Nitrogen-Fixing Plants

The role of biological agents as providers of combined nitrogen is discussed.

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Of the major metabolic processes going on in cells, the two most important in maintaining the productivity of the earth—photosynthesis and nitrogen fixation—are characteristic of the plant kingdom only. Photosynthesis has in the past received most attention, but the world population explosion has put a premium not only on carbohydrate synthesis but, probably even more so, on protein synthesis. About 50 percent of the world's population is now reported to be undernourished or actually starving (*1*). Undernourishment and starvation stem not only from food shortage but also from supply of the wrong type of food—food that is rich in carbohydrates and deficient in proteins, rather than the reverse. Protein deficiency results largely from nitrogen deficiency; nitrogen deficiency, from the inability to convert cheaply and efficiently the gaseous nitrogen of the air into combined nitrogen readily assimilable by most plant and animal species. The basic step, the conversion of gaseous nitrogen to the combined form, is the process of nitrogen fixation.

Nitrogen fixation may be carried out by biological agents, such as the root nodule-bearing legumes, or blue-green algae, or chemically, as in chemical fertilizer plants or in electrical discharges in the atmosphere. The total contribution of fixed nitrogen to the earth's surface has been estimated at about 100 million tons of nitrogen per year, 90 percent of which is considered to be biological in origin (*2*). The minor role of chemical-fertilizer nitrogen in the world as a whole is difficult to appreciate in the agriculturally well-advanced countries of Western Europe and North America, where about 70 percent of the total world chemical fertilizer plants and about 18 percent of the total world population are located, and where, as a result, ample cheap chemical-fertilizer nitrogen is available. It is against the background of the situation in the world as a whole that one should consider biological nitrogen fixation.

Studies are currently developing along three rather distinct lines, all of which have the ultimate aim of increasing the process and obtaining a more accurate assessment of its importance. First, there is the problem of getting agriculturally important legumes to fix nitrogen more efficiently. Second, we must find out what other nitrogen-fixing plants besides the legumes are present, how they fix nitrogen, and what their contribution to soil fertility is. Third, studies on the enzymic mechanism of biological fixation, at present proceeding at an unprecedented rate, may have practical application in the chemical fertilizer industry.

The Legume-Rhizobium Association

In agricultural ecosystems nodulated legumes are undoubtedly the preeminent nitrogen fixers, having been harnessed to man's agricultural needs. The bacterial partners—species of the genus *Rhizobium*—were classified by Fred, Baldwin, and McCoy in 1932 into 16 cross-inoculation groups, the members of which induce nodulation on a certain number of legume genera and on these alone (*3*). This classification has persisted because it is convenient and because there is no better alternative. Within recent years the evidence of transduction in bacteria has revived interest in the classification and breeding of rhizobial strains. The need for efficient nitrogen-fixing symbioses is greatest in tropical agriculture, where the well-established species of temperate areas are, on the whole, unsuitable—their growth habit provides little protection from the sun, and they tend to be overgrown by the long tropical grasses. Also, nitrogen fixation rates decrease rapidly at temperatures above the optimum for fixation.

Although rhizobia are common soil bacteria, it is the usual practice for the farmer to obtain from private industry or agricultural institutes rhizobial supplements for the plants he wants to nodulate. Such supplements are added to the soil either separately or along with the seed. It is remarkable that such important collections, comprising hundreds of rhizobial strains, are, on the whole, left in the hands of a few individuals and institutes often unable, because of lack of funds and personnel, to maintain and expand them. National Collections have been established for less-deserving causes!

The conditions which bring about the symbiosis are far from being well understood. In the legume rhizosphere, bacterial multiplication is nonselective, being stimulated by legume secretions. Among the secretions is tryptophan, which is converted to indoleacetic acid. The latter, together with polygalacturonase, an enzyme secreted by the legume in response to a specific rhizobial exudate, may help loosen fibrils of the root hair wall and allow the rhizobia, which may be in a swarming stage, to enter the root hair tips and penetrate the inner cortical cells of the root, often by way of infection threads (*4*). The infection threads, as seen under the electron microscope, comprise rhizobia embedded in mucilage and surrounded by the invaginated host cell wall and plasmalemma. Release of the rhizobia from the infection thread is by pinocytosis: deposition of cellulose around the infection-thread tip ceases, and the plasmalemma invaginates, pinches off the bacteria either singly or in groups, and releases them into the host cell still surrounded by a host-cell membrane (*5*). The multiplication of the bacteria and their increase in size (the enlarged nonmotile bacteria are called bacteroids) then result in swollen infected cells, in which hemoglobin develops. Such cells comprise the central infected tissue of legume nodules.

