methylselenide, or dimethylsulfide. These volatile products are slowly oxidized by molecular oxygen to give stable water-soluble species such as cacodylic acid. It is likely that cacodylic acid will represent the most abundant methylated arsenic compound in both freshwater and seawater. Methylarsenic compounds can be incorporated in phospholipids, as demonstrated by the isolation of a trimethylarsenic-containing phospholipid found as a natural product in fish, shellfish, and marine algae (*25*).

Conclusions

As we obtain more information on the movement of toxic elements in the biosphere, we will have a greater understanding of the environmental conditions required for the individual processes involved in biogeochemical cycling. In this article we have shown how oxidation-reduction conditions can be correlated with biomethylation processes. In recent years we have been able to establish biogeochemical cycles for mercury and arsenic (*3*). We can now formulate a biogeochemical cycle for tin in some detail (Fig. 4). The biomethylation of tin is interesting in two respects: (i) the use of tin by advanced industrial societies has more than doubled in the last 10 years (*26*); and (ii) the analytical methods to definitively establish whether methyl tin compounds accumulate in the food chain, including humans, have not yet been developed. Since methyl tin compounds are poisonous to the central nervous systems of higher organisms (*27*), we feel that it is critical to examine whether higher organisms provide a reservoir for some of the methyl tin compounds established as intermediates in the biogeochemical cycle for tin.

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Biological Nitrogen Fixation for Food and Fiber Production

What are some immediately feasible possibilities?

Harold J. Evans and Lynn E. Barber

Before the World War II period legumes were used extensively in the United States as a method of providing nitrogen to agricultural land. After this period, however, the supply of relatively inexpensive fertilizer nitrogen increased, values of land available for growth of legume cover crops rose, and interest in legumes and other biological nitrogen-fixing systems declined (*1*, *2*). It was recently estimated that commercial synthesis of fixed nitrogen in the United States required about 2.5 percent of our annual consumption of natural gas (*3*, *4*). The scarcity of appropriate sources of energy for the manufacture of nitrogen fertilizer and other problems associated with world food production have stimulated a reconsideration of recent agricultural practices (*2*, *5*). As a consequence, renewed interest in the possibility of increased dependence on nitrogen fixation has developed.

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Nitrogen-Fixing Systems

Some microorganisms that live in association with plants and others that exist under free-living conditions utilize solar energy stored as products of photosynthesis to biologically fix atmospheric nitrogen into compounds that may be used for the synthesis of protein and other products. Organisms that are capable of biologically fixing atmospheric nitrogen ordinarily require no other source, but those lacking this capacity are absolutely dependent on nitrogen from soil reserves or applied fertilizer. The best known of the nitrogen-fixing systems are the nodulated legumes. The beginning of our understanding of the symbiotic relationship between legumes and *Rhizobium* species was provided by Hellreigel and Wilfarth in 1888 (*6*), but inclusion of legumes in crop rotations as a method of supplying nitrogen was practiced in ancient agriculture (*7*). *Rhizobium* species with specificities for particular groups of legumes invade root hairs of plants such as alfalfa, clover, beans, and peas and induce root nodules that become packed with modified forms of the *Rhizobium* cells called bacteroids. If the legume cultivar and particular *Rhizobium* strain in

the nodule are compatible and the nodulated plant is provided with essential nutrients and a suitable environment, the nitrogen needs of most plants may be obtained from the atmosphere.

In addition to legumes, nitrogen is fixed by a variety of microorganisms and associations. These include bacteria located in soils, in decaying wood, and on the surfaces of plant roots; free-living blue-green algae in terrestrial and marine environments; and associations of blue-green algae with fungi, ferns, mosses, liverworts, and higher plants (*5*). Also there are nitrogen-fixing associations between obligately symbiotic actinomycetes and woody shrubs and trees such as *Ceanothus, Dryas, Myrica, Purshia, Comptonia*, and *Alnus*. Nodulated legumes are the primary nitrogen-fixing plants in agriculture, but most of the nitrogen for maintenance of our forests, woodlands, and freshwater and marine habitats is provided by a variety of nonleguminous nitrogen-fixing associations and free-living microorganisms. Our knowledge of the increasing number of organisms that are capable of fixing nitrogen is expanding rapidly and is the subject of several reviews (*8–11*).

Relative Fixation Capabilities

Biological nitrogen fixation by all the different types of organisms has been estimated to contribute to the earth about 175 million metric tons of nitrogen per year (*12*). Nodulated legumes grown for grain, hay, pasture, and other agricultural purposes account for almost half (80×10^6 metric tons) of the annual quantity of nitrogen fixed by biological systems (*5*). Microorganisms that live in symbiosis with green plants have an advantage because solar energy in the form of carbohydrates that are synthesized by plants is available for use in the nitrogen-fixing process. Free-living nitrogen-fixing blue-green algae and photosynthetic bacteria also possess built-in mechanisms for capturing solar energy, but free-living nitrogen-fixing bacteria that lack a capacity for photosynthesis must obtain their energy from organic materials synthesized by other organisms. In most soils, the population of microorganisms is enormous (*13*) and nitrogen fixation by nonphotosynthetic free-living bacteria is limited by the supply of carbon compounds that provide the energy. This is the fundamental reason why rates of fixation by nodulated alfalfa, clover, lupins, and soybeans range from 57 to 600 kilograms per hectare per year, whereas the free-living *Azotobacter* and *Clostridia* are estimated to fix from 0.1 to 0.5 kg ha^{-1} $year^{-1}$ (Table 1). Nodulated nonlegumes, blue-green algal associations, and free-living blue-green algae also produce their own carbohydrates and are capable of fixing nitrogen at high rates (Table 1).

