Journal of Industrial Microbiology & Biotechnology (1999) 22, 381–393

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# Advances in Biological Nitrogen Fixation*

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Recent work on the biochemistry of $N_2$ fixation including evidence supporting the current concept of electron transfer in $N_2$ fixation is discussed. MgATP combines specifically with the Fe protein of nitrogenase, and lowers its potential sufficiently (near -400 mv) so that it can reduce the MoFe protein of nitrogenase. The MoFe protein serves as an electron sink to reduce all nitrogenase substrates. The potential loss of energy via release of $H_2$ is discussed. There is a marked homology among the nitrogenase components from diverse sources, but the components from *Clostridium pasteurianum* often do not form catalytically active complexes with components from other organisms. The Fe protein from *C. pasteurianum* and the MoFe protein from *Azotobacter vinelandii* form a tightly binding, catalytically inactive complex.

## INTRODUCTION

It is a particular honor to be associated with the name of Charles Thom who is certainly one of the outstanding microbiologists of the century. In appreciation of this award, I would like to thank the Society for this recognition and to accept the award on behalf of my mentor Perry W. Wilson and all the students who have been my colleagues in research.

Until about a decade ago, biological nitrogen fixation was an area which seemed to have little appeal. In 1964, Bill Bulen and I organized a conference and invited most of the people who were actively working on the biochemistry of nitrogen fixation. The conference attracted about a dozen people to Butternut Lake in northern Wisconsin. It was very successful, as small conferences frequently are, and following this we had conferences at Sage Hen (the Sierra Mountains biological station of the University of California, Davis), Sanibel Island in Florida, and at the "White House" in the Isle of Thorns, near Sussex University in England. There were about two dozen people at the 1968 meeting at the Isle of Thorns, and that was representative of interest in the subject. In 1974, we had a fifth meeting that was labeled the First International Congress on Nitrogen Fixation at Pullman, Washington, and there were about 150 people present. Last year at Salamanca, Spain, the Second International Congress drew about 250 people, a number approx. 20 times the attendance at Butternut Lake. The Third International Congress will be held at Madison, Wisconsin, in June 1978. Why is there this sudden interest in biological nitrogen fixation?

Today, there is an increasing realization that we are facing an energy crisis, and many people are aware that the usual feedstock for chemical nitrogen fixation is natural gas and that the end of natural gas supplies is in sight. There also is a food crisis, and it is apparent that if people are to be fed, farmers must have adequate nitrogenous

*Address given upon receipt of the 1977 Charles Thom Award.

fertilizers to support plant growth. The energy crisis will continue to increase the cost of chemically fixed nitrogen, so agriculturalists must turn once more to the biological processes for fixed nitrogen.

The world has a production capacity of about 60 million metric tons of anhydrous ammonia a year, and about 50 million metric tons are produced. Of this, over 40 million metric tons are used as fertilizer worldwide. The biological process appears to fix two to three times the amount produced by chemical fixation, so the plants and bacteria support a very large operation. People also are interested in biological nitrogen fixation as a relatively nonpolluting source of fixed nitrogen. Fertilizer nitrogen often gets into the ground water, primarily in the form of nitrate, and some ground waters have nitrate concn approaching levels that are toxic to infants. The development of new methods, such as the acetylene reduction method, has facilitated laboratory and field work and has attracted research in areas that were difficult to tackle in the past.

Quite apart from practical considerations, biological nitrogen fixation is inherently interesting, and there has been sufficient progress to attract investigators to many new problems. The enzyme system has been purified, so that progress can be made in studies of the mechanism of the process. It now is possible to apply genetic techniques to alter the nitrogen fixation system, and this may yield improvements in the process. Today, free-living rhizobia can be induced to fix nitrogen apart from the host plant, and this has enhanced the possibilities for genetic work on these organisms. Recently, it has been shown that the lectins may have a role in the specificity of infection in leguminous plants by specific strains of rhizobia.

Another area of interest has been opened by the realization that the *Anabaena azollae* association, that is, the association between a simple water fern and a blue-green alga, constitutes a system that has been used successfully for $N_2$ fixation in the Far East for many years and may have great potential for application elsewhere. At the 1977 meeting of the American Society of Plant Physiologists, it was announced that an actinomycete that infects nonleguminous plants has been isolated, cultured, and transferred back to aseptic plants to reinfect them. Its reisolation has completed the entire infectivity cycle; i.e., Koch's postulates have been satisfied. This work of Torrey et al. (1977) marks a real breakthrough in the study of these organisms, and it should promote interest in studying the nonlegumes and their associated actinomycetes. The isolate was from *Comptonia peregrina*, the "sweet fern."

