentiful, V-nitrogenase is synthesized. When both are absent, Fe-nitrogenase is

produced. However, vanadium only represses transcription of the Fe-nitrogenase structural genes if the V-nitrogenase structural genes are present (97).

The fourth nitrogenase (St-nitrogenase) is completely different to the three convential nitrogenases and has been found only in *Streptomyces thermoautotrophicus* (98). Its components and properties will be described later (see the section Requirements for Catalysis and Substrate Reduction).

4.1. Nitrogenase Component Proteins. In 1960, Mo-nitrogenase was first isolated as a cell-free extract from *Clostridium pasteurianum* (99). Since then, many species have yielded Mo-nitrogenase. In general, the enzymes from all nitrogen-fixing bacteria are very similar (56). The Mo-nitrogenase has been isolated as a $\sim$300-kDa complex (100,101), which is more stable to O_2 than its individual component proteins. The individual components, the MoFe protein and the Fe protein, have also been separately purified (83,102). Neither has activity alone. In fact, all three of the related nitrogenases comprise two separately purifiable component proteins and the genes that encode the subunits of each protein are given the same letter designation. Thus, the homodimeric Fe protein of the Mo-nitrogenase is called Fe protein-1 and is transcribed from *nifH;* Fe protein-2 from the V-nitrogenase is encoded by *vnfH;* and Fe protein-3 from the Fe-nitrogenase is encoded by *anfH*. Each Fe protein is $\sim$60 kDa and has a single [4Fe–4S] cluster that bridges the two identical subunits. There is $\sim$90% sequence identity among Fe protein-1 and Fe protein-2, but only $\sim$60% identity when Fe protein-3 is compared with either Fe protein-1 or Fe protein-2.

The second component is $\sim$230 kDa and takes different forms for each of the three nitrogenases. For Mo-nitrogenase, it is a tetrameric $\alpha_2\beta_2$-MoFe protein encoded by *nifDK*; for V-nitrogenase, it is a hexameric $\alpha_2\beta_2\gamma_2$ VFe protein, encoded by *vnfDGK*; and for the Fe-nitrogenase, a hexameric $\alpha_2\beta_2\gamma_2$ FeFe protein encoded by *anfDGK*. Both the VFe and FeFe proteins have an additional 13-kDa γ-subunit, encoded by *vnfG* and *anfG*, respectively (90,91,94–96,103,104). There is no *nifG* gene and the function of the γ-subunit is assumed by a different protein. All three proteins contain two types of prosthetic group, both unique to nitrogenases. These are the cofactors (FeMo-, FeV-, or FeFe-cofactors) and the P cluster. The best characterized of the alternative nitrogenases are the V-nitrogenases from *A. vinelandii* (103) and *A. chroococcum* (89,104,105) and the Fe-nitrogenases from *A. vinelandii* (105), *Rhodobacter capsulatus* (93), and *Rhodospirillum rubrum* (106). In heterologous crosses, Fe protein-1 and Fe protein-2 cross-complement well with the MoFe protein and VFe protein, but not with the FeFe protein, whereas the Fe protein-3 is virtually ineffective with either the MoFe protein or the VFe protein (91,94–96,104).

In addition, Fe protein-1 has at least two noncatalytic roles related to nitrogen fixation, and presumably Fe protein-2 and Fe protein-3 do also (94). The first function is somehow involved in the early stages of FeMo-cofactor biosynthesis because mutant strains having a deletion in *nifH* produce neither Fe protein nor FeMo-cofactor, instead only a FeMo-cofactor-deficient apo-MoFe protein is produced (107,108). The second role involves insertion of separately biosynthesized FeMo-cofactor into these apo-MoFe proteins (109).

4.2. Comparative Genetics of the Three Nitrogenase Systems of *Azotobacter vinelandii*. In early studies, *K. pneumoniae* was the organism

of choice for identifying the genes whose products were involved with nitrogen fixation. Using genetic complementation, biochemical reconstitution and *in vivo* DNA-directed gene expression, 20 *nif* genes were identified (110). Later, using the cloned *K. pneumoniae nifHDK* genes as a heterologous probe, similar structural genes were identified in a variety of nitrogen-fixing organisms. Now, using DNA-sequence analysis of whole genomes, the *nif*-gene clusters of many organisms have been and are still being mapped. Often, substantial differences in organization are observed.

The cluster of 20 contiguous *nif* genes in *K. pneumoniae* (111) is shown in Fig. 3. By a series of deletion and complementation experiments, the function of most of these genes has been determined. The general functions are listed in the figure. Not all of these genes are found in all nitrogen-fixing organisms (110), in fact, *Clostridium acetobutylicum* has a minimal set of only nine *nif* genes (112). Nineteen of the 20 genes have been identified, cloned, and sequenced in *A. vinelandii*, but they are distributed in two unlinked clusters (113,114). The principal cluster includes the structural genes *nifHDK*; four of six FeMo-cofactor biosynthetic genes, *nifENVH*; *nifM*, which is involved in Fe-protein processing; *nifSU*, which are involved in providing S^{2-} and Fe, respectively, for cluster biosynthesis (115,116); and others (113). The minor cluster encompasses the other two FeMo-cofactor-associated genes, *nifBQ*, and the regulatory genes, *nifLA* (114).

A number of genes associated with both of the genetically distinct Mo-independent enzymes have also been identified. These are labeled as *vnf* (vanadium nitrogen fixation) for those genes assoicated with the V-nitrogenase and *anf* (alternative nitrogen fixation) for those associated with the Fe-nitrogenase. The known genes include the structural genes for both components of the V-nitrogenase, *vnfHDGK*, and the Fe-nitrogenase, *anfHDGK* (117,118), two *nifA*-like genes (119), and one *nifEN*-like sequence (120). Apparently, each system has its own specific NifA-like protein (either VnfA or AnfA) as a positive regulator. In contrast, the *nifEN*-like (called *vnfEN*) set of genes appears to have a common function in both the *vnf* and *anf* systems. The *nifEN*-gene products form a template on which FeMo-cofactor is biosynthesized before insertion into the separately biosynthesized apo-MoFe protein (121,122). The *vnfEN*-gene products probably work similarly for both the FeV-cofactor and the putative FeFe-cofactor (120).

The already complex genetic situation is further complicated by *nifMBVUS* being essential for all three nitrogenases (114,123). The common *nifM* requirement indicates that all three Fe proteins are so similar that a single NifM protein can process them all. Because *nifBV* are involved with the biosynthesis of the FeMo-cofactor of Mo-nitrogenase, their common requirement suggests similar cofactors in all three nitrogenases. Further, the absolute *nifB* requirement indicates that its function cannot be Mo-specific and it is now known to produce the Fe core of the FeMo-cofactor (124). The requirement for *nifB* but not *nifEN* is unexpected because *nifB* and *nifN* are fused in *Clostridium pasteurianum* (125), which expresses a Fe-nitrogenase (112). The requirement for *nifV*, which provides homocitrate, an organic component of FeMo-cofactor (126), clearly indicates that homocitrate is a constituent of all three cofactors, although its role in nitrogen fixation remains unclear. The products of *nifUS* are required for full activation of both component proteins of Mo-nitrogenase (127) with *nifS* and

nifU providing the means to deliver S^{2-} and Fe for cluster synthesis (115,116) for all three nitrogenases.

4.3. Requirements for Catalysis and Substrate Reduction. All three nitrogenases have the same requirements for catalytic activity; namely, magnesium adenosine triphosphate (MgATP), a low potential reductant, and an anaerobic environment. Under appropriate conditions, a variety of substrates, in addition to the physiological substrate, N_2, can be reduced. Figure 4 shows the inputs, substrates, and products of Mo-nitrogenase. During catalysis, N_2 is reduced to NH_3 and magnesium adenosine triphosphate (MgATP) is hydrolyzed to magnesium adenosine diphosphate (MgADP) and phosphate. Because MgADP is an inhibitor of nitrogenase catalysis by competing for the MgATP-binding sites on the Fe protein, this inhibition is circumvented in *in vitro* assays by using the creatine phosphate–creatine phosphokinase system to reconvert MgADP to MgATP. Reductants capable of supporting nitrogenase turnover have redox potentials more negative than -300 mV and include naturally occurring ferredoxins and flavodoxins *in vivo* and sodium dithionite, $Na_2S_2O_4$, *in vitro* (128). The optimal stoichiometry for nitrogenase function is 4 mol of MgATP hydrolyzed for each pair of electrons transferred to substrate; this ratio is independent of the substrate reduced.

Unlike with the alternative nitrogenases from the same organism, where heterologous crosses that involve components of the Fe-nitrogenase are inactive, the component proteins from Mo-nitrogenases, regardless of their microbial origin, form catalytically active enzymes as heterologous crosses (56). There is but notable exception, that of the *C. pasteurianum* proteins, which do not form active crosses. Carbon monoxide (CO) is a potent inhibitor of all nitrogenase-catalyzed substrate reductions, with the exception of H^+ reduction (129). Molecular hydrogen has a unique involvement with Mo-nitrogenase and with N_2 reduction in particular. It has four well-documented roles both *in vivo* and *in vitro*: (*1*) via hydrogenase, H_2 can act as a reductant for nitrogenase; (*2*) it is the product in the absence of other reducible substrates in an ATP-dependent, CO-insensitive reaction (130); (*3*) H_2 is a specific competitive inhibitor of N_2 reduction, affecting neither reduction of any other substrate nor its own evolution (131); and (*4*) under an N_2–D_2 atmosphere, HD is formed in a CO-sensitive, MgATP-requiring reaction (132–135).