Table 1. Relative rates of biological nitrogen fixation. The data are summarized average rates from Mishustin and Shil'nikova (*69*), Burns and Hardy (*12*), Silvester (*8*), and Becking (*70*).

Organism or system	N_2 fixed (kg^{-1} ha^{-1} $year^{-1}$)
Legumes	
Soybeans	57–94
Cowpeas	84
Clover	104–160
Alfalfa	128–600
Lupins	150–169
Nodulated nonlegumes	
Alnus	40–300
Hippophae	2–179
Ceanothus	60
Coriaria	150
Plant-algal associations	
Gunnera	12–21
Azollas	313
Lichens	39–84
Free-living microorganisms	
Blue-green algae	25
Azotobacter	0.3
Clostridium pasteurianum	0.1–0.5

New methods for detecting biological nitrogen-fixing activity have revealed loose associations between nitrogen-fixing bacteria and the roots of a variety of grasses. These so-called associative systems do not produce nodules, but in some cases the bacteria live underneath a mucilaginous sheath on the root surface. Dobereiner *et al.* (*14*) discovered *Azotobacter paspali* in association with the roots of a sand grass, *Paspalum notatum*. From laboratory experiments in which acetylene reduction was used to detect nitrogenase activity, Dobereiner *et al.* (*14*) concluded that this association was capable of fixing about 90 kg ha^{-1} $year^{-1}$. More recently, the occurrence of a nitrogen-fixing *Spirillum lipoferum* on the roots of *Digitaria decumbens* which reduced acetylene at relatively high rates was reported (*15*). In this association some bacteria were observed inside root cells. The discovery of nitrogen-fixing organisms on roots of grasses including maize and wheat has led to excitement and speculation about the possibility of providing nitrogen for important food crops by use of the associative systems.

Several different methods, including variations of the acetylene reduction assay (*16*), have been employed to assess fixation by these systems, and it is difficult to evaluate and compare the results. Some of the rates of acetylene reduction and nitrogen fixation that have been observed with associative systems are presented in Table 2, which also includes information on methods used for measurements. In upland tropical habitats high rates of acetylene reduction with several systems have been reported. Approximately 3000 nanomoles of acetylene reduced per gram of dry root per hour has been considered sufficient nitrogen-fixing activity to supply about 100 kg ha^{-1} per 100-day growing season (*17*), which would be sufficient for growth of maize. However, most of these results in tropical habitats were based on acetylene reduction by excised plant roots that had been incubated overnight under limited oxygen. This technique has been found to lead to an increase in the number of nitrogen-fixing bacteria and to increased rates of acetylene reduction (*18*). Low rates of fixation estimated from acetylene reduction of roots in soil cores were observed with many of the plants in upland temperate areas. Although these rates are low, they may be sufficient for appreciable benefit to uncultivated grasses. In the more anaerobic conditions of the wet temperate and tropical areas, acetylene reduction rates observed with rice and other aquatic plants were significantly higher than rates observed with root samples from more aerobic environments. In Florida, inoculation with *Spirillum* of some tropical grasses grown with little or no added fertilizer nitrogen has resulted in higher yields of plant dry matter than were obtained from control plots that received a culture medium only (*19*). Since no nitrogen-fixation rates were reported, and since some microorganisms produce growth-stimulating hormones, it is not possible to decide whether the yield increases from *Spirillum* inoculation were due to nitrogen fixation.

Properties of the Nitrogen-Fixing Process

Before we can assess some of the problems that might be encountered in improving the nitrogen-fixing capacities of organisms or developing the nitrogen-fixing capabilities of additional organisms, it is necessary to consider some of the complexities of the system. Several reviews of this field have been published (*20–22*).

Nitrogenase. The enzyme complex referred to as nitrogenase, in combination with the necessary reactants, catalyzes the reduction of nitrogen to ammonia. Cell-free preparations containing active nitrogenase have been obtained from about 20 different organisms (*20*). These

include anaerobic, facultative anaerobic, aerobic, and photosynthetic bacteria, blue-green algae, and the bacteroids from the nodules of soybeans and lupins. Not all of the preparations have been purified to homogeneity, but there is convincing evidence (*20, 21*) that nitrogenases from different organisms have many common properties. All highly purified preparations have two major protein components. The larger components from several sources (molybdenum-iron proteins) have molecular weights ranging from 200,000 to 270,000 and contain 1 or 2 atoms of molybdenum, 17 to 36 iron atoms, and 14 to 28 acid-labile sulfur atoms per protein molecule. The molybdenum-iron protein consists of four subunits of two types (*23*). In addition to the molybdenum-iron protein, nitrogenase activity requires an iron protein, which has a molecular weight ranging from 55,000 to 67,000 depending on the source. This component has four atoms each of iron and acid-labile sulfur per mole of protein. There is some disagreement (*24–26*) about the constitution of the functional unit of the nitrogenase complex. Smith *et al.* (*25*) believe that it is made up of a one-to-one combination of the iron and molybdenum-iron proteins, but Orme-Johnson (*26*) favors a unit of two iron proteins for each molybdenum-iron protein. The high sensitivities of the purified nitrogenase components to oxygen complicate the han-

Table 2. Estimates of rates of acetylene reduction and nitrogen fixation by microorganisms associated with nonnodulated plants.

