Improving Nitrogen Fixation

Extending or imitating this unique natural process may provide a key to easing the global food shortage

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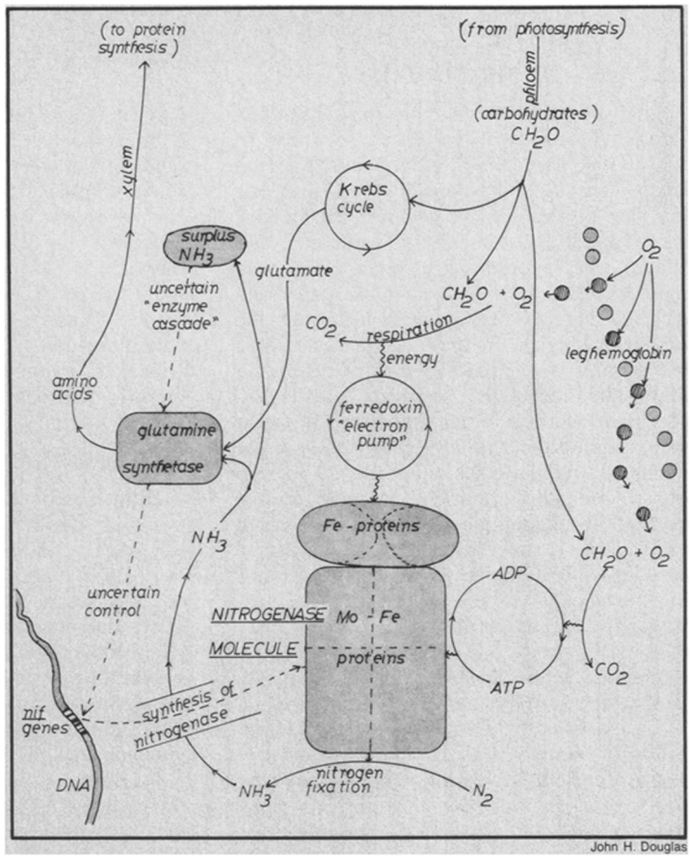
Another in our continuing series of articles on the New Green Revolution

The world's greatest food problem is not a lack of carbohydrates, the body's fuel, but rather a lack of proteins, the body's building blocks. Most people on earth simply cannot afford enough of the high-quality protein found in meat, and the grains that provide the bulk of their diet are all lacking in some essential amino acids—the protein constituents. In either case, the ultimate source of the protein eaten is nitrogen in the atmosphere, which first must be "fixed" (joined into compounds a plant can use) and then added to the products of photosynthesis to make amino acids. Only recently have scientists begun to understand the complex biological processes involved in this vital transformation well enough to begin manipulating them for nutritional improvement.

In nature, nitrogen is fixed by a variety of bacteria and blue-green algae that can either exist independently in the soil or form a close association (symbiosis) with some higher plant. The independent microorganisms must rely on the haphazard supply of nutrients brought to them by ground water. Those that form an obligatory symbiosis with certain plants (called legumes) infect their roots, causing the growth of large nodules, in which the bacteria can utilize nutrients coming down the plant's vascular system, and in turn, release fixed nitrogen for the plant to use in protein synthesis. In a looser, "associative" symbiosis, microorganisms cluster around the root system of a plant and apparently rely on leakage of nutrients (though some recent studies have found bacteria inside the root cells).

Few people realize just how extensive and important this process really is. Conservatively, microorganisms fix 175 million tons of nitrogen each year, around the world, including 90 million tons on agricultural soil. What nature doesn't provide must be offset by the addition of fixed-nitrogen fertilizer, now produced at a rate of 40 million tons a year. Because of the energy crisis, the price of this artifically provided nitrogen was six times higher in 1975 than four years earlier, which means that the biologically fixed nitrogen on agricultural soil is now saving farmers roughly $24 billion a year.



Nitrogen fixation: Carbohydrates descend from a plant's leaves and yield their energy via respiration. The energy passes through a complicated series of reactions to the bacterial enzyme nitrogenase, which catalyzes the "fixing" of atmospheric nitrogen to a more useful form, ammonia, NH_3. Some ammonia is used to form protein, but if too much accumulates, a "feedback loop" (dotted lines) shuts down the process.