Substrate reduction is accomplished by a series of sequential associations and dissociations of the two proteins and, during each cycle, two molecules of MgATP are hydrolyzed and a single electron is transferred from the Fe protein to the MoFe protein (11,136). The dissociation step is rate limiting at $\sim 6\ s^{-1}$ (11). Although the kinetics of all the partial reactions have been measured, little is known about the details of the mechanism, especially the pathway of electron flow within the MoFe protein, the binding of substrates, the nature of reaction intermediates, etc. However, the structures of the *Azotobacter* Fe protein (137,138), the MoFe protein (9,139–143) and variants (144–146), and 2:1 complexes (10,147,148) have been solved (see the section Structures of the Mo-Nitrogenase Component Proteins and Their Complex) and point out possibilities for all these processes. Earlier mutagenesis studies showed that Arg-101of the Fe protein (149) and Asp-161 of the α-subunit of the MoFe protein (150) were involved in docking the Fe protein onto the MoFe protein.

Despite intensive research effort, the precise role(s) of MgATP hydrolysis are still unclear (151). The consensus view is that MgATP is hydrolyzed to promote electron transfer and to provide the energy needed for completion of the catalytic cycle. Consistent with this view, MgATP is hydrolyzed only when the Fe protein is complexed to the MoFe protein. A currently popular role for MgATP (and its hydrolysis) is to ensure the unidirectionality of electron transfer, so preventing the electron delivered to the MoFe protein from returning to the Fe protein. This situation is often thought of as "gating" of electron flow, which is controlled by MgATP binding and hydrolysis. The "gate" is opened and an electron transferred to the MoFe protein when reduced Fe protein with bound MgATP complexes with the MoFe protein. After MgATP hydrolysis, the "gate" closes preventing back-flow of the electron. However, the order in which electron transfer and MgATP hydrolysis occur is not yet certain (152–154). The binding of MgATP to the Fe protein induces a conformational change, which is reversed on MgATP hydrolysis. This reversible conformational change could be the basis for "electron gating" to ensure delivery to, and accumulation of, multiple electrons within the MoFe protein prior to their delivery to substrate. This so far uncharacterized conformational change in the Fe protein affects the redox potential (155), the shape of the electron paramagnetic resonance (epr) signal (97,156,157), and the accessibility to chelating agents of the iron (158) in the [4 Fe–4 S] cluster of the Fe protein (see Iron Compounds). These observations, together with details from the Fe-protein crystal structure, which shows well-separated binding sites for the [4Fe–4 S] cluster and the nucleotide, are the basis of a hypothesis by which MgATP binding and hydrolysis coordinates and regulates the unidirectional flow of electrons to the MoFe protein (159).

The following sequence of events occurs during each electron-transfer cycle (11). First, the Fe protein is reduced and binds two molecules of MgATP, which results in a contraction of the Fe protein and induces the conformational change that makes for a competent interaction with the MoFe protein (138). Second, the two proteins form a complex, which effects changes in the midpoint potential of the metal–sulfur clusters that energetically favor movement of electrons toward the FeMo-cofactor. The resulting redox potentials are −620 mV for the Fe protein's [4Fe–4S] cluster; −390 mV for the P cluster; and −40 mV for the FeMo-cofactor (158). Third, complex formation results in electron transfer and also triggers MgATP hydrolysis. At this stage, MgATP is hydrolyzed whether or not an electron is transferred. Although phosphate release is usually the work-producing step (when energy transduction occurs), phosphate release from the nitrogenase complex does not drive the dissociation of this complex into its component proteins (152). Fourth, this hydrolytic conversion of the Fe protein from its MgATP-bound state to its MgADP-bound state somehow causes complex dissociation. Dissociation of the complex is the rate-limiting step in nitrogenase catalysis when dithionite is used as reductant (11). Finally, the free Fe protein is rereduced, undergoes nucleotide exchange, and is ready to reenter the catalytic cycle.

This complex intercommunication between the two proteins, which is synchronized by sequential conformational changes induced by MgATP binding, component protein interaction, and MgATP hydrolysis, allows electrons to be accumulated within the MoFe protein for substrate reduction. However, it is

still unclear how, when, and where the eight electrons necessary for the reduction of each N_2 are stored within the MoFe protein and how the required protons are delivered to the bound substrate. Furthermore, because crystals of an altered Fe protein–MoFe protein complex are capable of exchanging MgADP for MgATP (147), dissociation of the complex is not an absolute requirement for nucleotide binding and exchange.

4.4. Electron Transport to Nitrogenase. As mentioned above, a source of low potential reducing equivalents is required to rereduce the Fe protein during the nitrogenase catalytic cycle. Although the ultimate donor of these low potential electrons within the cell has not been identified for most nitrogenases, both flavodoxins and ferredoxins (with molecular weights ranging from 6 to 24 kDa) are capable of serving this function *in vitro*. In some bacteria, eg, *A. vinelandii*, several redundant systems, involving several flavodoxins and ferredoxins, can apparently support nitrogenase catalysis. The ferredoxins contain at least one [4Fe–4S] cluster similar to that found in the nitrogenase Fe protein, whereas flavodoxins do not contain iron atoms, but instead rely on a flavin prosthetic group for redox activity.

These ferredoxins and flavodoxins obtain their electrons from different sources. In *C. pasteurianum*, the appropriate ferredoxin is reduced by the pyruvic phosphoroclastic system, which is responsible for pyruvate metabolism and, therefore, links the ability to reduce N_2 directly to cell metabolism. In the heterocystous cyanobacteria, the primary electron donor is also a ferredoxin, but it receives electrons produced by photosynthesis. In *K. pneumoniae*, a flavodoxin is the ultimate electron donor and its fully reduced (hydroquinone) flavin is oxidized by one electron to the semiquinone. Rereduction is accomplished by a pyruvate-flavodoxin oxidoreductase, which couples the oxidation of pyruvate, yielding acetyl-CoA and CO_2, to the reduction of the bound flavin (161,162). This last enzyme system is not found in other nitrogen-fixing microbes and so they must have other means of providing the required reducing equivalents to nitrogenase.

4.5. Structures of the Mo-Nitrogenase Component Proteins and Their Complex

The Fe Protein. Because of their similar properties, all three Fe proteins likely have similar structures, however, only the Fe protein of Mo-nitrogenase has been structurally characterized (137,138). The X-ray derived structure shows that the subunits of the Fe protein from *A. vinelandii* Mo-nitrogenase are made up of a single domain, which comprises an eight-stranded β-sheet flanked by nine α helices (Fig. 5; **PDB code: 1FP6; 138**). The lone [4Fe–4S] cluster symmetrically bridges the two subunits, each of which provides two cysteinyl residues (numbered 97 and 132 in the *A. vinelandii* primary sequence) to the [4Fe–4S] cluster, which occupies a solvent-exposed position at one end of the dimer interface.

Each subunit has a single nucleotide-binding site in the channel between the subunits. The subunits have two consensus amino acid sequences that are common to other nucleotide-binding proteins. One is the Walker A motif (or P-loop), GXXGXGKS (where X represents any amino acid residue), for residues 9–16, which forms a β-strand–loop–α-helix structure and provides the direct interactions with the nucleotide phosphates. The other sequence, DXXG

(residues 125–128) is the Walker B motif, which includes Asp-125 that interacts with the nucleotide-bound Mg^{2+} ion. The first Fe-protein structure showed partial occupancy of the nucleotide-binding sites with about half an MgADP molecule bound per Fe protein. Its presence was a surprise because no nucleotide had been added during crystallization, so it must have copurified with the Fe protein. A second major surprise was that the nucleotide is bound *across* the subunit–subunit interface with the adenosine interacting with one subunit and the terminal PO_4^{3-} with the other subunit (137). Two later structures showed a different nucleotide orientation; it was bound parallel, not perpendicular, to the subunit interface (10,138), but here excess nucleotide was present during crystallization to result in full occupancy of the nucleotide-binding sites. One structure was from a 2:1 complex of the Fe protein with the MoFe protein (10), the other was of the Fe protein alone (138). Apparently, the orientation assumed by the nucleotide in the structure depends on when it was added and whether sufficient is present to saturate the binding sites with two nucleotides per Fe-protein molecule.

Nucleotide binding to the Fe protein is cooperative and it induces changes in the properties of the [4Fe–4S] cluster (151,162). These include both a change in both the epr spectral line shape and a lowering of the redox potential by ca. −100 to ca. −400 mV. Although the bound nucleotide does not contact the [4Fe–4S] cluster, the effect of nucleotide binding is propagated through a significant structural change in the Fe-protein backbone, which occurs when nucleotide binds. This region, called switch II by analogy to G proteins, involves the Asp-125 residue, which interacts with the nucleotide-bound Mg^{2+}, through to the cysteine-132 residue, which bounds the [4Fe–4S] cluster. Thus, nucleotide binding results in a change in conformation at the cluster and a change in its electronic and redox properties (151). A similar mechanism, through switch I, allows communication between the nucleotide-binding site and the area of the Fe-protein surface that interacts with the MoFe protein during complex formation. It involves residues 59–68 and includes Asp-39. This loop may indicate contact with the MoFe-protein surface and so initiates MgATP hydrolysis and electron transfer to the MoFe protein.

The MoFe Protein. The deduced amino acid sequences of the α- and β-subunits of the VFe and FeFe proteins show significant (55%) identity with one another, but less (32%) with those of the MoFe protein (118). Even so, the conservation of both the domain structures around the eight strictly conserved cysteine residues and the constant spacing between them (94,164,165) indicate that all three protein types have the same general structural features. Subtle but important differences are, however, likely to exist. For example, when the FeV-cofactor center is extracted from the VFe protein and used to reconstitute an apo-MoFe protein, the resulting hybrid protein, unlike both parents, can no longer fix N_2 even though it continues to catalyze the reduction of other substrates (166).