The original suggestion (*6*) that the site of nitrogen fixation within the infected area is the membrane envelope

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surrounding the bacteroids is no longer tenable. Klucas and Burris (*7*) have shown consistently that if the nodules are exposed to $^{15}N_2$ for short periods and the distribution of ^{15}N in the various nodule fractions is assayed, the highest labeling is found in a soluble fraction, not in the membrane. It was fairly clear that, until nitrogen-fixing cell-free extracts of symbiotic systems became available, the exact nitrogen-fixing site would remain uncertain. Bergersen (*8*) was able to demonstrate nitrogen fixation in soy bean homogenates supplied with oxygen, but Koch, Evans, and Russell (*9*) were the first to achieve fixation in extracts containing no intact cells. They found that, if soy bean nodules are crushed anaerobically in a solution of polyvinylpyrrolidone (which removes phenolic compounds, thus preventing phenol oxidase activity) the nitrogen-fixing system in the nodules is preserved. They then separated various nodule components by centrifugation, tested each for nitrogen fixation, and found maximum activity in the bacteroid fraction. Like cell-free extracts of free-living bacteria, bacteroid extracts fix nitrogen under anaerobic conditions when (i) a source of adenosine triphosphate (ATP) and (ii) reducing power (sodium dithionite) are available. Other evidence supporting the view that the bacteroids are the nitrogen-fixing sites has been obtained by Kennedy *et al.* (*10*). They showed that, over the first 25 minutes of exposure to $^{15}N_2$, the bacteroid fraction is more highly labeled with ^{15}N than the supernatant or membrane fraction is. Furthermore, in serradella nodules, where the nitrogen-fixing sequence is $N_2 \rightarrow NH_3 \rightarrow$ glutamic acid, the glutamic acid extracted from the bacteroid fraction after exposure to $^{15}N_2$ is the most highly labeled. Thus, few major differences seem to exist between the mechanisms of fixation in symbiotic and in free-living systems. The nodules may be regarded as specialized ecological niches providing essential microenvironmental conditions and specific metabolic products which allow the bacteroids to fix nitrogen.

Studies on whole-plant physiology suggest that iron and molybdenum are both implicated in symbiotic nitrogen fixation. For example, legumes require higher concentrations of molybdenum for growth on elemental nitrogen than for growth on nitrate nitrogen, and when the supply of molybdenum is limiting, that which is present accumulates in the bacteroid tissue (*11*). Studies on cell-free extracts of free-living nitrogen-fixing organisms have confirmed the need for both elements. Such findings have had practical application; molybdenum is now added routinely to many soils where nodulated legumes grow. In some areas of Australia, additions of as little as 70 grams of molybdenum per hectare (1 ounce per acre) have increased legume yields spectacularly. Cobalt, another element required for legume growth on elemental nitrogen, is essential for cobamide coenzyme synthesis in rhizobia (*12*), and the addition of small quantities of cobalt to field crops of nodulated legumes has increased yields by 29 to 77 percent (*13*).

Studies on legumes have rightly centered around the 200-odd species commonly used in agriculture, where they contribute, on the average, 100 to 200 kilograms of nitrogen per hectare per year. However, the 12,000-odd species distributed throughout the nonagricultural soils which constitute nine-tenths of the land surface should not be forgotten. Many are bushes and trees which have not yet been examined for nodules, but as 89 percent of those so far examined are nodule-bearing (*14*), there is little doubt that they also contribute appreciably to soil fertility.

Table 1. Nitrogen-fixing genera of nonleguminous plants.

Bacteria	Blue-green algae	Root-nodulated angiosperms	Root-nodulated gymnosperms
Azotobacter	*Anabaena*	*Alnus*	*Ceratozamia*
Bacillus	*Anabaenopsis*	*Casuarina*	*Cycas*
Beijerinckia	*Aulosira*	*Ceanothus*	*Encephalartos*
Chlorobium	*Calothrix*	*Cercocarpus*	*Macrozamia*
Chromatium	*Chlorogloea*	*Comptonia*	*Podocarpus*
Clostridium	*Cylindrospermum*	*Coriaria*	*Stangeria*
Derxia	*Fischerella*	*Discaria*	
Desulfovibrio	*Hapalosiphon*	*Dryas*	
*Klebsiella**	*Mastigocladus*	*Elaeagnus*	
Methanobacterium	*Nostoc*	*Hippophäe*	
Pseudomonas	*Scytonema*	*Myrica*	
Rhodomicrobium	*Stigonema*	*Purschia*	
Rhodopseudomonas	*Tolypothrix*	*Shepherdia*	
Rhodospirillum	*Westiellopsis*		
Spirillum			

* This genus is the nitrogen-fixing endophyte of *Psychotria* leaf nodules.