Plant	Rate	Method of estimation	Reference
	Tropical grasses—upland habitats		
Digitaria decumbens	Maximum* 60–404 nmole $C_2H_4 g^{-1}$ hour†	C_2H_2, roots‡	(*15, 71*)
Paspalum notatum	Maximum 80–283 nmole C_2H_4 g^{-1} $hour^{-1}$	C_2H_2, roots‡	(*71, 72*)
	1–32 nmole C_2H_4 g^{-1} $hour^{-1}$ (90 kg N ha^{-1} $year^{-1}$)	C_2H_2, roots	(*14*)
	10–22 μg N_2 per core per 17 hours	^{15}N, C_2H_2, cores	(*15*)
Panicum maximum	23–299 nmole C_2H_4 g^{-1} $hour^{-1}$	C_2H_2, roots‡	(*71, 72*)
Pennisetum purpureum	Maximum 215–954 nmole C_2H_4 g^{-1} $hour^{-1}$	C_2H_2, roots‡	(*71–73*)
Sorghum	Mean 1053 nmole C_2H_4 g^{-1} $hour^{-1}$	C_2H_2, roots‡	(*73*)
Sugarcane (*Saccharum officinarum*)	Maximum 5.5 mole C_2H_4 g^{-1} $hour^{-1}$ (2 kg N ha^{-1} $year^{-1}$)	C_2H_2, roots	(*74*)
	25–780 μg N g^{-1} per 30 hours	$^{15}N_2$	(*75*)
Maize (*Zea mays*)	Mean 74–7124 nmole C_2H_4 g^{-1} $hour^{-1}$ (2.4 kg N ha^{-1} day^{-1})	C_2H_2, roots‡	(*17, 73*)
Gramineous ecosystem	4–12 kg N ha^{-1} $year^{-1}$	C_2H_2, in situ	(*76*)
Other grasses	Maximum 269–730 nmole C_2H_4 g^{-1} $hour^{-1}$	C_2H_2, roots‡	(*71*)
	Maximum 1.5 nmole C_2H_4 g^{-1} $hour^{-1}$	C_2H_2, roots	(*74*)
	1–500 nmole C_2H_4 g^{-1} $hour^{-1}$	C_2H_2, soil-plant system	(*77*)
Rice (*Oryza sativa*)	Maximum 7 g N ha^{-1} per wet season	C_2H_2, roots, $^{15}N_2$	(*78*)
	Tropical grasses—wet habitats		
Rice (*Oryza sativa*)	Maximum 24–30 μmole C_2H_4 g^{-1} $hour^{-1}$ (70 kg N ha^{-1} $year^{-1}$)	C_2H_2, in situ	(*76, 79*)
	1.8–6.1 μmole C_2H_4 g^{-1} $hour^{-1}$	C_2H_2, in situ	(*77*)
	Maximum 52 kg N ha^{-1} per wet season	C_2H_2, roots, $^{15}N_2$	(*78*)
	Temperate plants—upland habitats		
Agrostis tenuis	Mean 4.0 mole C_2H_4 ha^{-1} day^{-1} (37 g N ha^{-1} day^{-1})	C_2H_2, cores	(*80*)
Digitaria sanguinalis	3–10 g N ha^{-1} day^{-1}	C_2H_2, cores	(*81*)
Panicum virgatum	1.3 kg N ha^{-1} per 120 days	C_2H_2, cores	(*82*)
Gramineous ecosystem	Maximum 11 kg N ha^{-1} $year^{-1}$	C_2H_2, in situ	(*76*)
Grassland	0.6–1.5 kg N ha^{-1} per 28 days	$^{15}N_2$, cores	(*83*)
	0.002–0.38 g ha^{-1} $hour^{-1}$	C_2H_2, cores	(*83*)
Pioneer weed	1.2 kg N ha^{-1} $year^{-1}$	C_2H_2, soil cores	(*84*)
Annual grass	0.7 kg N ha^{-1} $year^{-1}$	C_2H_2, soil cores	(*84*)
Prairie	3.5 kg N ha^{-1} $year^{-1}$	C_2H_2, soil cores	(*84*)
Maize (*Zea mays*)	Maximum 298 nmole C_2H_4 per core per 18 hours	C_2H_2, 2-kg cores	(*85*)
Pasture, prairie	Maximum 1 kg N ha^{-1} per season	C_2H_2, small soil cores	(*86*)
Wheat field	4.1 kg N ha^{-1} $year^{-1}$	$^{15}N_2$	(*87*)
Prairie grass	3–9 kg N ha^{-1} $year^{-1}$	C_2H_2, soil-grass cores	(*88*)
Lawn	4.8 kg N ha^{-1} $year^{-1}$	$^{15}N_2$	(*87*)
Nonlegumes (mixed)	5–260 g N ha^{-1} per 28 days	C_2H_2, undisturbed cores	(*83*)
	2.1 kg N ha^{-1} $year^{-1}$	$^{15}N_2$	(*87*)
Dicots	Maximum 18 kg N ha^{-1} $year^{-1}$	C_2H_2, intact plant, soil	(*89*)
	Temperate plants—wet habitats		
Glyceria borealis	12.6 μmole N_2 g^{-1} day^{-1} (60 kg N ha^{-1} $year^{-1}$)	C_2H_2, roots, sediments	(*90*)
Typha sp.	1.2 μmole N_2 g^{-1} day^{-1}	C_2H_2, roots, sediments	(*90*)
Zostera marina	12 nmole C_2H_4 g^{-1} $hour^{-1}$	C_2H_2, plant tissue, sediment	(*91*)
† *Thalassia testudinum*	32 nmole C_2H_4 g^{-1} $hour^{-1}$ (500 kg N ha^{-1} $year^{-1}$)	C_2H_2, $^{15}N_2$, plant and sediment	(*91*)
Juncus balticus	0.8 kg N ha^{-1} day^{-1}	C_2H_2, cores	(*92*)
Wet mixed forest	Maximum 1 kg N ha^{-1} day^{-1}	C_2H_2, cores	(*93*)
	Plants inoculated with spirillum		
Maize (*Zea mays*), greenhouse	Maximum 2186 nmole C_2H_4 g^{-1} $hour^{-1}$ (734 g N ha^{-1} day^{-1})	C_2H_2, roots‡	(*18*)
	Maximum 15 g N ha^{-1} day^{-1}	C_2H_2, intact plants	(*18*)
Millet (*Pennisetum americanum*)	Maximum 42 kg N ha^{-1} per season	Yield, inoculated − uninoculated plants	(*19*)