Only the MoFe protein has been structurally characterized. Extensive spectroscopic studies of the MoFe protein, the application of cluster-extrusion techniques (84,167), X-ray absorption spectroscopy (168), and later X-ray diffraction (9,10,139–144) showed that the MoFe protein contains two types of prosthetic groups (protein-bound metal clusters), each of which contains ∼50% of the

MoFe protein's total Fe and S^{2-} content. Sixteen of the 30 Fe atoms and 14 of the 32 S^{2-} constitute one type of prosthetic group in the form of two P clusters, the remaining 14 Fe atoms and 18 S^{2-}, together with both Mo atoms, comprise the two FeMo-cofactors. The distribution of these cluster types within the protein, as originally predicted by sequence and mutagenesis studies (169,170) and confirmed by X-ray techniques, is shown in Fig. 6. These clusters are distributed in pairs, one FeMo-cofactor, which is a [Mo–Fe_7–S_9] cluster with an attached *R*-homocitrate molecule, and $\sim$1.5 nm away, one P cluster, which has a [Fe_8–S_7] composition, to each αβ dimer. One pair is at either end of the protein and they are separated by $\sim$7.0 nm. Their exact composition was only established after their structures were modeled within the MoFe-protein structures from both *A. vinelandii* **(9, 142; PDB code: 3MIN)** and *K. pneumoniae* **(0143; PDB codes: 1QGU, 1QH1 and 1QH8)**.

The MoFe protein is often treated as a dimer of dimers with each αβ-dimer functioning independently of the other, even though active αβ dimers have never been isolated and there is evidence of long-range interactions between its two Fe-protein binding sites (171). The two αβ dimers interact primarily through helices in the β-subunits. An approximately 1-nm wide channel passes through the center of the tetramer. The α- and β-subunits have similar polypeptide folds, that consist of three domains. In the α-subunit, these three domains meet to form a shallow cleft, which the FeMo-cofactor occupies $\sim$1 nm below the protein's surface. It is covalently bound by only two amino acid residues (cysteine-275 and histidine-442, using *A. vinelandii* numbering) from the α-subunit and no close involvement with the β-subunit. In contrast, the P cluster occupies the α- and β-subunit interface with both subunits providing equal numbers of ligating cysteinyl residues (172,173; see Fig. 7).

The P cluster has a biologically unique structure (142,174). It consists of a [4Fe–4S] subcluster that shares one of its sulfides with a [4Fe–3S] partial cube. This shared sulfide is hexacoordinated by the six central Fe atoms. The [4Fe–4S] subcluster is terminally ligated by the γ-S of both α-Cys-62 and α-Cys-154, whereas the [4Fe–3S] partial cube is terminally ligated by the γ-S of both β-Cys-70 and β-Cys-153. Two other cysteinyl residues, α-Cys-88 and β-Cys-95, form μ_2-sulfide bridges between the subclusters (9,142). In the dithionite-reduced (as isolated) state of the MoFe protein, the P clusters exist as a biologically unique [8Fe–7S] cluster in the all ferrous state. On oxidation by redox-active dyes, the P cluster rearranges such that two of the four Fe atoms of the [4Fe–3S] partial undergo a change in ligation and lose contact with the central hexacoordinated sulfide. One Fe atom binds the γ-O of β-Ser-188 and the other binds to the deprotonated backbone amide-N of the already bound and bridging α-Cys-88. Both of these latter ligands are protonated in their unbound state and deprotonated in their Fe-bound state, which raises the possibility that a two-electron oxidation of the P cluster during catalysis releases two protons from the P cluster, thus providing a mechanism for coupling electron transfer to proton transfer (142).

The VFe and FeFe protein also have the equivalent of P clusters, which have similar properties to those found in the MoFe protein (175,176), consistent with the relatively high sequence identity and the similar genetic basis of their biosynthesis. Although the catalytic role assigned to the P cluster involves

initially accepting electrons from the Fe protein for storage and future delivery to the substrate via the FeMo-cofactor centers, this role has yet to be proved.

The FeMo-cofactor is the origin of the biologically unique, characteristic $S = 3/2$ epr spectrum of the MoFe protein. The cofactor can be extracted into an organic solvent, usually *N*-methylformamide (NMF), in which it is stable. After isolation, it still shows the $S = 3/2$ epr signal, although significantly broadened, and the redox activity of the MoFe protein (177–180), but it is ineffective in catalyzing the reduction of N_2, however, it can reconstitute all activity and spectroscopic properties of an apo-MoFe protein when added to an appropriate extract (84). Using either the unique epr signal or X-ray absorption spectroscopy (xas) as a monitor of ligand binding, only one thiolate was found to bind (and only to Fe) per isolated FeMo-cofactor, consistent with the later discovery that only one cysteinyl binds FeMo-cofactor to its protein matrix (181,182). The dithionite-reduced VFe protein from both *A. vinelandii* and *A. chroococcum* exhibits spectroscopy consistent with the presence of a FeV-cofactor, which has now been extracted into NMF (166). Similarly, a FeFe-cofactor likely exists because a $S = 3/2$ epr signal has been elicited from the purified FeFe protein from *R. capsulatus* (183).

Again, a definitive description of the composition and structure of the FeMo-cofactor had to await a high resolution structure of the MoFe protein. Now, it is known that the FeMo-cofactor consists of two subclusters, one [Mo–Fe_3–S_3] and one [Fe_4–S_3], which were originally described as being bridged to one another by three nonprotein sulfides (9,139–143). More recently, a very high resolution structure (184) has shown the presence of a single light atom (C, N, or O) within the central cavity of the FeMo-cofactor and equidistant to all six of the central Fe atoms (Fig. 8). It is unknown whether this light atom plays a mechanistic or a structural role or both. The FeMo-cofactor has only two covalent bonds to the protein. These are provided by the γ-S of α-Cys-275 to the terminal Fe atom and by the imidazole δ-N of α-histidinyl-442 to the Mo. The octahedral coordination of Mo is completed by the three μ_3-sulfides and by the 2-hydroxyl and 2-carboxyl groups of (*R*)-homocitrate. The structure shows that only the homocitrate-Mo apex is surrounded by water molecules. Moreover, the homocitrate is positioned between the FeMo-cofactor and the P cluster and may be involved in electron and/or proton transfer to bound substrate.

In addition to the two covalent bonds to FeMo-cofactor, indirect (hydrogen-bonded) interactions occur through α-Gln-191 and α-Gln-440, both of which hydrogen bond to the homocitrate, and α-Arg-359, α-Arg-96, and α-His-195, all of which bond to various sulfides. Many of these interactions were predicted through mutagenesis studies (185–189). In fact, site-directed mutagenesis of the *A. vinelandii nifD* gene produced the early evidence for FeMo-cofactor as the site of substrate reduction because a single amino acid substitution at either α-Gln-191 (by Lys or Glu) or α-His-195 (by Asn) results in simultaneous changes in both substrate specificity and the epr spectrum (185).

Although FeMo-cofactor is clearly implicated in substrate reduction catalyzed by the Mo-nitrogenase, efforts to reduce substrates using the isolated FeMo-cofactor have been mostly negative or equivocal. Thus, the FeMo-cofactor's polypeptide environment must play a critical role in substrate binding and

reduction. Also, the different spectroscopic features of protein-bound versus isolated FeMo-cofactor clearly indicate a role for the polypeptide in electronically fine-tuning the substrate-reduction site. Site-directed amino acid substitution studies have been used to probe the possible effects of FeMo-cofactor's polypeptide environment on substrate reduction (185,186,188–190). Catalytic and spectroscopic consequences of such substitutions have provided information concerning the specific functions of individual amino acids located within the FeMo-cofactor environment (191–193).

The crystal structures of three variant MoFe proteins are now available. The first is of the αH195Q-MoFe protein, which has residue αHis-195 replaced by Gln **(145; PDB code: 1FP4)**. This variant has been used widely in reactivity studies. Its structure was found to be virtually identical to that of the native MoFe protein, including the >NH→S hydrogen bond, which was originally formed between a central FeMo-cofactor μ_2-sulfide and the gkε-N of the imidazole of αHis195 and is here replaced by a similar bond with the amide-N of glutamine (145). The second structure was of the *nifV*$^-$ MoFe protein isolated from a mutant that could no longer biosynthesize homocitrate **(144; PDB code: 1H1L)**. Again, the structure was essentially identical to that of the native protein, except that now citrate replaces homocitrate as a ligand to Mo. Both structures confirm that the substitutions introduced, at least, in these variant MoFe proteins, have only a local and not a global effect on structure. The third structure was of a "so-called" apo-MoFe protein, which is really a FeMo-cofactor-deficient form of the MoFe protein **(146; PDB code: 1L5H)**. Here, the three domains of the β-subunits and two of the three domains of the α-subunit are essentially unchanged, but the third α-subunit domain shows significant structural changes. These changes create a funnel that leads to the FeMo-cofactor-binding site within the α-subunit and is lined with positively charged residues from its entrance all the way down to αHis-442. This last residue probably serves both as the initial docking point for the negatively charged FeMo-cofactor and as the trigger for the conformational changes that close the funnel and bury the FeMo-cofactor within the α-subunit.

The 1:2 MoFe Protein–Fe Protein Complex. Because the primary structure of the Fe protein shows considerable similarities to those of "nucleotide switch" proteins, like ATPases and GTPases, similar trapping techniques have been applied to nitrogenase. Using AlF_4^- together with ADP, a stable nitrogenase complex was formed and was comprised of two Fe-protein molecules with one MoFe-protein molecule. The 0.3-nm resolution crystal structure of this complex (Fig. 9) confirmed predictions of earlier modeling studies of their docking. The Fe protein undergoes a significant conformational change, whereas the MoFe protein is little changed from its uncomplexed structure (10).