Nonleguminous Nitrogen-Fixing Plants

The legumes are far from being the only nitrogen-fixing plant group of any importance. In natural and in agricultural ecosystems one or more of the following may be present: (i) nonleguminous root-nodule-bearing plants; (ii) plants bearing leaf nodules; (iii) blue-green algae; (iv) free-living nitrogen-fixing bacteria and possibly some fungi, actinomycetes, and yeasts. Nonleguminous genera for which reliable evidence of fixation is available are listed in Table 1.

Among the angiosperms, 13 root-nodule-bearing genera, comprising over 200 species, are known and many more probably remain to be discovered. Ecologically these woody perennials are characteristic pioneer plants of areas low in combined nitrogen, for there they are at a competitive advantage. Thus, Crocker and Major (*15*) noted the abundance of *Alnus* plants in recently deglaciated areas in Alaska and conjectured, on the basis of ecological data, that *Alnus* fixed nitrogen, unaware at the time that this had previously been established in laboratory culture. Similar ecological indications led to tests on *Shepherdia*, another root-nodulated plant of this area, and it was subsequently shown to fix nitrogen. Other nodulated genera such as *Arctostaphylos* are yet to be tested. Such plants are not restricted to Alaska and deglaciated areas. The United States genera include *Alnus, Ceanothus, Cercocarpus, Comptonia, Elaeagnus, Myrica,* and *Purschia.* In Britain, *Alnus, Myrica,* and *Hippophaë* are common.

The plants are divided among the families Betulaceae, Casuarinaceae, Coriariaceae, Ericaceae, Myricaceae, Rhamnaceae, and Rosaceae, but not all genera or species in all families fix nitrogen. Even the occurrence of nodules within known nodule-bearing genera has not yet been assessed properly, but a project for making such studies has been started under the auspices of the International Biological Programme.

Nodules occur laterally on the roots as perennial coralloid masses ranging from a few millimeters to 10 to 15

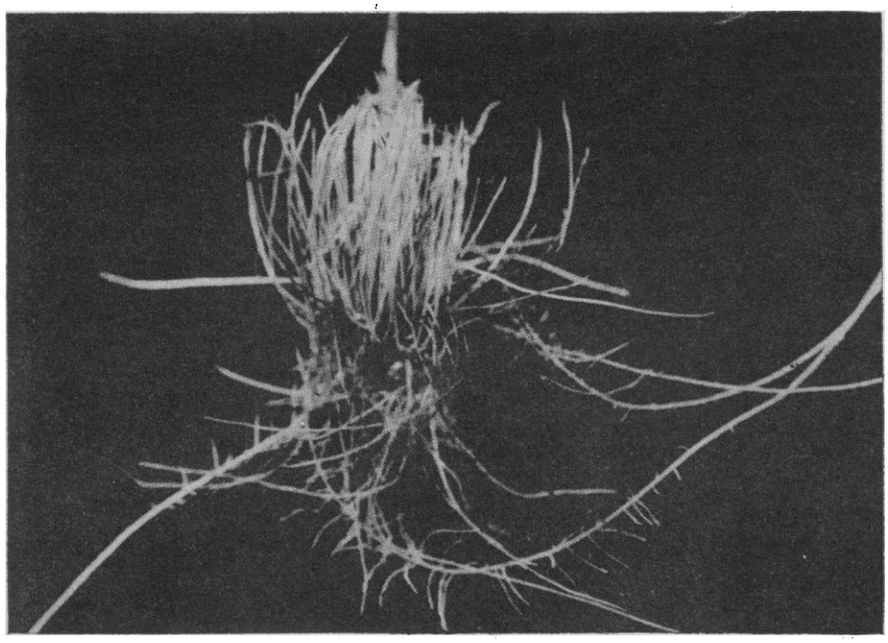

Fig. 1. The root system of a young *Myrica gale* plant, showing the presence of white upwardly growing nodule roots (× ⅔). [From W. D. P. Stewart (*25*)]

centimeters in diameter. Some species, particularly those of marshy ground—for example, *Myrica gale*—have white, upwardly growing roots originating from the nodule lobes (Fig. 1), and although these roots may be aerating in function they also occur in species of nonwaterlogged areas. The nodule endophytes which cannot be isolated routinely in laboratory culture seem, from electron-microscope studies on nodule tissues, to be actinomycetes (*16*). These infect the cortical tissues rather than the intravascular tissues as in legumes, and, while mainly filamentous, show various morphological forms, not yet correlated with the cycle of nitrogen-fixing activity of the nodules. There are resemblances to the situation in legumes in that the endophyte is surrounded by a membrane envelope and hemoglobin is reported to be present (*17*).

As Bond (*18*) has emphasized, the basic physiology of the nodules is similar to that of legume nodules. However, when detached, nonlegume nodules fix nitrogen considerably longer than legume nodules do, and it may be that the nonlegume endophytes fix nitrogen in the free state. Fiuczek (*19*) claims to have found evidence of this, but this finding has yet to be confirmed by other workers.