As energy prices continue to rise, so will the cost of fertilizer. The problem lies in the nature of the atmospheric nitrogen molecule, N_2: It is exceedingly stable, which means simply that to break the molecule apart and combine its atoms into other compounds requires the addition of considerable energy. Not surprisingly, the commercial process (Haber-Bosch process) used to fix nitrogen must operate at more than 1,000 degrees F. and 200 atmospheres pressure; fully half the cost of producing a ton of fertilizer goes to pay for the energy used. To relieve this expense, scientists from many disciplines are now seeking ways of extending natural fixation or of imitating it to develop a more efficient artificial process.

The key to biological nitrogen fixation is one of the most extraordinary enzymes found in nature—nitrogenase. Its detailed structure is unknown, but two kinds of very large, metal-containing proteins seem to be included. Under an electron microscope, one fraction (known to contain iron) appears as two connected ellipsoids; the other (containing iron and molybdenum) looks like four little blocks. Like other enzymes, nitrogenase acts as a catalyst, somehow officiating at the transformation of nitrogen from its low-energy, atmospheric state to a high-energy molecule of ammonia. How the reaction takes place no one knows, but an enormous expenditure of energy is involved.

Two separate biochemical cycles are apparently involved in charging up the nitrogenase system with enough energy to do its job. Some evidence indicates that the iron-containing fraction of a nitrogenase molecule receives energetic electrons from a cycle of reactions involving the compound ferredoxin, which pumps them to the molecule from sites where respiration is taking place. Also, 15 molecules of ATP (adenosine triphosphate, the "universal energy currency" of living organisms) are consumed for each nitrogen molecule fixed—more than in any other known enzyme reaction.

The overall process of nitrogen fixation and protein systhesis can thus be tentatively understood: Carbohydrates descend from photosynthesis in the leaves by way of the phloem. Some are burned up in respiration to supply energy to the nitrogenase, giving off carbon dioxide as a by-product. Others are modified, and their products, such as glutamate, combine with the newly created ammonia—in the presence of another enzyme, glutamine synthetase—to eventually form several amino acids, which are carried up the xylem to form plant proteins.

But complications arise, and it is here that scientists are concentrating on how

to improve the performance of the overall system. First, oxygen must be kept away from the nitrogenase. This is apparently accomplished by a pink-colored compound called leghemoglobin, which binds stray oxygen molecules the way a similar substance does in human blood. (Besides protecting nitrogenase, leghemoglobin apparently helps transport the reactive oxygen molecules to where they can do some good, reacting with carbohydrates during respiration.) Also, a complex and little-understood feedback system exists so that when enough ammonia has been produced, nitrogen fixation shuts down. Apparently, a surplus of ammonia molecules triggers an "enzyme cascade" that blocks the action of glutamine synthetase, which in turn somehow inhibits the appropriate genes from making new nitrogenase.

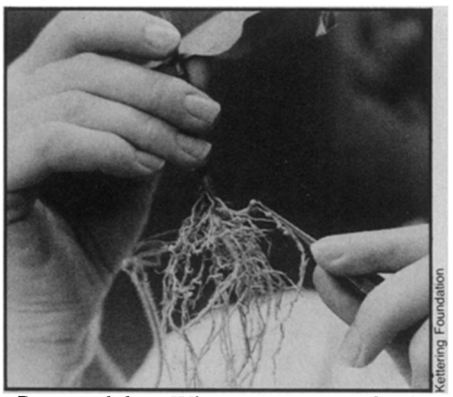
Both the oxygen blockage and the ammonia feedback system have enormous practical consequences. One of the ways scientists have hoped to improve the amino acid content of grains is by establishing a new kind of bacterial symbosis in their root systems. But now it appears that two of the key problems will be how to protect the nitrogenase in these bacteria from oxygen in the soil and how to keep the ammonia-feedback loop from shutting down the whole operation. Creating nodule growth seems very complicated, while associative symbiosis would probably be very inefficient with most grains.

Another early dream must also be reexamined—whether it will be possible to produce commercial fertilizer in a low-temperature process using cell cultures of nitrogen-fixing organisms or a synthetic catalyst analogous to nitrogenase. Similar ideas have worked effectively in imitating other natural systems. Cornstarch, for example, is commercially manufactured using a synthetic enzyme. If the Haber-Bosch process could be run at a couple of hundred degrees lower temperature, a 25 percent cost savings would be realized. But the problem with building biological reactors for the much more complicated process of nitrogen fixation is that an enormous amount of expensive carbohydrates would be needed in order to fuel the ferredoxin and ATP energy-donating reactions. Since plants have their own built-in supply of such fuel, they enjoy a privilege man does not share, that of evolving an effective but extremely wasteful system, which burns up about 40 carbohydrate molecules for each molecule of nitrogen fixed.