The conformational change of the Fe protein involves a 13° rotation of both subunits, resulting in a more compact structure. Interestingly, small-angle X-ray scattering data indicate that the complex maintains this structure in solution (194). Docking of the two proteins occurs along the Fe protein's twofold symmetry axis, which bisects its single [4Fe–4S] cluster, and the pseudosymmetric αβ-interface of the MoFe protein. The more compact conformation of the Fe protein results in its [4Fe–4S] cluster protruding further into the protein–protein interface and approaching to within ∼1.4 nm of the P cluster of the MoFe protein.

This interaction results in the P cluster being situated equidistant between the Fe protein's [4Fe–4S] cluster and the FeMo-cofactor. This arrangement is consistent with the suggestion that electrons are transferred from the [4Fe–4S] cluster through the P-cluster to the FeMo-cofactor.

About 20 intimate contacts occur between the surfaces of the Fe protein and MoFe protein. One of significance involves the Arg-100 residues of the Fe protein. In some bacteria, Arg-100 is modified in a facile, reversible manner to regulate nitrogenase activity. Obviously, modification of these Arg-100 residues would introduce steric hindrance to the protein–protein interface, prevent complex formation, and so suppress activity and regulate the enzyme. Each of the four Fe-protein subunits in the complex has an associated Mg^{2+}-ADP-AlF_4^- moiety, which is bound parallel to the interface between the subunits. Although different from the orientation of the bound MgADP in the original structure of the isolated Fe protein, this orientation is compatible with that in the more recent crystal structures of the Fe protein (see the section The Fe Protein).

Two other structures of a 2:1 Fe–MoFe protein complex have been solved. The first involves a variant (L127Δ) Fe protein, which has Leu-127 deleted from the switch-II region (see the section the Fe Protein). The effect of this deletion is to mimic the binding of nucleotide, so causing the Fe protein to be permanently in the MgATP-bound state and to form a tight, but inactive, 2:1 complex with the MoFe protein (**147; PDB code: 1G20 and 1G21** for the structures with or without MgATP present). This structure closely resembles that of the MgADP.AlF_4^--stabilized complex as far as the MoFe-protein component and the protein–protein interfaces are concerned. However, a significant difference is the more open conformation adopted by the complexed L127Δ Fe protein, which closely resembles the conformation of the uncomplexed nucleotide-free wild-type Fe protein. The wild-type Fe protein in the second 2:1 complex structure, which resulted from chemical cross-linking the two wild-type component proteins through Glu-112 and βLys-400, adopts a structure that is even more open than when it is uncomplexed (**148; PDB code: 1M1Y**). Again, the MoFe-protein structure is effectively unchanged. However, the relative orientation of the two components is different as is the different interface area. This cross-linked complex might represent an "initial encounter" state that then proceeds to form the "electron-transfer competent" state, which is likely represented by the MgADP.AlF_4^- stabilized complex.

4.6. The *Streptomycetes thermoautotrophicus* Nitrogenase. This enzyme system also consists of two component proteins, but the two components bear no relationship to those components described above. The larger component protein (called St1) is an $\alpha\beta\gamma$ heterotrimer of $\sim$100 kDa that contains Mo, Fe, and sulfide. The smaller component (St2) is a homodimeric manganese-containing superoxide oxidoreductase rather than a Fe protein. The St2 component oxidizes superoxide ($O_2^{\bullet -}$) to O_2, and then transfers the resulting electron to the St1 component, where N_2 is reduced by eight electrons to two NH_4^+ and one H_2. This reaction involves the hydrolysis of less MgATP per N_2 reduced than does the conventional Mo-nitrogenase. Another difference is in the form of the proximal electron donor. Intead of either flavodoxin or ferredoxin, which are the *in vivo* electron donors to the conventional Fe protein, a molybdenum-containing CO

dehydrogenase (St3) provides superoxide by coupling the oxidation of CO to CO_2 to the reduction of O_2 (98).

$$CO + H_2O \longrightarrow CO_2 + 2\,H^+ + 2\,e^-$$

$$2\,O_2 + 2\,e^- \longrightarrow 2\,O_2^{\bullet -}$$

This nitrogenase is also unique in both its lack of reaction with O_2, CO, H_2, and MgADP, all of which are potent inhibitors of N_2 reduction catalyzed by the conventional nitrogenases, and in its inability to catalyze the reduction of acetylene to ethylene.

4.7. Regulation of Nitrogen Fixation. Both the synthesis and activity of nitrogenase are under tight genetic control in nitrogen-fixing cells, mainly because nitrogenase is a principal cellular component having a high energy demand. Regulation responds to three environmental factors: fixed-nitrogen status, O_2 tension, and metal-ion availability. The presence of fixed nitrogen, eg, nitrate, is a significant regulatory factor, and bacteria utilize the fixed nitrogen until it is totally depleted rather than synthesize nitrogenase to fix their own (195). This control mechanism is important for free-living nitrogen fixers, eg, *A. vinelandii*, but less so for symbiotic organisms, eg, *B. japonicum*, which are adapted to export fixed nitrogen to their host. Of primary importance is O_2 tension, because both nitrogenase proteins are extremely sensitive to oxidative damage (196). Finally, because nitrogenase consists of metal-containing proteins, the availability of certain metal ions becomes regulatory, particularly in those organisms that biosynthesize the "alternative" nitrogenases (164,197,198).

Regulation of nitrogen fixation can be exerted at several different levels in different organisms (199). For example, in photosynthetic bacteria, added NH_3 causes a reversible post-translational modification through which the Fe protein is ADP-ribosylated and consequently inactivated (200). In strict aerobes, the enzyme can undergo conformational protection to guard against O_2 damage by forming a protein aggregate, which dissociates to resume fixation when the O_2 stress is relieved. In most filamentous cyanobacteria, *nif*-gene expression is developmentally regulated, such that nitrogenase is only expressed in the specialized, nonphotosynthetic heterocysts (201). However, the most uniform, although by no means all-encompassing, mechanism is one of transcriptional regulation of the *nif* genes themselves by the specific regulatory genes, *nifLA*, which encode the positive regulatory protein, NifA, and the negative repressor, NifL, respectively (202).

NifA proteins are of two types. One type consists of oxygen-sensitive proteins, which are found, eg, in the rhizobia, *Rhodobacter*, and *Azospirillum*. These proteins have an iron-binding motif on a linker between the central and C-terminal domains that forms a redox-sensitive site to sense changes in O_2 tension. The second type, exemplified by the *A. vinelandii* and *K. pneumoniae* NifA proteins, are not oxygen sensitive and do not have this motif. The *nifL* gene product is the negative regulator, which modulates NifA activity in response to changes in O_2 and fixed-nitrogen status (203). Regulation of *nifA* expression varies among organisms and depends on preferred physiology and ecology. For example, fixed-nitrogen status is usually more important for free-living

nitrogen-fixers, whereas O_2 tension is an overriding concern of organisms involved in symbiotic associations with plants.

In enteric bacteria, expression of the *nif* genes is under general nitrogen regulation by the *ntr* system. This system consists of essentially a sensor–activator pair of proteins, encoded by *ntrB* and *ntrC*. When fixed nitrogen is limiting, the sensor protein (NtrB), phosphorylates (and so activates) the activator protein, NtrC, which then interacts with, and allows expression from, all *ntr*-regulated promoters, including that of the *nifLA* transcription unit. When fixed nitrogen is sufficient, NtrB dephosphorylates (and so deactivates) NtrC, which, in turn, switches off the *nifLA* promoter and so stops *nif* transcription. The activity of the NtrB sensor is modulated through the activity of the gene products of the *gln* (glutamine-producing) system. Regulation of nitrogen fixation is obviously a complex, demanding process.

5. Chemical Systems

Nitrogen has a dissociation energy of 941 kJ/mol (225 kcal/mol) and an ionization potential of 15.6 eV, both indicative of the difficulty in either cleaving or oxidizing N_2. For reduction, electrons must be added to the lowest unoccupied molecular orbital of N_2 at -7 eV, which can only occur in the presence of highly electropositive metals, eg, lithium. However, lithium also reacts with water. Thus, such highly energetic interactions are unlikely to occur in the aqueous environment of the natural enzyme system. Even so, highly reducing systems have achieved some success in N_2 reduction, even in aqueous solvents.

5.1. Dinitrogen-Reducing Systems. The binding of N_2 to a metal center is the first step in activating molecular nitrogen toward reduction. Since the first compound of this type, $[Ru(NH_3)_5(N_2)]Br_2$ [15246-25-0], was synthesized (204), many transition metals have been found to form similar compounds (205–207). However, many dinitrogen compounds are so stable that they are unreactive toward reduction and so have little chance to form the basis of a catalytic system.

Nonaqueous Systems. The first nonbiological nitrogen-reducing system was reported in 1964 (208,209) at about the same time that the first metal–N_2 complexes were prepared and when the first active nitrogen fixing cell-free bacterial extracts were being produced. It used titanium tetrachloride ($TiCl_4$ [7550-45-0]), or dichlorobis(η^5-cyclopentadienyl)titanium(IV) (cp_2TiCl_2 [1271-19-8]) together with either ethylmagnesium bromide or lithium naphthalenide as reductant in ethyl ether (208–211). Although the mechanism of reduction remains unclear, the conversion of N_2 into nitride is likely. These systems are not catalytic because solvolysis is needed to liberate NH_3 and in doing so, the active species is destroyed. Later, a truly catalytic effect was demonstrated using a mixture of $TiCl_4$, metallic Al, and $AlBr_3$ at 50°C, when NH_3 was obtained at 200 mol/g.atom Ti via the catalytic nitriding of aluminum (212).