*Maximum followed by a range of values indicates the range in maximum values observed in different experiments. †Nanomoles of C_2H_4 per gram per hour refers to nanomoles of C_2H_4 formed from C_2H_2 reduction per gram of dry roots per hour. ‡The roots were incubated before exposure to C_2H_2.

dling of these proteins in the laboratory (*21*). The half-life of molybdenum-iron protein in air ranges from 4 to 10 minutes. The iron protein is even more labile, being essentially instantaneously inactivated by contact with air. Although components of nitrogenase from different sources exhibit similarities, molybdenum-iron and iron proteins from different organisms are not always active in reconstitution experiments. In general, combinations of two components from different aerobes, or from different anaerobes, seem more compatible than combinations from an aerobe and an anaerobe (*20, 22*).

Reactions of the nitrogenase complex. The basic requirements for nitrogenase catalysis and some relationships to metabolic systems are outlined in Fig. 1. Energy in the form of a reductant and adenosine triphosphate (ATP) is essential for the nitrogenase reaction. Bulen and associates (*27*) discovered that sodium hydrosulfite ($Na_2S_2O_4$) could be used as a nonphysiological electron donor for the reaction and this greatly simplified in vitro assays. In their experiments (*27*), ATP was supplied continuously by a regenerating system consisting of creatine phosphokinase, creatine phosphate, and a small amount of Mg ATP. During the nitrogenase reaction, energy from ATP and electrons from the reductant cause a conformational change in the iron protein and convert it to a very powerful reductant with an oxidation-reduction potential of about −400 millivolts (*28, 29*). This fully reduced iron protein transfers electrons to the molybdenum-iron protein, which in turn reduces N_2. During this process four or five molecules of ATP are hydrolyzed per two electrons transferred. Reduction of N_2 to 2 moles of NH_3 therefore requires 12 to 15 moles of ATP in laboratory experiments (*30*).

In addition to N_2, several other substrates including H^+ function in the nitrogenase reaction (Fig. 1). Even though the nitrogenase reaction, in vitro, is allowed to proceed under pure N_2, about 25 to 30 percent of the total reductant provided to the reaction is expended in the reduction of H^+ to H_2 (*27, 31*). The mechanisms of reduction of N_2 and H^+ obviously differ because N_2 reduction is inhibited by CO whereas H_2 evolution is not (*12*). From kinetic experiments, Rivera-Ortiz and Burris (*32*) concluded that an infinite concentration of N_2 would not completely suppress H_2 evolution. In contrast, a high concentration of acetylene (C_2H_2) essentially inhibits H_2 evolution. This inhibition creates an error when the C_2H_2 reduction assay is used to estimate nitrogen fixation (*32, 33*). Schrauzer (*34*) argues that diimide and hydrazine are hypothetical intermediates in the N_2 reduction process and suggests that H_2 evolution resulting from a disproportionation of diimide may be an obligate step in that process. Reduction of N_2 to diimide is considered the thermodynamically unfavorable step in the N_2 reduction process. Production of H_2 from diimide therefore would be expected to help shift the equilibrium toward reduction of N_2.

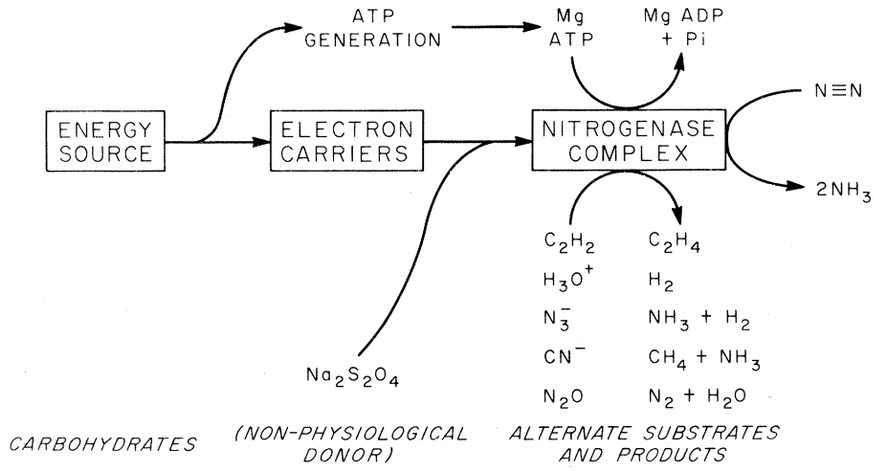

Fig. 1. Generalized scheme illustrating the relationship between sources of energy from organisms and reactions catalyzed by nitrogenase. Energy is supplied by oxidation of carbohydrates or other materials. This produces ATP and electrons. Nitrogenase catalyzes the reduction of nitrogen gas (N ≡ N) to NH_3. Alternate substrates are also reduced to products by the nitrogenase complex. Sodium hydrosulfite may be used as an electron donor for in vitro nitrogenase reactions in laboratory experiments.

In vitro experiments have provided information about ways of influencing the allocation of electrons in the nitrogenase-catalyzed reaction. An increase in the ratio of molybdenum-iron protein to iron protein results in an increase in the proportion of electrons transferred to H^+ and a decrease in N_2 reduction (*35*). An increase in the ratio of ATP to ADP (adenosine diphosphate) in reaction mixtures favors the allocation of electrons to N_2 rather than H^+ (*36*). When considering the energetics of nitrogen fixation, it is important to remember that the ATP consumption per two electrons transferred in the nitrogenase reaction is approximately the same regardless of whether N_2 or H^+ serves as the acceptor (*30*). Unless organisms possess mechanisms for utilizing H_2 from the nitrogenase reaction, large amounts of energy may be wasted (Fig. 2).