The importance of these plants as nitrogen sources is only now being appreciated fully. Allen and Allen (*20*) recently reviewed their quantitative significance, and it may be concluded that nonlegumes contribute as much nitrogen to the soils in which they occur as leguminous crops do. This conclusion is supported by the work of Daly (*21*), who noted in Canada that, under *Alnus rugosa*, soil nitrogen may be built up at the rate of 150 kilograms per hectare per year. In Britain, in sand-dune systems dominated by *Hippophaë rhamnoides*, Stewart and Pearson (*22*) record a total nitrogen accretion of 179 kilograms per hectare per year.

The nodulated nitrogen-fixing gymnosperms are of two main types. There is *Podocarpus*, in which the symbiont (a phycomycete) occurs intracellularly in the cortex, and there are the nodulated cycads, such as *Macrozamia* and *Encephalartos*, in which the microbial partner (a blue-green alga) occurs in a distinct air space in the nodule cortex. With the renewed interest in nodulated nonleguminous plants, and because it grows well in poor soils, *Podocarpus* has recently received a good deal of attention. There have been reports of no nitrogen fixation and of very slight or moderately high fixation (*23*), but even when fixation has been demonstrated it has clearly been insufficient to supply the total nitrogen requirements of the plant. The cycads, although dominant in the Carboniferous period of the geological time scale, are now of restricted distribution, and the endophytes (species of *Nostoc* or *Anabaena*) fix nitrogen when they are isolated in pure culture.

Those plants which bear leaf nodules have been the most neglected of all higher plants, from the point of view of nitrogen-fixation studies. Leaf nodules occur particularly in *Psychotria* and *Pavetta* of the Rubiaceae, and in *Ardisia* of the Myrsinaceae. Nitrogen-fixing glands and buds are also reported in other families, including the Verbenaceae and Myoporaceae. The nodules are subepidermal cavities filled with mucilage and bacteria. Over 40 years ago it was reported that these nodules fix nitrogen, but Silver *et al.* (*24*) were the first to provide unequivocal evidence of nitrogen fixation. They showed that (i) nodule homogenates of *Psychotria bacteriophylla* and (ii) the isolated endophyte (a species of *Klebsiella*) fix nitrogen, and that nonnodulated plants grow unsatisfactorily even when combined nitrogen is supplied, possibly because the bacteria supply an essential growth factor as well. Should all nodule-bearing species fix nitrogen (and this has yet to be established), this group may be important in some tropical and subtropical regions. *Psychotria* and *Pavetta* are most abundant in Africa, where the plants grow well on poor soil, but they also occur eastward to tropical Australia. *Ardisia* is indigenous in South American countries such as Colombia, Peru, and Venezuela, and its range stretches north to Panama. With modern methods, a rapid check of the nitrogen-fixing capacity of nodulated leaves should be possible, but because of the abundance of free-living nitrogen-fixing organisms on the leaf surfaces of tropical species it will be less easy to ensure that, if fixation is established, it is actually due to the nodule bacteria and not to surface contaminants.

Although blue-green algae fix nitrogen endophytically in cycad root nodules, and in other associations such as some lichens, they are most abundant as free-living nitrogen-fixing organisms. Vegetatively they are prokaryotic organisms, the cells of which comprise an outer zone of photosynthetic lamellae (the chromatoplasm) and an inner region of ill-defined nuclear material (the centroplasm). The lamellae are the main sites of metabolic activity; studies made with cell-free extracts have shown that the enzymes for photosynthesis, respiration, hydrogenase activity, and nitrogen fixation all occur on, or are associated with, the lamellae (*25*).

Over 40 nitrogen-fixing species of blue-green algae are known from pure culture studies (*25*). All are filamentous

and belong either to the order Nostocales (for example, *Nostoc, Calothrix,* and *Aulosira*) or the order Stigonematales (for example, *Fischerella* and *Westiellopsis*). Such genera characteristically have large empty-looking cells called heterocysts (Fig. 2). Unlike ordinary vegetative cells, heterocysts do not fix nitrogen (*26*), perhaps because their photosynthetic lamellae are disorganized. Thus they are not the "root nodules" of blue-green algae, and their relationship to nitrogen fixation seems phylogenetic rather than physiological. Heterocysts provide a "rapid-scan" method for identifying potential nitrogen-fixing blue-green algae, and the International Biological Programme hopes to use this criterion to obtain information on the distribution of nitrogen-fixing blue-green algae in nature.

In tropical rice paddy fields, under waterlogged conditions unsuitable for legume growth, blue-green algae abound, and possibly they have been the main source of combined nitrogen in paddy fields for thousands of years. Despite the need for nitrogen fertilizer in Southeast Asia (over half the world's population lives on rice as staple diet), little attempt, with two notable exceptions (*27*), has been made to investigate scientifically the further application of this biological source of fertilizer. This is partly because rice paddies and associated blue-green algae are characteristic of underdeveloped countries (Japan is an exception) having neither the scientific approach, the equipment nor the funds needed to exploit this nitrogen source to the full. In the few paddy fields where the contribution of blue-green algae has been assessed, the fixation rate is of the order of 30 to 50 kilograms of nitrogen fixed per hectare per year (*25, 27*).