A number of reaction products have been isolated from the cp_2TiCl_n–N_2–reductant system, where $n = 1$ or 2, all of which assume an intense blue color (λ_{max} 600 nm) in solution. The relationship among these products is unclear (213–215), but the lability of the cp ring may be an important complicating

factor. When $cp^*_2TiCl_2$ [11136-36-0], where $cp^* = \eta^5\text{-}C_5(CH_3)_5$, is used, two distinct interconvertible N_2 complexes are formed (216). X-ray crystallography shows one compound to be a bridged mono-N_2 complex, $[cp^*_2Ti]_2(N_2)$ [11136-46-2], which has a linear Ti−N=N−Ti bridge and an N−N bond length of 0.116 nm (217). The second is a tri-N_2 complex of Ti that is unstable above −80°C. Both zirconium analogues are thermally stable and the structure of $[cp^*_2Zr(N_2)]_2(N_2)$ [54387-50-7] shows a similar linear Zr− N=N−Zr bridge (N−N bond is 0.118 nm) plus one end-on terminal N_2 ligand (N−N bond length is 0.1115 nm) on each zirconium atom (218).

The mono-N_2 Ti complexes apparently cannot produce hydrazine or ammonia directly, but in the presence of excess reductant do give NH_3. In contrast, the tri-N_2 complexes of both Zr and Ti react directly with HCl to liberate two N_2 molecules and produce hydrazine at a ratio of 0.9 mol/mol of complex from the third (215). The structure of the tri-N_2–Zr complex gives no clue to the basis for this reactivity because no significant differences are evident either among the terminal and bridging N_2 ligands or when compared to the structure of other metal–N_2 complexes that do not produce hydrazine or ammonia on protonation. Related Ti(III)–N_2 complexes also show this variation in requirements for the production of hydrazine or ammonia (219,220).

Early on, it appeared that no direct connection existed between the highly reducing systems that produce either ammonia or hydrazine from N_2 and the well-defined metal–N_2 complexes. However, just as metal–N_2 compounds were isolated from the reducing systems, so too have a number of metal–N_2 compounds been degraded to either ammonia or hydrazine. The mononuclear, tertiary phosphine complexes of Mo(0) and W(0) are the best studied examples. The reaction of *trans*-$M(N_2)_2(dppe)_2$, where dppe = 1,2-bis(diphenylphosphino)ethane (221) and M=Mo or W, [25145-64-6] and [41700-58-7], respectively, with excess acid does not proceed past the hydrazido(2−), ie, =N−NH_2, stage (222).

$$trans\text{-}Mo(N_2)_2(dppe)_2 + 2\,HBr \longrightarrow [Mo(=N\text{-}NH_2)Br(dppe)_2]Br + N_2$$

However, when either *cis*- or *trans*-$M(N_2)_2(PR_3)_4$ (where PR_3= $P(C_6H_5)(CH_3)_2$ or $P(C_6H_5)_2(CH_3)$ and M=Mo or W, respectively) is used, followed by treatment with acid, ammonia yields of up to 2 mol/mol of complex are produced (222–224). These and related data have been used to suggest a possible stepwise sequence for the reduction and protonation of N_2 on a single molybdenum atom in nitrogenase (209,223,224). However, acidification leads to complete destruction of the complex. This limitation has been circumvented by using both the stabilizing effect of the chelating phosphine, called triphos, $(C_6H_5)P[CH_2CH_2P(C_6H_5)_2]_2$, and the lability of a simple phosphine. These changes resulted in a cyclic system in which the product, $MoBr_3$(triphos), can be used to regenerate the starting Mo–N_2 complex again (225).

$$2\,Mo(N_2)_2(PR_3)(triphos) + 8\,HBr \longrightarrow 2\,MoBr_3(triphos) + 3\,N_2 + 2\,NH_4Br + 2\,PR_3$$

Further improvements have resulted in a cyclic process for the electrochemical synthesis of NH_3 (226) with the preferred catalyst being *trans*-$W(N_2)_2(dppe)_2$. The choice of acid is important to provide both a ligand during part of the cycle and an effective leaving group when the catalyst is reformed. In practice, a sulfonic acid is used.

Production of NH_3 in these Mo and W systems has certain features in common with the Ti and Zr systems. Each has more than one bound N_2 ligand in the metal's coordination sphere, but only one is reduced in a process initiated by acid addition. Protonation of N_2 occurs as the phosphine or other N_2 ligands, which stabilize the lower oxidation states of the metals, are successively replaced by acid counterions. The latter favor the higher oxidation states, and this ligand exchange encourages the transfer of more metal electron density to N_2, thus promoting further protonation. Because the chelating phosphines, eg, dppe, are much less easily replaced, $M(N_2)_2(dppe)_2$ does not produce ammonia readily, whereas using the more easily displaced, simple phosphines, all six metal electrons are used to produce ammonia. In the mixed-phosphine system, ammonia is formed but only three electrons per atom of molybdenum are used. In the binuclear zirconium(II) system, only four electrons are available and so only hydrazine is formed. Here also, electron flow from metal to N_2 could be encouraged by loss of N_2 and coordination of the appropriate acid anion. Similarly, only hydrazine is produced from other dinuclear Ta and Nb compounds, which have bridging N_2 (227).

These ammonia- and hydrazine-forming reactions appear to be conducted under mild conditions. However, the reducing power, eg, of magnesium metal, which has $E^0 = -2.4\,V$, is built into these systems during the preparation of the metal–N_2 complexes. The complete degradation of the simple phosphine complexes of Mo and W during ammonia formation does not favor catalysis. An advantage is offered by the electrochemical and the mixed-phosphine systems and by the zirconocene system, where the product of acid degradation is the starting material for preparation of the metal–N_2 complex. It remains to be seen if significantly milder reductants can effect the synthesis of these or similarly reactive nitrogen-containing species and if these can be operated cyclically or catalytically for long periods.

In addition, a range of higher valent metal–N_2 complexes and multi- (and sometimes mixed) metal complexes have been synthesized and studied. An early example involved a Ti_2–N_2–Ti_2 core that involved both end-on and side-on (unusual at that time) N_2 bonding (228). Other examples include $\{[Co(N_2)(PMe_3)_3]_2[Mg(thf)_4]\}$ thf = tetrahydrofuran, which contains a Co–N=N–Mg–N=N–Co core with coordinated N_2 that is susceptible to hydrolysis to NH_3 and N_2H_4 (229). Similar cores, eg, W–N=N–Na and Mo–N=N–Mg–N=N–Mo, were generated with W and Mo complexes (230–233). The latter core reacts with $FeCl_2$ to give a $\{[Mo(N{=}N)_3]Fe\}$ core. Also, a large number of compounds with Mo/W bridged by N_2 to Ti/Zr/Hf/Nb/Ta has been synthesized (234). Both V(II) and V(III) complexes containing V–N=N–V cores are known but only the V(II) compound produces NH_3 and /or N_2H_4 on protonation (235,236). Rare earth and actinide complexes of N_2 (237–239) all involve side-on N_2 binding to two metal ions. It is unknown if these complexes produce NH_3 and/or N_2H_4 on protonation.

Similar side-on N_2 coordination occurs in $\{[Li(thf)_3]_2(N_2)\}^{2+}$, $\{[(PhLi)_3Ni]_2(N_2)\}$, and $\{[(PR_3)Au]_6(N_2)\}^{2+}$, where the N_2 is bound to three metal ions (240–242), and in some Ti and Zr compounds. In fact, $\{Ti[N(SiMe)_2]_2\}_2(N_2)_2$ has two side-on bridging N_2 moieties (243–246). The question of why some complexes prefer side-on versus end-on N_2 binding has been discussed elsewhere (206).

As described in the protonation of $M(N_2)_2$ $(dppe)_2$ above, the single metal atom provides all six electrons for N_2 reduction to NH_3. Recently, two metal ions have been employed to do the same job. Using amido-molybdenum complexes of the type, [Mo(N*t*-Bu $Ph)_3$], N_2 is initially bound at low temperature (−35°C) and is then cleaved as the temperature is raised to 28°C (247).

$$2[Mo(Nt-Bu\,Ph)_3] + N_2 \longrightarrow [Mo(Nt-Bu\,Ph)_3(N_2)] \longrightarrow$$
$$[(t-Bu\,PhN)_3Mo-N=N-Mo(Nt-Bu\,P[(t-Bu\,PhN)_3M_O-N=$$
$$N-Mo(Nt-Bu\,Ph)_3] \longrightarrow 2\,[(t-Bu\,PhN)_3MoN]$$

A related system involves $[(ML_3)_2(N_2)]$, where $L = CH_3OCH_2CH_2OCH_3$, in which the bridging N_2 is cleaved on ultraviolet irradiation to give $[(L_3Mo)N(ML_3)]$ (248). In contrast, the W analogue that is produced by reduction under N_2 forms oligomers of the type, $\{[(WL_3)(N_2)]_nWL_3\}$, containing 1, 2, or 3 bridging N_2 molecules (249). This use of bulky ligands to protect the N_2 bound to Mo from forming relatively unreactive nitrogen-bridging dimers has led to the development of a catalytic nitrogen-reduction process (250). Using weak reductants and selected proton sources in nonaqueous solvents, several intermediates in a potential catalyzed process were identified. Then, using a somewhat more powerful reductant, the catalyzed reduction of N_2 to NH_3 was driven efficiently to give a 60 + % yield based on the reducing equivalents available.

Aqueous Systems. Many reports indicate that strong reducing agents produce minute amounts of ammonia from N_2 in aqueous solution in the presence of derivatives of transition metals. However, spurious results are easily obtained because (*1*) low metal concentrations are used and contaminating species may occur; (*2*) the Nessler test for ammonia is not specific; (*3*) the system may scavenge traces of ammonia or nitrogen oxides, which are subsequently reduced, from the N_2 gas; and (*4*) nitrogen-containing substances added to the reaction mixture may be degraded to ammonia. Only those systems that have been substantiated through the use of $^{15}N_2$ are discussed herein.