Provision of energy for support of nitrogen fixation. Photosynthesis is the ultimate source of energy for support of biological nitrogen fixation. The free-living microorganisms lacking photosynthetic capabilities must obtain their energy from external sources. Energy for nitrogen fixation by the bacteroids in legume nodules is provided by the plant. The rate of nitrogen fixation by legumes, as expected, is influenced strikingly by factors that affect photosynthesis (*37–39*). Decreasing light intensity or decreasing photosynthesis by partial defoliation results in decreased rates of nitrogen fixation by soybeans. When photosynthesis in soybeans was increased by grafting two soybean shoots on a single nodulated root (*40*) or by increasing the carbon dioxide content of the air over soybean plants in field plots (*5*), rates of nitrogen fixation were strikingly stimulated. The major product of photosynthesis transported from leaves to nodules of legumes is sucrose (*41*). Sucrose is not utilized directly by isolated bacteroids but is hydrolyzed in the nodule by invertase of plant origin (*38*). Pathways of carbohydrate metabolism in nodule bacteroids are not completely understood, but it is known that bacteroids contain some of the glycolytic enzymes and utilize a variety of organic acids. The addition of succinate to bacteroids isolated from soybean nodules and to free-living rhizobia under appropriate conditions stimulates nitrogen fixation (*42, 43*).

The metabolism of carbohydrates or other carbon substrates provides the electrons and ATP to drive the nitrogen-fixing process. In *Clostridium pasteurianum* and perhaps other anaerobic microorganisms, the phosphoroclastic breakdown of pyruvate reduces the carrier protein, ferredoxin (or flavodoxin), which serves as the natural electron do-

nor for nitrogenase (*44*). The phosphoroclastic reaction also synthesizes acetylphosphate, which is capable of transferring its high-energy phosphate to ADP, thus forming ATP. In the aerobic nitrogen-fixing organisms, including azotobacters and legume nodule bacteroids, it seems obvious that ATP for the nitrogenase reaction is generated by oxidative phosphorylation (*22, 45, 46*); however, details of the physiologically important pathways for provision of reductant are not clear (*22, 44*). It has been possible to reconstitute electron transfer sequences from NADPH (reduced nicotinamide adenine dinucleotide phosphate) to ferredoxin and flavodoxin and then to purified nitrogenase extracted from either soybean nodule bacteroids or *Azotobacter vinelandii*. Rates of reactions with these systems are relatively low, and reconstitution requires the addition of NADPH-ferredoxin reductase from organisms that do not fix nitrogen (*44*). The most interesting recent information concerning electron transport in aerobic nitrogen fixers was supplied by Haaker *et al.* (*47*), who showed that the ratio of reduced to oxidized pyridine nucleotides in *Azotobacter* was inadequate to maintain a sufficiently negative oxidation-reduction potential to support nitrogen fixation. Their evidence indicates that the energy state and integrity of cell membranes control the supply of electrons to nitrogenase.

Regulation. Ammonia is known to repress the synthesis of nitrogenase in free-living nitrogen-fixing bacteria and inhibit nodulation of legumes (*48, 49*). Streicher *et al.* (*50*) have shown that active glutamine synthetase is necessary for nitrogenase synthesis in *Klebsiella pneumoniae*. Their results are consistent with the conclusion that glutamine synthetase without an attached adenyl group plays a role in the induction of nitrogenase synthesis. High concentrations of ammonia in media result in the addition of an adenyl group to glutamine synthetase and repression of nitrogenase synthesis. More recently, Shanmugam and Morandi (*51*) have shown that regulation of nitrogenase synthesis is more complex than was originally believed. In addition to ammonia, glutamine and asparagine participate in nitrogenase regulation. O'Gara and Shanmugam (*52*) have suggested that amino acids supplied by the host legume may repress genes involved in the assimilation of ammonia in *Rhizobium* bacteroids, thus promoting the excretion of ammonium into the plant cytosol (Fig. 3).