Paddy fields represent just one ecological niche of nitrogen-fixing algae. Generally, the more extreme the physical conditions are, the more likely one is to find blue-green algae, provided light, water, and carbon dioxide are available at some period. Two examples will suffice. (i) In Antarctica, on rock and soil surfaces, particularly under alkaline conditions, *Nostoc* species fix nitrogen alone, or in lichen symbiosis, at temperatures near zero. *Nostoc* accumulates in such areas to depths of 15 centimeters (6 inches) as algal peat and is probably the main source of biologically fixed nitrogen in this region (*28*). (ii) At the other end of the temperature scale there are the nitrogen-fixing algae of hot-spring regions. For example, in Yellowstone Park, where the color of large thermal areas is due largely to the presence of blue-green algae, nitrogen fixation occurs at temperatures up to 55°C in streams dominated by the nitrogen-fixing alga *Mastigocladus* (*29*).

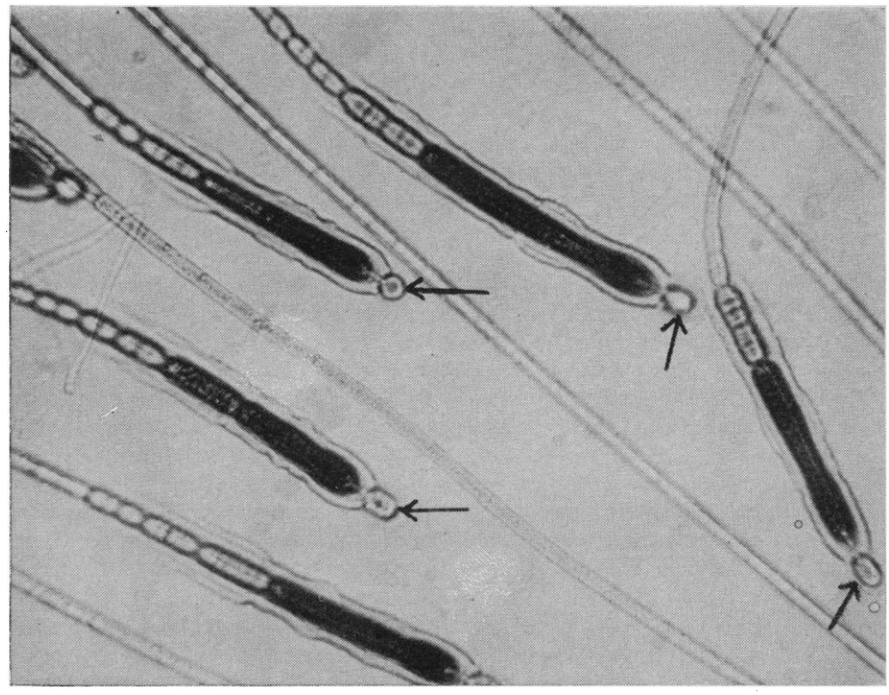

Fig. 2. Filaments of the blue-green alga *Gloeotrichia natans* (15 microns broad at base), showing (arrows) the presence of basal heterocysts.

Blue-green algae may be important in the marine environment. Many nitrogen balance sheets of the earth have been prepared but most are based on terrestrial measurements and ignore the fact that this represents only one-fifth of the earth's surface. In the four-fifths covered by the sea, the important terrestrial nitrogen-fixers—the nodulated plants—are absent, and concentrations of inorganic combined nitrogen are, on the average, about 100 times lower than on land. The nitrogen required for marine productivity comes mainly from wash-off from the land, from combined nitrogen in the atmosphere, or from *in situ* biological nitrogen fixation. Wash-off from the land is unimportant in the oceans as a whole but contributes appreciably in coastal areas, where productivity is greatest. There is less combined nitrogen in the atmosphere, per unit volume, above the oceans than above land masses. What, then, is the contribution of *in situ* biological nitrogen fixation?