Aqueous systems are known that reduce N_2 to either hydrazine or ammonia. Aqueous or aqueous–alcoholic solutions of Na_2MoO_4 [7631-95-0] or $MoOCl_3$ [13814-74-9], with $TiCl_3$ as reductant and Mg^{2+} at pH 10–14, produce some N_2H_4 at 25°C and 0.1 MPa (1 atm) N_2. However, at 50–100°C and 5.1–15.2 MPa, N_2, yields of hydrazine reach 100 mol/mol Mo plus some NH_3. Either V(II) or Cr(II) are equally effective as reductants. The reaction mixture is heterogeneous, and hydroxide-bonded polynuclear entities may furnish the reducing capacity of Ti(III) through Mo(III) to N_2. This system's efficiency is ∼ 1% of the biological systems (251). This system was developed into a catalytic process that uses Na amalgam coated with phophatidylcholine in the presence of a methanolic solution of Mo(III), Mg^{2+}, and a tertiary phosphine. Later, a methanolic solution of

Mo(III) and Ti(III) that was buffered by guanidine was used to electrocatalytically reduce N_2 at a Hg electrode (252).

Both Mo and Ti may be replaced by V(II), which, at alkaline pH, lower temperatures, and 10.1 MPa (100 atm) N_2 pressure, quantitatively produces 0.22 mol N_2H_4/mol of V within minutes. A four-electron reaction is proposed, one electron from each V(II) in a tetramer. At room temperature or higher, both NH_3 and H_2 are produced because the hydrazine is further reduced by other V(II) ions present (211).

$$\left(V^{2+}\right)_4 + N_2 + 4\,H_2O \longrightarrow 4\,V^{3+} + N_2H_4 + 4\,OH^-$$

A related homogenous aqueous–alcoholic system, which is composed of V(II) complexes of catechol or its derivatives, reduces N_2 to ammonia and H_2. Only catecholates are active in this system, which requires a pH of 9–11. This system has been likened to nitrogenase by suggesting that both use a sequence of two four-electron reductions to evolve one H_2 for every N_2 reduced (253).

Another aqueous nitrogen-reducing system was developed using the basic components of Mo-nitrogenase, namely, iron, molybdenum, sulfide, and thiol groups. About 3–5 μmol of NH_3 are produced from ~ 5 mmol Na_2MoO_4, 2.5 mmol thioglycerol, 0.1 mmol $FeSO_4{\cdot}5H_2O$, and 0.25 g $NaBH_4$ in 50 mL of borate buffer (pH 9.6) under 13.7 MPa (135 atm) N_2 (254). In the absence of molybdate, no NH_3 is obtained. Yields up to 0.04 mol NH_3/mol Mo are obtained with a molybdenum–cysteine complex under 0.1 MPa (1 atm) N_2. Specific stimulation of activity by ATP is reported, but acids may produce the same effect (255). These molybdothiol systems are suggested to produce diazene, N_2H_2, which disproportionates to give N_2H_4 that is then reduced to NH_3 (254). More efficient are (*1*) the $[MoO(CN)_4(H_2O)]^{2-}$ [55493-45-3] and $NaBH_4$ system, which gives up to 0.3 mol NH_3/mol complex (256), and (*2*) the 6:1 MoO_4^{2-}–insulin mixture with $NaBH_4$, which produces 65 mol ammonia/mol Mo in 30 min at 23°C under 0.1 MPa N_2 (257).

All the chemical nitrogen-reducing systems require further development to become important as nitrogen-reduction methods, but insight into both the activation of N_2 toward reduction as well as into the induction of internal redox reactions is gained through them. The first step in enzymic N_2 reduction undoubtedly involves the binding of N_2 to a transition-metal site. There is nothing in the known enzymology that precludes either changes in the metal coordination sphere or protonation of bound N_2, or both, as initiators of the redox process. Even so, these inorganic systems for N_2 reduction are not expected to provide a useful means of ammonia production for the near future.

5.2. Nitrogenase Structural Models. Structural modeling of nitrogenase initially concentrated on the nitrogen-binding prosthetic group, ie, the FeMo-cofactor, which contains one Mo, seven irons, and nine sulfides, plus (*R*)-homocitrate on the Mo. The arrangement of these atoms was partially indicated by the results of X-ray absorption spectroscopic (xas) studies of both the MoFe protein and the isolated FeMo-cofactor (168,258) and later clearly defined by the X-ray structure data (see the section Structures of the Mo-Nitrogenase Component Proteins and Their Complex). Now, a complete set of structural features, with which

any synthetic model must be compatible, includes an interstitial light atom (C,N, or O) in the center of the cavity.

The early prototypes of Mo–Fe–S clusters (259,260) consisted of two $[MoS_4(FeSR)_3]$ cube-like structures triply bridged via the two Mo atoms by sulfide or thiolate groups. These complexes have Mo–Fe and Mo–S distances that are quite close to those occurring in the MoFe protein, but obviously the atomic ratio of molybdenum/iron is different. Many other variations on the Mo–Fe–S cluster theme have been synthesized (261,262). Of note are the single-cubane clusters, which are prevented from bridging to one another by a catecholate ligand (to mimic homocitrate) bound to the Mo atom. These clusters bind CN^-, N_3^-, and N_2H_4 individually, but they do not bind N_2, and although they also exhibit a $S = 3/2$ epr signal, the clusters do not duplicate the metal ratio of FeMo-cofactor. Also of interest are the compounds with two bridged ($MoFe_3S_4$) cores, some of which do bind N_2H_4 as a second bridge between the Mo atoms. Again, none of these Mo–Fe–S cluster compounds bind N_2 to form a stable complex, although certain of them have been used as catalysts in systems that produce NH_3 from N_2 (263). Related compounds include the double $[MoFe_3S_4]$ cubanes, which are bridged either at two Fe–S edges (264) or by single ions, eg, oxide or sulfide (262). Although these chemically synthesized $[MoFe_3S_4]$ cubane clusters exhibit close structural similarity to the Mo-containing subcluster of FeMo-cofactor, there is neither a complete topological model nor a complete compositional model of the FeMo-cofactor available currently.

More recently, a shift in emphasis has resulted in attempts to synthsize the 8Fe–7S-containing P cluster. Reaction of an edge-bridged $[MFe_3S_4]$ (M = Mo or V) double cubane [containing an inert tripodal ligand (L) on M] with hydrosulfide yields a close topological, but not compositional, model of the P cluster in its as-isolated state (see Fig. 10) and contains both a μ_6-S and two μ_2-S entities that simulate the two μ_2-S (from cysteinyls) bridges (262,265).

$$[LMS_3\,Fe_2(PR_3)_2](\mu_2 \cdot Fe_2S_2)[Fe_2(PR_3)_2S_3ML] + 3\,HS^- \longrightarrow [LMS_3Fe_3(SH)](\mu_2 \cdot S)_2(\mu_6S)[Fe_3(SH)S_3ML]$$

An 8Fe–7S topological model of the P cluster has been synthesized by a complicated reaction of an iron–amide complex with sulfur, a thiol, and a thiourea analogue (266). Although it contains two [4Fe–4S] cubanes linked through a common μ_6-S, the remaining ligation is different from that of the P cluster. In place of the two μ_2-S (from cysteinyls) bridges are two μ_2-N (amide) bridges and the terminal ligation is not from cysteinyl analogues, but from a combination of amides and thioureas. As with the FeMo-cofactor, we continue to wait for an exact replicate of the P cluster.

6. Outlook

The mature Haber-Bosch technology is unlikely to change substantially in the foreseeable future. New catalysts without iron have been introduced commercially, including a graphite-supported ruthenium catalyst. As discussed above,

recent research has indicated that mixed nitrides of Mo with Fe, Co, or Ni are more active at low ammonia concentrations than iron catalysts and a barium-promoted carbon-supported Co catalyst is more active than the convential commercial catalyst at higher operating temperatures. These results are, however, likely not to have a large impact on commercial ammonia production, which currently operates at close to the theoretical limit of efficiency.

A variety of small-scale innovative nitrogen-fixing systems have come to light recently, but their utility for commercial NH_3 production appears limited. One such systems involves a solid-state H^+-conducting reactor, which converts H_2 into H^+ for electrochemical reaction with N_2 (101 kPa) at the cathode at 570°C (267). Another consists of a two-compartment cell with a solid polymer electrolyte and Ru electrodes. Under 101 kPa N_2 and at temperatures of $\sim$ 100°C, NH_3 is formed in very low yield from N_2 and H_2O (268). A third system uses a composite of perchlorate-doped polymerized 3-methylthiophene and TiO_2, which slowly forms crystals of $[NH_4]ClO_4$ on exposure to N_2 and moisture under ambient conditions (269). This last system exhibits similarities to the natural photofixation that occurs on minerals like rutile in desert sands (270). Other systems involve the simultaneous reductive and oxidative photocatalytic N_2 fixation that occurs either in hydrous iron(III) oxide-loaded Nafion films (271) or on zinc-doped gallium phosphide semiconductors (272).

To be successful, such systems must be operated continuously at ambient conditons, preferably using a renewable energy resource (qv), eg, solar, wind, or water power, or other off-peak electrical power, and be located near or in irrigation streams. Such systems might produce and apply ammonia continuously, eg, directly in the rice paddy, or store it as an increasingly concentrated ammoniacal solution for later application. In fact, the Arc process for N_2 oxidation was reconsidered (273) some years ago for areas where fertilizer production capacity of a few t/year can make a significant impact on agricultural production. However, this research effort was not adopted.