The new understanding of the nitrogenase and glutamine synthetase cycles has, however, revealed some exciting new possibilities for promising research:

- Joyce K. Gordon, a graduate student working with University of Wisconsin bacteriologist Winston J. Brill, discovered that the feedback loop to shut off nitrogenase production could be short-circuited by blocking the action of glutamine synthetase with methionine sulfoximine. Brill and his colleagues then searched for a mutant strain of the bacteria in which glutamine synthetase regulatory properties were altered, to see if these would also be unable to stop fixing nitrogen, no matter how much surplus ammonia was formed.

They succeeded (as did other researchers), and now Raymond C. Valentine, a biochemist at the University of California at San Diego, has developed what he calls a "nodule in a test tube." By purposely manipulating the genes of one bacterial strain, he created mutants that will produce directly measurable amounts of ammonia from nitrogen bubbled into a bag of sugar water. Although ammonia yields from the apparatus have increased by more than a factor of eight just since January, the process remains rather inefficient, and Valentine says that



Root nodules: Where nitrogen is fixed.

commercial versions will probably have to use blue-green algae, rather than bacteria, since algae can manufacture their carbohydrates by photosynthesis.

- Manipulation and transfer of the genes that control nitrogen fixation (called "*nif* genes") is proceeding at several laboratories. This genetic engineering uses recently developed techniques such as infection by a bacteriophage (a virus that carries DNA material from one bacterium to another) and transfer of plasmids (small, extra-chromosomal pieces of DNA that can reproduce themselves and move from one bacterium to another). Geneticist Ray A. Dixon of the University of Sussex first succeeded in transferring *nif* genes between two separate species of bacteria—from the natural parent, *Klebsiella pneumoniae*, to the familiar intestinal microorganism, *Escherichia coli*. Since then, successful transfer of nitrogen-fixing ability among more distantly related bacteria and algae has been accomplished.

Using similar techniques, scientists should be able to introduce *nif* genes to the cells of higher plants, but several problems immediately arise. The structure of nitrogenase is so complex that even if the correct genetic instructions were passed to a cell, there is no guarantee that a functioning molecule would be constructed. The task is a little like giving a carpenter an electronics schematic diagram—no matter how well constructed, a wooden computer won't work. Similarly, even in nitrogen-fixing microorganisms, if the soil is too deficient in molybdenum or if other ingredients are missing, nitrogenase synthesis doesn't take place.

- More and more attention is being focused on the supply of nutrients to the microorganisms; it may be that the best way to improve nitrogen fixation is by the indirect route of improving photosynthesis. Most plants evolved during a period when the atmosphere contained much more carbon dioxide and much less oxygen than now. As a result, the common "C_3" method of photosynthesis (so-called because compounds with three carbon atoms are used) is now rather inefficient. Oxygen can compete with carbon dioxide for access to the cycle, and when it succeeds, newly completed carbohydrates are just converted back to carbon dioxide. (The process is called "dark respiration.") A few plants of tropical origin (including sugar cane) use a separate, four-carbon photosynthesis process. Much new attention is now being paid to these "C_4" plants.

Brazilian microbiologist Johanna Döbereiner found the nitrogen-fixing bacteria *Spirillum lipoferum*, surrounding the roots of several tropical C_4 grasses, and presumably living in an associative symbiosis. Apparently these bacteria can receive enough nutrition from the plant without having to form nodules; also some sort of internal mechanism seems to protect their nitrogenase from oxygen—perhaps through a high level of internal respiration, to use up the oxygen. Rex Smith of the University of Florida has reported establishing new kinds of associative symbioses in locally grown plants, but he says that so far none of the systems will produce ammonia at soil temperatures below about 98 degrees F.—which limits their adoption to very few areas of the United States.

Further indication that nitrogen fixation is limited by nutrient supply was discovered by Ralph Hardy of Du Pont. By exposing some soybeans (a C_3 plant) to a carbon-dioxide-rich atmosphere more like that in which they evolved, he found that the bacteria living in the nodules fixed more nitrogen in a week than they would have fixed during a whole three months normal growing season. Hardy calls the ability of nitrogenase and photosynthetic compounds to mistake oxygen for the nitrogen or carbon dioxide they need "an evolutionary disaster"—at least for people. So far, no C_4 legumes have been discovered.

Fortunately, new techniques of manipulating cell cultures taken from higher plants may someday make possible the improvement of photosynthesis and development of a more efficient support system for nitrogen fixation. □