In tropical and subtropical oceans an important bloom-former is the blue-green alga *Trichodesmium*. Studies in the Arabian Sea, the Indian Ocean, the South Atlantic, and the Sargasso Sea have shown, many times, that such blooms assimilate $^{15}N_2$ (*30*). One cannot be sure whether the blue-green algae or associated microorganisms actually fix the nitrogen, for the test samples are impure, but this is immaterial. What is important is that nitrogen-fixing blooms are now known which may cover areas of hundreds of square kilometers. *Nostoc* is an important planktonic blue-green alga in the Indian Ocean, and also in the subantarctic seas, where it reaches its maximum development (*31*). This marine form has not yet been tested for nitrogen fixation, but *Nostoc* is known to be a nitrogen-fixing genus. In inshore waters, *Calothrix* dominates the group of nitrogen-fixing blue-green algae characteristic of high tide level of temperate rocky shores, where it fixes approximately 2.5 grams of nitrogen per square meter per year (*32*), about one-tenth the amount fixed by a good leguminous crop under agricultural conditions. In the tropics, *Calothrix* may be replaced by the nitrogen-fixing genera *Tolypothrix* and *Rivularia*, whereas in brackish areas *Nostoc* is common. The nitrogen fixed by these algae is rapidly assimilated by associated non-nitrogen-fixing plants (*33*). Marine nitrogen fixation has been largely neglected in the past, and an accurate quantitative

assessment of fixation rates, particularly by planktonic species, is now required.

Until about 1950, *Azotobacter* and *Clostridium* were regarded as the only two genera of nitrogen-fixing bacteria and, because of the low numbers of isolates usually obtained from agricultural soils, were considered unimportant. Recent evidence suggests that a reassessment is necessary.

First, it has been recognized that most nitrogen-fixing bacteria are strict anaerobes—they would not have been isolated under the aerobic culture conditions commonly used in the past. The number of known nitrogen-fixing genera has now increased to at least 15 (see Table 1), and some of the more recently isolated bacteria may prove to be particularly useful physiological tools. For example, *Derxia gummosa*, from Indian soils, is one of the most efficient nitrogen-fixing species known, in terms of nitrogen fixed per unit weight of carbohydrate consumed (*34*). Second, aerobic bacteria such as *Azotobacter* fix nitrogen most rapidly at oxygen tensions below 20 percent (*35*). With these two points in mind, it has been possible to isolate many more nitrogen-fixing strains and to show that these are present in nature in much higher numbers per unit weight of substrate than had previously been supposed.

The contribution of free-living bacteria to soil fertility is uncertain, but Knowles (*36*), on the basis of ^{15}N measurements, recently reported fixation rates ranging from 0.1 kilogram to 73.0 kilograms per hectare per year under anaerobic conditions and up to 34.6 kilograms under aerobic conditions in some Quebec soils. The general importance of free-living bacteria in soils will be known only when many such tests have been carried out, with strict controls and measurement of seasonal variation. It is hoped that the acetylene reduction technique, described below, may provide a quick and sensitive method for indirectly measuring, *in situ*, nitrogen fixation by free-living organisms.

Meanwhile, there are arguments for and against the view that the level of bacterial fixation is important. Supporting this view is the discovery of many nitrogen-fixing strains, particularly in the tropics, where *Beijerinckia* is important in lateritic soils and where nitrogen-fixing organisms of the phyllosphere possibly supply nitrogen to the rain forests (*37*). Second, the bacteria remain in the soil throughout the year and are potentially capable of fixing nitrogen whenever environmental conditions permit. Third, appreciable nitrogen fixation by bacteria has been found to occur in some soils. Arguing against the view that bacteria are of widespread importance is the fact that most are heterotrophic and inefficient users of carbohydrate, only about 1 milligram to 10 milligrams of nitrogen being fixed per gram of carbohydrate utilized. Readily assimilable carbohydrate is in short supply in most soils, and there is severe competition for it, not only by nitrogen-fixing heterotrophs but also by non-nitrogen-fixing forms. Several workers have shown that nitrogen fixation by soil microorganisms increases markedly when carbohydrates such as sucrose are added to the soil. Furthermore, in habitats where concentrations of carbohydrates are high, concentrations of combined nitrogen are often high as well, so that, although bacterial growth may be stimulated by the carbohydrate, fixation may be inhibited by the combined nitrogen. Heterotrophic bacteria probably play their main role by fixing small quantities of nitrogen over long periods and over wide areas, rather than by supplying large quantities over short periods as agricultural legumes do.

The photosynthetic bacteria, because of their requirements for light and anaerobic conditions, are of local importance only—for example, in the anoxic sediments of shallow lakes. The role of other suspected nitrogen-fixing groups—the actinomycetes, fungi, and yeasts—is uncertain, and more critical work has yet to be carried out before their contribution can be assessed properly (*25*). Of particular interest, however, is the evidence for fixation by actinomycetes (*38*), which are considered to be the endophytes of root-nodule-bearing angiosperms, and the evidence for nitrogen fixation by mycorrhizae associated with *Pinus* seedlings (*39*).

Points of Biochemical Interest

Although production of chemical nitrogen fertilizer has doubled in the last decade, so also has the number of known nonleguminous nitrogen-fixing species. Thus, the percentage contribution of synthetic fertilizer nitrogen is probably no greater than was supposed 10 years ago. If a large increase in the percentage contribution of chemical nitrogen fertilizer is to be achieved, more efficient ways of producing it must be developed.