Other important contributions could be made by improving the utilization of applied nitrogen fertilizer. Less than 50% of the nitrogen applied is actually assimilated by plants. To this end, slow-release fertilizer can make an impact as can development of nitrification and denitrification inhibitors (see Controlled Release Technology, Agricultural). All such strategies would prevent ammonia losses to the atmosphere and ground water. However, the effects of such inhibitors on the nitrogen cycle are unclear.

To exploit the benefits of biological nitrogen fixation in agriculture, we must concern ourselves with farming practices, environmental constraints, the rhizobial microsymbiont, and the host legume. In the shorter term, the introduction (or increased use) of nodulated legumes would improve soil quality and also provide a nutritious source of food. Similarly, legume crop yields would improve from soil inoculation with effective and competitive strains of rhizobia and technologies, which are already in place, could be used to combat many environmental constraints, like soil acidity and phosphorus deficiency.

In the medium term, careful matching of the most effective rhizobial strains with the appropriate cultivar would be required. These associations could also be manipulated to start fixation earlier or to continue it later into the plant's growth. If so, a substantial benefit could accrue. Another significant benefit

would result if the microbes could fix N_2 in the presence of fixed-nitrogen sources. In rice paddies, cyanobacteria, which are remarkably self-sufficient, should continue to be exploited, together with the *Azolla–Anabaena* association, to enhance the vitally important production of rice.

In the longer term, the continuing discovery of new nonleguminous (associative) symbioses indicates avenues through which both nitrogen fixation and the delivery of fixed nitrogen to crop plants may be enhanced. The development of associations, possibly with the principal food crops that do not enter into nitrogen-fixing symbioses, could have dramatic effects on both fertilizer usage and food production. Genetic manipulation of nitrogen fixation appears to be the ultimate solution both for reducing fossilfuel energy inputs to fertilizer production and for increasing food supplies (274). An understanding of the recognition and infection processes followed by genetic manipulation could result in new or enhanced symbiotic associations, because many plants appear already to have most of the genes necessary to produce a nodule-like structure. Perhaps the best possibility is to transfer the ability to fix N_2 to those bacteria that already reside within the cells of plants. The success of transferring the *nif* genes from one bacterial genus to another has opened up the possibility of transfer to a crop plant. However, genetic transfer is not enough. Possibly nitrogenase could be relocated to the chloroplasts of leaves where, if properly protected from the O_2 evolved by photosynthesis, it could take advantage of directly available reducing equivalents produced from sunlight (275). Whatever happens, plants will remain our principal source of fixed nitrogen for the foreseeable future.

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WILLIAM E. NEWTON
Virginia Polytechnic Institute and State University

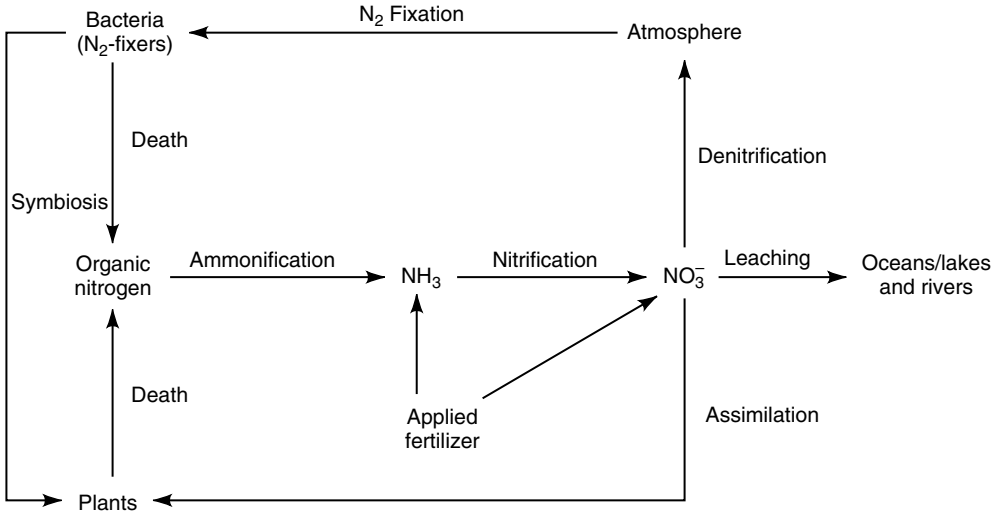

Fig. 1. Biological pathways and processes involved in the nitrogen cycle.

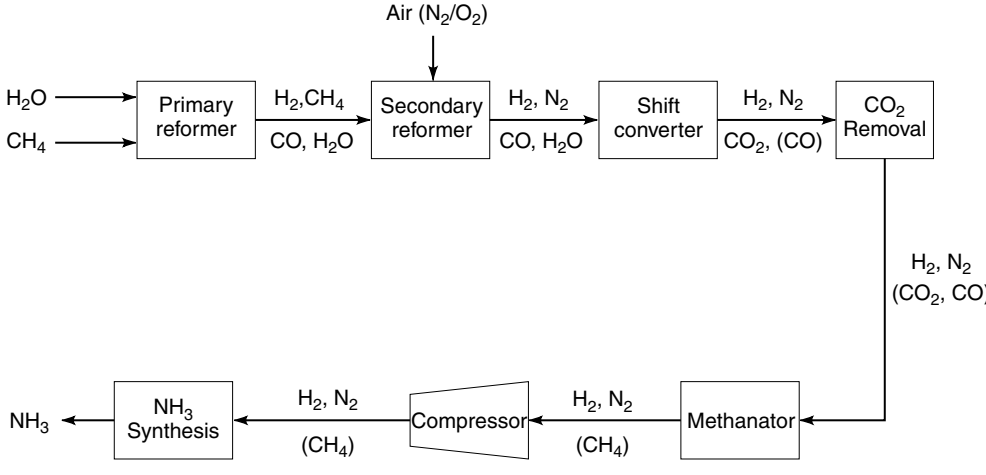

Fig. 2. The Haber-Bosch process. Gases in parentheses are minor constituents of the mixture.

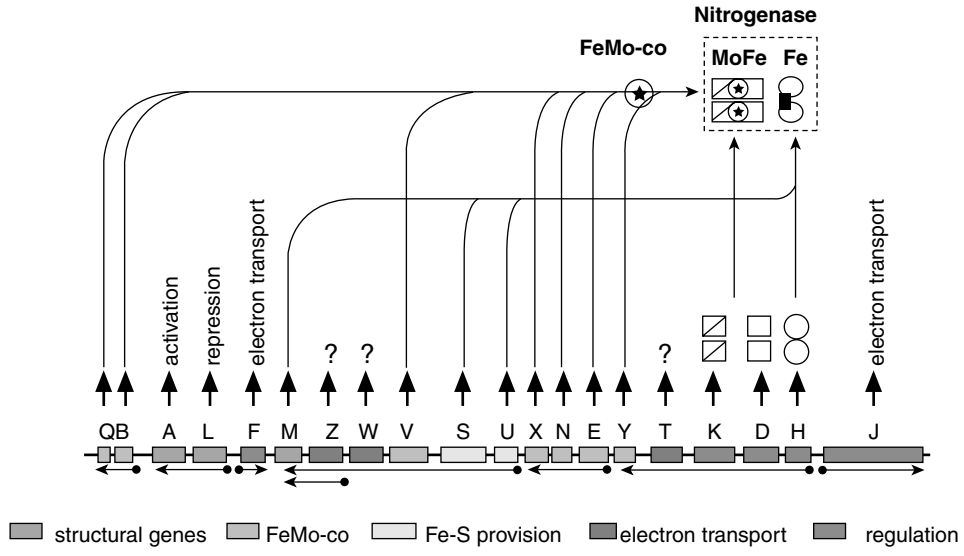

Fig. 3. The nif-gene cluster of *K. pneumoniae*. The function of the various gene products is shown if known. The FeMo-co is the FeMo-cofactor, MoFe is the MoFe protein, and Fe is the Fe protein. See text for additional explanations.

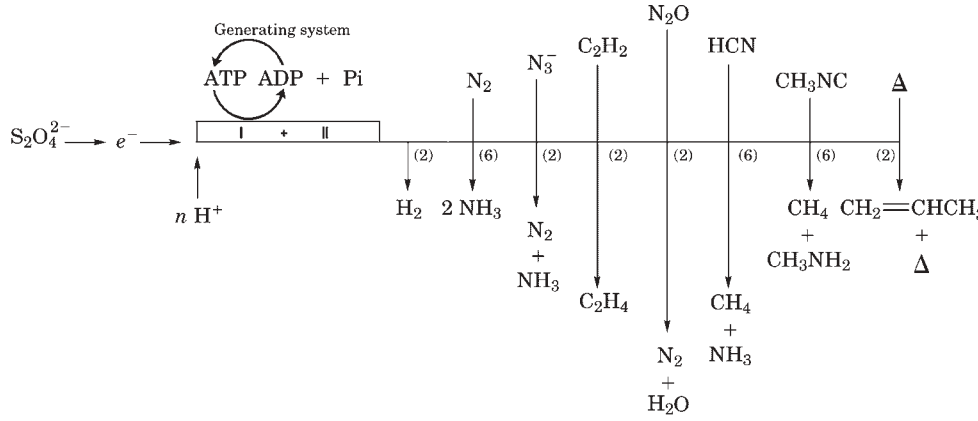

Fig. 4. Inputs, substrates, and products of Mo-nitrogenase catalysis, where I is the MoFe protein; II the Fe protein; and Pi is inorganic phosphate. The generating system is composed of creatine phosphate and creatine phosphokinase to recycle the inhibitory MgADP produced during catalysis to MgATP. The numbers in parenthesis represent the number of electrons required for the reaction shown. △ is cyclopropene; cyclopropane.