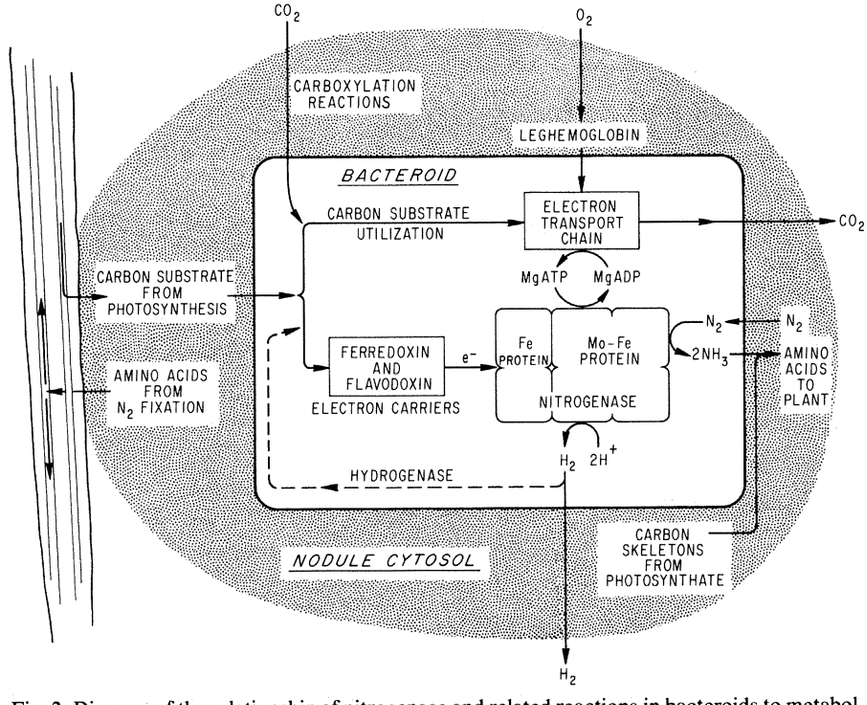

Fig. 2. Diagram of the relationship of nitrogenase and related reactions in bacteroids to metabolic processes in legume nodules. Energy to support nitrogen fixation is supplied by the plant. The oxidative degradation of carbon substrates through a series of enzymes and coenzymes produces high-energy phosphate (ATP) and reductant. Leghemoglobin in legume nodules participates in the transport of O_2 from outside the nodules to the bacteroids in a way that maintains an exceedingly low O_2 concentration at the bacteroid surface. Ammonia, the produce of nitrogen fixation in bacteroids, is excreted into the plant cytosol, where amino acids and amides are synthesized. These compounds are secreted into the root xylem elements for transport to various parts of the plant.

Adaptations Favoring Nitrogen Fixation

Respiratory protection. All purified nitrogenase preparations are extremely sensitive to O_2. The mechanisms of protection of nitrogenase in anaerobes are obvious, but the mechanisms that aerobes have evolved for the protection of the enzyme vary and some are incompletely understood (*21*). *Azotobacter* species and other members of this family exhibit unusually high respiratory rates, which is believed to be an essential aspect of a respiratory protection mechanism. The respiratory rates of azotobacters are usually sufficient to lower the O_2 content of the medium to noninhibiting levels. When N_2 is the sole source of nitrogen, growth of *Azotobacter chroococcum* is inhibited by vigorous aeration, but growth of this organism is much less sensitive to O_2 when NH_4^+ is supplied (*21*). According to Yates and Jones (*45*), *Azotobacter* possesses a branched electron transport chain, a portion of which generates ATP under conditions of high O_2 concentration, while the other part synthesizes ATP at lower O_2 tensions. Adenosine triphosphate is synthesized at a lower rate when the O_2 concentration is high. The concept that nitrogenase in aerobic organisms is protected from O_2 by a conformational mechanism has been discussed (*21*). It is based in part on the pioneering work of Bulen *et al.* (*27*), who prepared a relatively stable particulate nitrogenase complex from *A. vinelandii*. The sensitivity of the enzyme to O_2 returned, however, when the particulate complex was broken down and nitrogenase released in a soluble form. There is no convincing evidence for the existence of O_2-insensitive particulate nitrogenase complexes in anaerobic or facultative anaerobic bacteria or in bacteroids of legume nodules.

Synthesis of gum. Many free-living nitrogen-fixing bacteria such as *Derxia* and *Azotobacter* species synthesize large quantities of gum. There is evidence that gum or slime may diminish the rate of diffusion of O_2 and in this way help protect nitrogenase from damage (*53*). An adequate supply of oxidizable carbohydrates is obviously essential for either the synthesis of gum or the maintenance of a respiratory protection mechanism.

Heterocysts. Many blue-green algae are capable of both nitrogen fixation and photosynthesis. In these microorganisms the nitrogenase system is vulnerable to O_2 produced during photosynthesis. In most of the blue-green algae, the major part of nitrogenase activity is found in heterocysts, which are specialized enlarged cells lacking the O_2-evolving por-

tion of the photosynthetic apparatus. This adaptation appears to accommodate the nitrogen-fixing process. Fixation of nitrogen by the few blue-green algae that do not form heterocysts occurs only with very low oxygen concentrations or under conditions of limited illumination (*11*).

Root nodules. The legume root nodule is a highly developed plant organ that possesses several characteristics that appear to be particularly compatible with the symbiotic nitrogen-fixing process. The relationship between a nodule bacteroid, with its essential nitrogen-fixing apparatus, and leghemoglobin, which is present in the plant cytoplasm (or cytosol), is illustrated in Fig. 2. Actually the bacteroids in nodules are located in many individual packets, each with three or four bacteroids surrounded by a membrane of plant origin (*38*). The mass of bacteroids enclosed in these membranes makes up about 30 percent of the total nodule weight. The bacteroid tissue is supplied with water and carbohydrate (or other carbon substrates) from the plant (*54*) and with atmospheric carbon dioxide, which serves as a substrate for carboxylation reactions (*55*). Ammonia, the first product of the nitrogenase reaction, is excreted from the bacteroids into the cytosol, where it is converted into glutamine, asparagine, and a variety of amino acids (*54*). Of particular interest is a group of highly specialized cells that surround the xylem elements of the root and function as secretory glands in the delivery of asparagine, glutamine, and other products of fixation into the xylem elements, where they are transported to various parts of the plant (*54*).

The mechanisms that nodulated legumes have evolved to protect the nitrogenase system from oxygen damage are highly specialized and effective. The occurrence near the periphery of nodules of a barrier to the diffusion of free O_2 has been demonstrated by Tjepkema and Yocum (*56*) by use of a microelectrode. Within the nodule tissue the group of bacteroids is surrounded by a cytosol containing many proteins. A major protein is leghemoglobin, which is present in soybean nodules at a concentration of about 1 millimolar (*57, 58*). In air, leghemoglobin in soybean nodules is about 20 percent saturated with O_2, but the dissociation of O_2 from oxyleghemoglobin is so low that the concentration of free O_2 at the bacteroid surface is about 10 nanomolar (*57*). Leghemoglobin participates in a facilitated diffusion process in which O_2 from the outside of the nodule is transferred to the bacteroids through oxyleghemoglobin in a way that continuously maintains an exceedingly low concentration of free O_2. At the bacteroid surface, O_2 is accepted by a terminal oxidase system with an unusually high oxygen affinity. This system is reported to be more efficient in the synthesis of ATP from ADP than an alternate bacteroid terminal oxidase that operates at higher O_2 concentrations.