Synthetic nitrogen fertilizer is still produced largely by the Haber-Bosch process, which involves passing N_2 and H_2 over a catalyst (generally a complex of iron oxides) at temperatures near 500°C and pressures of about 1000 atmospheres. More economic processes, such as naptha-reforming in the presence of air, have been introduced lately, but none really compares with biological nitrogen fixation, where the morphologically insignificant bacteria and blue-green algae, either alone or in symbiosis with higher plants, convert molecular nitrogen to ammonia at room temperature. It is the hope of finding out exactly how plants do this which has stimulated so much interest in the mechanism of biological nitrogen fixation (*40*). Some of the major well-established findings may be mentioned briefly here.

In the late 1950's, although excellent work on nitrogen fixation, performed with ^{15}N as tracer, had been carried out, studies on the mechanism of fixation were stagnating because no methods for extracting active nitrogen-fixing enzymes from the organisms had been found. Reports of cell-free extracts appeared from time to time, but it was not until 1960 that the preparation of consistent nitrogen-fixing cell-free extracts was reported (*41*). Since then, rapid advances have been made, particularly by Mortenson at Purdue, by Bulen and his co-workers, by Burris's and Wilson's groups at Madison, and by Hardy's group. Extracts may be prepared by vacuum-drying the cells and allowing the enzymes to diffuse out into a suitable buffer solution. Other methods are disruption of the cells by means of a pressure cell or an ultrasonic probe. All have yielded satisfactory extracts, the specific method used depending on the organism, the available facilities, and what is required.

Early advances were made with *Clostridium*, crude extracts of which fixed nitrogen only when pyruvate was added and strict anaerobic conditions were maintained. The role of pyruvate was to supply reducing power and energy by way of the phosphoroclastic reaction which converts pyruvate to the high-energy precursor acetyl phosphate, to carbon dioxide, and releases electrons in the process (*40*). The next step was to dispense with pyruvate, supply the energy and reducing power

separately, and still achieve fixation. Initial tests in which exogenous ATP was added were disappointing, for fixation was inhibited. This proved to be a concentration effect, for it was shown subsequently that small quantities, added at intervals or as an ATP-generating system, stimulated fixation. Molecular hydrogen, acting through a hydrogenase system, supplied electrons in the presence of ATP and ferredoxin (*42*).

The isolation of *Clostridium* ferredoxin was reported in 1962 (*43*), although other ferredoxins had been isolated previously from other tissues under a variety of names. The need for ferredoxin in the clostridial nitrogen-fixing system (dependent upon H_2 and hydrogenase as electron donor) was demonstrated when removal of ferredoxin inhibited the system and subsequent addition of purified ferredoxin reactivated it (*42*). Studies by Blomstrom *et al.* (*44*) suggest that *Clostridium* ferredoxin has an active site of seven non-heme iron atoms arranged linearly and bonded by sulfide bridges from cysteine residues and by labile sulfide. Recently another electron carrier, flavodoxin, has been isolated from iron-deficient *Clostridium* cultures (*45*). This protein, of molecular weight 14,600, is more electro-positive than ferredoxin and may replace ferredoxin in iron-deficient cultures.

It is clear that, in *Clostridium*, pyruvate, ferredoxin, and ATP-independent hydrogenase play no direct role in nitrogen reduction; they simply supply the necessary energy and reducing power. In *Azotobacter* (*46*), which has no phosphoroclastic system or ferredoxin, and also in *Clostridium* (*47*), the nitrogenase can fix nitrogen when an exogenous electron donor (sodium dithionite) and ATP are supplied. Associated with nitrogenase activity is ATP-dependent hydrogen evolution, reductant-dependent adenosine triphosphatase activity and production of inorganic phosphate. Hydrogen evolution occurs when electrons generated from dithionite in the presence of ATP are not utilized in nitrogen reduction (*48*).

As only the nitrogenase is active directly in nitrogen reduction, attention has recently been confined to its characterization. Thus, Mortenson, studying *Clostridium*, separated two fractions which, when recombined, fix nitrogen in the presence of an extract from ammonia-grown cells. One fraction, of molecular weight about 90,000, contains both iron and molybdenum; the other is an iron-containing fraction of molecular weight about 40,000 (*49*). The *Azotobacter* nitrogenase has also two rather similar components which fix nitrogen only when they are combined (*50*). Such findings imply that the nitrogen-fixing complexes in aerobic and anaerobic bacteria are of similar type. It seems only a matter of time before a two-enzyme system is obtained from root nodule extracts, for findings that the various nitrogenases are all oxygen-sensitive, require ATP, use the same electron donors, and have a cold-labile ferro-enzyme all suggest a common system. The similarity will be established with certainty when fixation is demonstrated on combining nitrogenase proteins of different organisms.