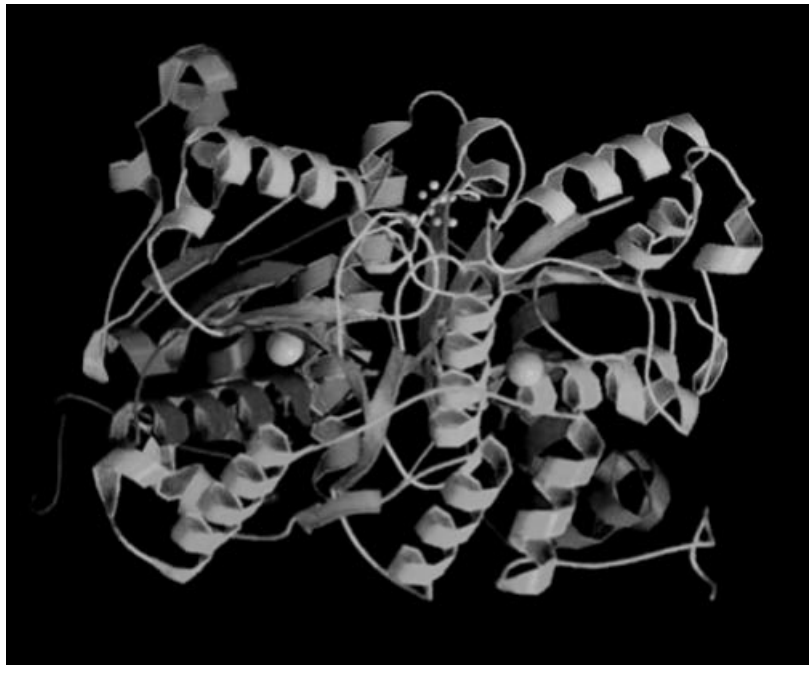

Fig. 5. Ribbons diagram of the Fe protein dimer from *A. vinelandii* showing the overall polypeptide fold. The α-helical regions are shown as coils; β-sheet regions are shown as arrows; the remaining regions are shown as threads. The [4Fe–4S] cluster (at the top) is bound between the two subunits. **(130; PDB code: 1FP6)**.

Fig. 6. Ribbons diagram of the MoFe protein $\alpha_2\beta_2$-tetramer from *A. vinelandii* viewed down the pseudotwofold symmetry axis. α-Helical regions are shown as coils; β-sheet regions are shown as arrows; the remaining regions are shown as threads. Each half of the molecule, which may be visualized by drawing a line through the center from the 1 o'clock

to the 7 o'clock position, represents an αβ-dimer, each encompassing one P cluster and one FeMo-cofactor. The β-subunits make all contacts among the αβ-dimers and are arranged symmetricaly around the central channel. The α-subunits, which do not contact one another, are located at the far left and far right of the molecule with their FeMo-cofactors at just above 3 o'clock and just below 9 o'clock (cluster of spheres), whereas the P clusters are at 10 o'clock and 4 o'clock **(142; PDB code: 3MIN)**.

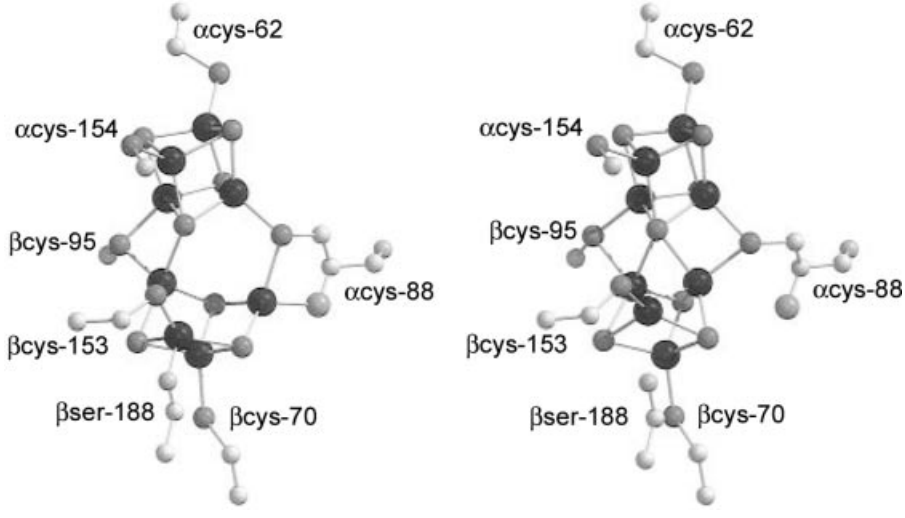

Fig. 7. The structure of the P cluster of *A. vinelandii* nitrogenase MoFe protein in its two-electron-oxidized (P^{OX}; left) and dithionite-reduced (P^{N}; right) states with its ligating amino acid residues (αcys-62, αcys-88, αcys-154, βcys-70, βcys-95, βcys-153, and βser-188), which are provided by both subunits. The Fe atoms are shown as the darkest largest spheres and the S atoms are the medium-sized dark-gray spheres **(142; PDB code: 3MIN and 2MIN)**.

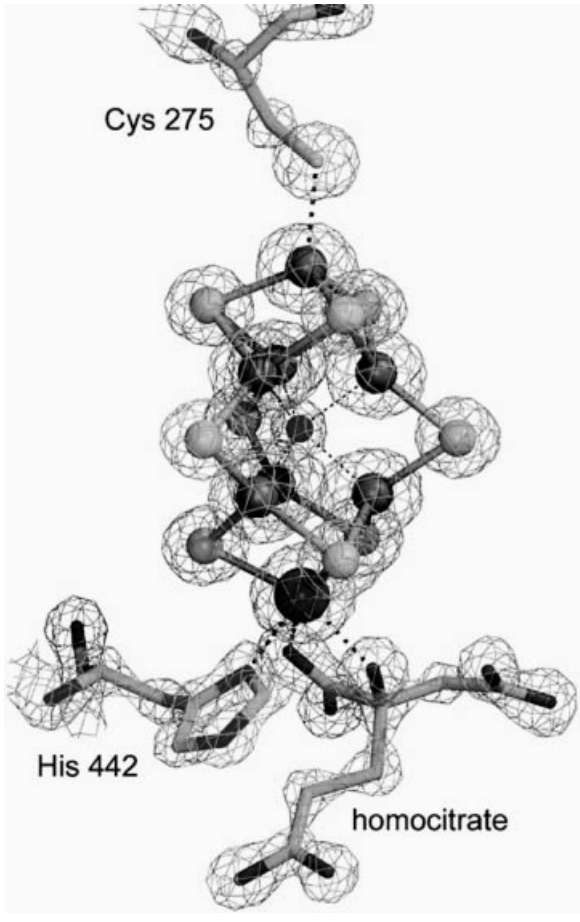

Fig. 8. View of the FeMo-cofactor prosthetic group of the nitrogenase MoFe protein with the two directly bonded amino acid residues. The molybdenum atom (largest dark sphere) is coordinated to α-His-442 and homocitrate (at the bottom). The iron atoms and interspersed sulfur atoms are the intermediate-sized dark and light spheres, respectively. The "light atom" in the central cavity is likely to be C, N, or O **(183; PDB code: 1M1N)**.

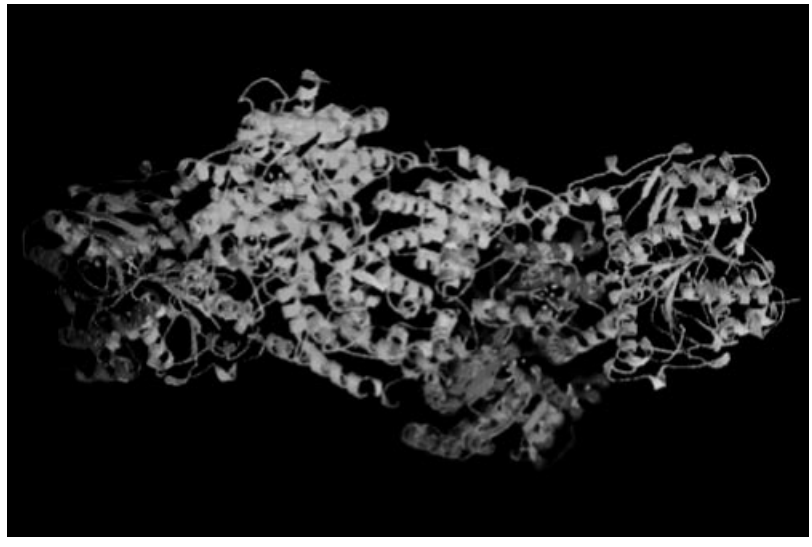

Fig. 9. The structure of the 2:1 Fe protein–MoFe protein complex of the *A. vinelandii* nitrogenase stabilized by MgADP plus AlF_4^-. The Fe protein molecules are docked at the far right and far left of the complex, one at each αβ-subunit interface of the MoFe protein to align their [4Fe–4S] clusters with the P clusters **(10; PDB code: 1N2C)**.

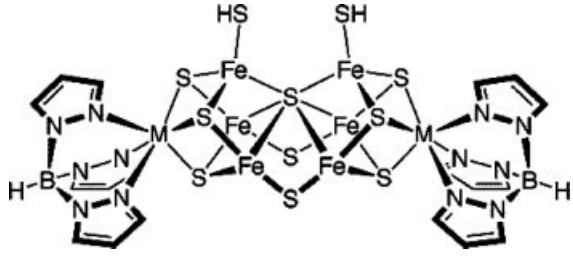

Fig. 10. A topological model for the P cluster in its P^N state, containing both the characteristic μ_6-S and two μ_2-S entities that simulate the two μ_2-S-cysteinyls. Its composition also approaches that of the P cluster, but has two terminal M–L (M = Mo, V) groups as replacements for two Fe–S-cys groups (262,265).