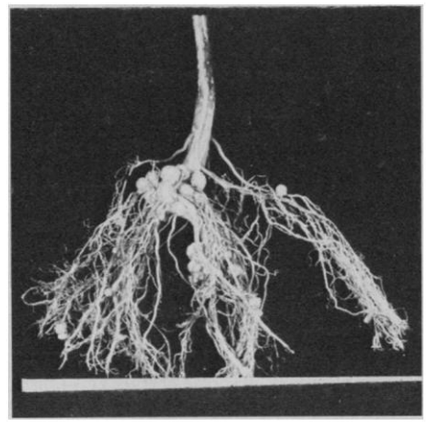

Fig. 3. Nodulated root system of soybean (*Glycine max*).

Capacity to recycle hydrogen. Schubert and Evans (*59*) have reported that the majority of nodulated legumes examined in an initial survey lost 30 to 60 percent of the energy supplied to nitrogenase as evolved H_2. In more recent experiments where soybeans were grown with selected *Rhizobium* strains and subjected to high light intensities before harvest, energy losses from H_2 evolution averaged 29 percent (*60*). A few legumes and several nodulated nonlegumes apparently have evolved mechanisms whereby the H_2 from the nitrogenase reaction is recycled and utilized in the electron transport system. They do not lose H_2 and therefore are more efficient. The nodules that do not evolve H_2 exhibit a capacity to take up H_2 supplied from an external source. From this evidence (*60*) and that of Dixon (*61*) it seems clear that utilization of the H_2 from nitrogenase in some legumes takes place through a sequence of reactions, the first of which activates H_2 through a hydrogenase (Fig. 3). Current evidence suggests that the *Rhizobium* cells contain the genetic information for hydrogenase synthesis, but the effects of the host legume on the expression of hydrogenase activity in nodules requires further examination. Recent results (*62–64*) show that *Azotobacter* and blue-green algae also possess hydrogenases that participate in an H_2 recycling process.

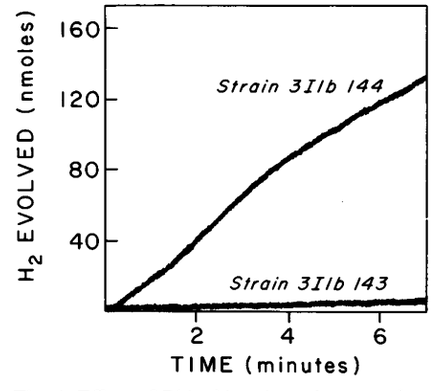

Fig. 4. Effect of *Rhizobium japonicum* strains on H_2 evolution from nodules of soybeans (*Glycine max* 'Anoka'). Plants were grown in a greenhouse under bacteriologically controlled conditions and were supplied with a nutrient solution lacking combined nitrogen. Eight replicate soybean cultures were inoculated with strain 311b 143 and another eight cultures with strain 311b 144. After growth periods of 28 and 37 days, nodules were removed and a 0.28 gram sample from each culture of plants was assayed for H_2 evolution in air by an amperometric method (*59*). Consistent differences were observed with samples from all eight replicate soybean cultures. The data are from Jennings *et al.* (*65*).

Schubert and Evans (*60*) have reported that inoculation of the 'Anoka' cultivar of soybeans with *Rhizobium japonicum* strain 110 produces nodules that lose little or no H_2 during periods when the nitrogenase system is operative. Recently Jennings *et al.* (*65*) in our laboratory have discovered that strains 3IIb 6, 3IIb 142, and 3IIb 143 of *R. japonicum* also produce nodules on soybeans that efficiently utilize energy supplied to the nitrogenase system (Fig. 4). Obviously, the necessary steps should quickly be taken to provide growers of legumes with combinations of *Rhizobium* strains and legume cultivars that produce nodules which efficiently utilize energy instead of wasting it as evolved H_2.

Some Proposed Approaches

Some of the rationale for increasing our dependence on biological nitrogen fixation has been summarized in review articles (*5, 22, 66*) and U.S. government–sponsored reports (*1*). These have helped generate enthusiasm for work toward increasing our dependence on biological nitrogen fixation as a means of meeting future food demands. Hopes have been stimulated that substantial grant funds may become available, and both experienced and inexperienced research groups seem to be gearing up with a variety of approaches. Some of the goals proposed are as follows: (i) transfer nitrogen-fixing genes from bacteria directly to higher plant cells, thus endowing the plant with a capability to use atmospheric N_2; (ii) transfer nitrogen-fix-

ing genes into a harmless bacterium that is capable of invading plant cells and establishing an effective nitrogen-fixing system such as a nodule; (iii) use protoplast fusion methods to create new symbiotic associations between microorganisms and higher plants; (iv) select or develop by genetic means nitrogen-fixing bacteria that are capable of living on roots of cereal crops such as corn and wheat and providing adequate fixed nitrogen for normal plant growth; (v) develop, by genetic manipulation, *Rhizobium* strains that are insensitive to soil ammonium and nitrate concentrations that normally inhibit legume nodulation and nitrogen fixation; and (vi) develop, by use of plant-breeding methods, legumes that have increased photosynthetic capabilities and therefore greater capacities for provision of energy for the nitrogen-fixing process in nodules. Other suggested novel ways of creating new or improved nitrogen-fixing biological systems have been tabulated by Hardy (*67*).