The nitrogenase systems show not only a remarkable similarity but also a unique versatility, for they are now known to reduce various compounds other than nitrogen. Early evidence that the nitrogen-reducing system is nonspecific was obtained by P. W. Wilson's group, which showed that N_2O, H_2, and CO competitively inhibit nitrogen fixation (*51*). Mozen and Burris in 1954 were the first to show actual reduction of a compound other than N_2, when they observed N_2O assimilation by *Azotobacter* and soy bean nodules; their observations with soy bean nodules were extended by Hoch *et al.*, who clearly demonstrated the formation of N_2 from N_2O (*52*). Later the Wisconsin group established the reduction of azide and acetylene by nitrogen-fixing cell-free extracts (*53*). Dilworth (*54*) independently observed reduction of acetylene and demonstrated that it is reduced to ethylene, while the reduction of cyanide and isocyanide has also been established (*55*). The evidence that the nitrogenase carries out these reductions is convincing. For example, Dilworth's studies with *Clostridium* extracts show that the same system (H_2, hydrogenase and ferredoxin, and an ATP-generating system) is required for nitrogen reduction and for acetylene reduction. Both reductions are inhibited by low CO concentrations and neither occurs in extracts of NH_3-grown cells. Acetylene reduction is now known to be characteristic of all groups of nitrogen-fixing organisms—symbiotic forms, bacteria, and blue-green algae (*56*)—and it is probable that this technique will become routine procedure in scanning for nitrogen-fixing systems in the laboratory and in the field.

In addition to acetylene and cyanide, other analogs are also reduced, and Hardy and Jackson (*57*) in particular have followed up the early studies by routinely assaying for the reduction of a variety of these. The electron transfers range from 2 to 12 and occur in two-electron stages. By analogy this implies that nitrogen reduction also occurs two electrons at a time and suggests that the enzyme-bound intermediate between N_2 and $2NH_3$ are diimide (HN=NH) and hydrazine (H_2N-NH_2).

The exact mechanism by which nitrogen is reduced on the nitrogenase is not known, but, as various workers have pointed out, there are probably two active sites—the electron-activating site and the substrate-binding site. The specific function of each fraction of the nitrogen-fixing complex is not clear, but both fractions must be present in order for the complex to carry out any of its known reactions. Recently Bui and Mortenson (*58*) obtained evidence that the molybdoferroprotein combines with ATP to form a high-energy intermediate which may be involved in electron activation. Much more information must be accumulated before current speculation on this topic [see a forthcoming review by Hardy (*59*) on the biochemistry of nitrogen fixation] becomes well-established fact. In the meantime, the intact nitrogen-fixing organisms continue to do in nature as they have done in the past—supply the bulk of the fixed nitrogen added each year to the surface of our planet.

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NEWS AND COMMENT

Housing: Defense Department Starts New Research Program

One of the durable political realities in government support of research is that the Department of Defense (DOD) is accorded a relatively large degree of sovereignty in picking what it wants to research, where, and under what circumstances. A case in point is one of the latest DOD ventures into research, a small but ambitious program in what might seem to be an area remote from military affairs—the design and construction of three- and four-bedroom middleclass-style houses.

Ironically, when the Commerce Department sought to establish a similar program during the Kennedy administration, Congress, egged on by lobbyists from established producers of building materials, flatly refused to appropriate the requested funds (*Science*, 28 June 1963). Since then, of course, times have changed a good deal; government is deep in research areas that once drew automatic congressional ire. But perhaps even more important is the fact that DOD, though not free of congressional impediments and restraints, enjoys a latitude of operation that is not available to other government agencies. National security, though not an infallible talisman for securing appropriations, is nevertheless a very good one, and along with it comes not only money but also a beneficent congressional indifference and permissiveness toward what happens to the money. In the case of its housing program, DOD did not seek congressional permission; rather, it merely informed several relevant congressmen of its intention to go ahead, and, in the absence of any opposition from them, went ahead. And for the relatively minor amount of money involved at this point —$200,000 for preliminary studies—it did not have to seek a specific authorization or appropriation, but was able to draw this sum from general funds.

In many respects DOD's presence in housing research is quite logical, since the Department is very probably the country's biggest landlord and homebuilder: It owns some 370,000 units of housing for service families and every year constructs, as replacements and additions, another 8000 to 10,000 units. Since the home-construction business regularly and deservedly wins honors as one of the most antiquated and technology-resistant segments of the economy, Defense cannot be faulted for looking out for itself in attempting to keep down costs and hold up quality. Not without justification, it has been said that, because of restrictive building codes, work-stretching union regulations, and the fragmentation of the industry among thousands of small contractors, home-building today is the one major industry in which an 18th-century workman could show up at a job site, work with his own tools, and earn his pay.