In our opinion, the probability of success with any approach should be carefully evaluated, taking into consideration less spectacular attacks on the problems for which a wealth of background information may already be available (*49, 68*). Several examples of opportunities for increasing the input of biologically fixed nitrogen into agricultural and other environments were included in a recent report (*1*). Most of the legume cultivars now being used in the United States were developed on land containing large populations of indigenous rhizobia of unknown nitrogen-fixing effectiveness. Coordinated research programs should be established in which plant breeders and specialists in rhizobial microbiology could develop nitrogen-fixing combinations of highly efficient *Rhizobium* strains and legume cultivars. These programs need to take into account problems of survival and competitiveness of rhizobial strains under adverse environmental conditions. There is a need to develop rhizobial strains with capabilities to colonize and infect legume roots and to fix nitrogen in soils with appreciable contents of fixed nitrogen. The search should be continued for sources of fertilizer nitrogen that may be utilized by legumes without inhibition of the nitrogen-fixation process. Management practices need to be developed that would allow economical use of legumes for provision of nitrogen in crop rotations of different types. A rhizobial technical research center is needed to provide leadership and expertise in the technology of aquisition, production, storage, distribution, and evaluation of rhizobial strains.

There is strong evidence indicating that the availability of carbon substrates to legume nodules is a major factor controlling the nitrogen-fixing process. Clarification of the metabolic processes that provide the energy for nitrogen fixation in nodules and definition of the environmental and physiological conditions in the field that limit the supply of photosynthate to nodules are areas of research that need attention. Selection or development of legume cultivars with growth and morphological characteristics that may contribute to increased yields of photosynthate could lead to an increased capacity to supply the energy for the nitrogen-fixing process. Research should be continued into the selection or development of legume cultivar–rhizobial strain combinations that recycle H_2 evolved from the nitrogen-fixing process rather than waste energy through H_2 evolution.

Associations of blue-green algae with plants such as *Azolla* or free-living blue-green algae possess the capability of making major contributions of fixed nitrogen to agricultural land and other environments. Development of cultural and management systems to take advantage of these capabilities would seem to have a high probability of success.

Enormous areas in the country are used for the production of forest trees and other species that are essential for maintaining ecological balance and controlling soil erosion. In these areas nitrogen is fixed by blue-green algae associated with lichens, liverworts, and mosses and by symbiotic associations between unidentified endophytes and woody species such as *Alnus, Ceanothus, Purshia, Myrica*, and *Dryas*. If the biology of some of these systems were better understood, the endophytes of the nonleguminous symbionts could be cultured and inocula prepared and used to increase nodulation and nitrogen fixation.

Conclusions

Some of the arguments for increasing our dependence on biological nitrogen fixation as a means of furnishing a part of the nitrogen requirements for production of food and fiber crops include the following: (i) biological nitrogen fixation takes place in fields, forests, and other places where nitrogen is utilized, and therefore energy consumption associated with industrial ammonia synthesis and transportation of fertilizer nitrogen is minimized; (ii) increasing the dependence on biological nitrogen fixation is an alternative for parts of the world where funds are limited for construction of ammonia synthesis factories and where sources of energy are inadequate; (iii) accumulation of ammonia, the product of biological nitrogen fixation, helps regulate the process and minimizes the possibility of accumulating excesses of nitrates in soils and water; and (iv) fixed nitrogen in the nodules of legumes and woody species is transported directly to the plant and is not immediately subject to losses.

Tabulation of the relative capacities of different systems to fix nitrogen reveals that nodulated legumes, nodulated nonleguminous woody species, free-living blue-green algae, and blue-green algal associations with plants are the major contributors. In terrestrial and aquatic environments where oxygen is limited, associations of microorganisms with the roots or rhizomes of plants may make contributions of fixed nitrogen that are sufficient to maintain the productivity of plants that are adapted to these conditions. In considering associations between roots of grasses and nitrogen-fixing bacteria in well-aerated soils, most of the relatively high rates of fixation were obtained by use of an unreliable method that allowed nitrogen-fixing bacteria to multiply on root surfaces before the tests. There is no doubt that nitrogen fixation occurs in root environments of nonnodulated species, but this type of fixation in well-aerated environments is sporadic, difficult or impossible to reproduce, and for the most part fails to occur at rates that are sufficient to meet the requirements of intensive crops.

Nodules of the nitrogen-fixing symbionts seem especially well adapted for the nitrogen-fixing process. The location of endophytes within plant nodules isolates them from the large population of microorganisms in the soil and prevents competition for energy. Microorganisms within nodules are provided with carbon substrates from the plant, and the products of nitrogen fixation are exported from nodules to the plant by a specialized system. Leghemoglobin in legume nodules (and perhaps other proteins in nodules of nonlegumes) participates in the transport of O_2 to the nitrogen-fixing bacteroids at an extremely low and nondamaging concentration. The periphery of legume nodules possesses a barrier to diffusion of free O_2. In some nodules a hydrogenase participates in utilizing the H_2 produced during nitrogen fixation, so that it is recycled rather than lost.

In considering the probabilities of success in increasing nitrogen fixation and extending the process to other organisms, we believe that researchers must

seriously assess the complexities of the fixation process. To develop new and economically important nitrogen-fixing organisms it will probably be necessary to transfer dozens of supporting genes as well as the known nitrogen-fixation gene cluster.

A nitrogen-fixing endophyte that exists within the protected environment of a nodule would have the benefit of a highly developed system for supplying carbon substrate and regulating the O_2 supply. Thus, it would be expected to be more reliable than any casual or associative symbiosis relying on occasional or stress-induced exudation of photosynthate from, for example, a grass root.

A summary of the status of biological nitrogen-fixation research in the United States has emphasized the urgent need for improving our research and technical capabilities for dealing with nodulated legumes (*1*). Some of the more spectacular approaches should be pursued as long-term possibilities of fundamental interest, but in our opinion, research toward improving the nitrogen-fixing capabilities of nodulated legumes and nonlegumes and of algal systems has the greatest probability of producing economic benefits to society in a relatively short period of time.

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