Another factor that has attracted people is the increased specific funding for research on biological nitrogen fixation. Each recent report on the world food crisis and what must be done about it has stressed the importance of photosynthesis and biological nitrogen fixation as two key research areas. In the bill which is currently before the U.S. Congress (designed to support competitive grants for research in the agricultural sciences), four different plant areas are targeted for attention: photosynthesis, nitrogen fixation, plant stress, and genetic engineering of plants. This again emphasizes concern for advancing our knowledge of biological nitrogen fixation.

## Discussion

Having presented some background information, I should now like to consider recent progress on the mechanism of biological nitrogen fixation. A working scheme for electron transfer in biological nitrogen fixation is shown in Fig. 1. It starts with the transfer

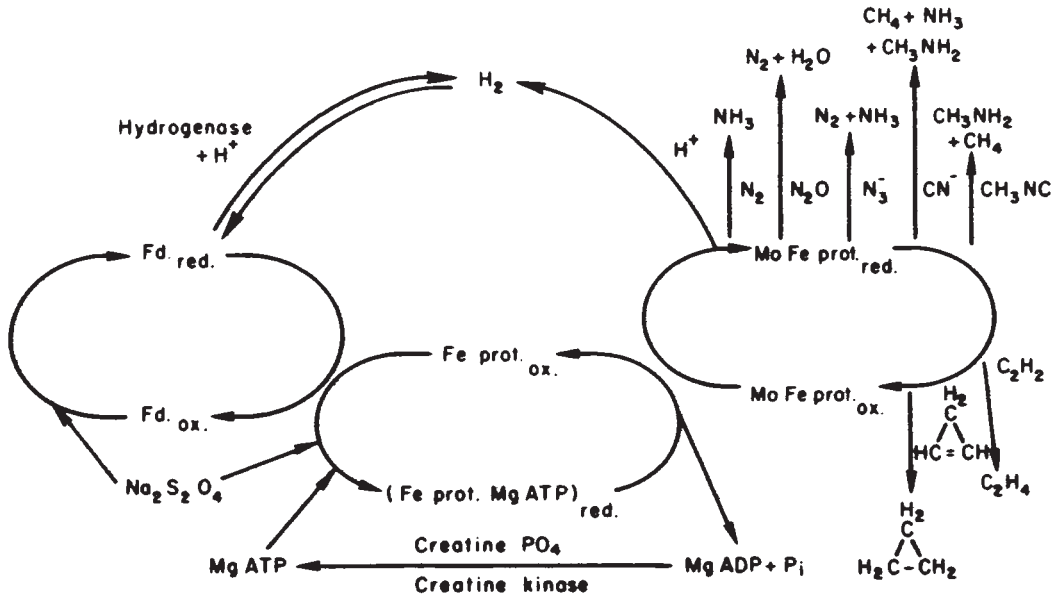


FIG. 1. Working scheme for electron transfer in biological $N_2$ fixation.

of electrons to the iron-protein (Fe protein). In biological nitrogen fixation, one must have an Fe protein and a molybdenum-iron protein (MoFe protein) to drive the reaction. The Fe protein can be reduced by sodium dithionite (the reductant normally used in the laboratory), or it can be reduced by the usual physiological donor, reduced ferredoxin (Fd.). The Fe protein can bind magnesium ATP specifically; the MoFe protein does not bind ATP. The Fe protein when it binds MgATP gains the capacity to transfer electrons to the MoFe protein to place it in the fully reduced state. The MoFe protein in turn can transfer the electrons to nitrogen, $N_2O$, and other substrates that will be noted later. Information on electron transfer from the ferredoxin through the Fe protein to the MoFe protein and thence to the substrates has been derived primarily from studies of electron paramagnetic resonance (EPR) changes in these various components of nitrogenase.

Some of the properties of the MoFe proteins are given in Table 1. The MoFe protein has a molecular weight of about 220,000. It can be dissociated into four subunits, and it is easy to demonstrate that most MoFe proteins have two types of subunits; one of the subunits from *Clostridium pasteurianum* has a molecular weight of about 60,000 and the other about 51,000. There has been a controversy as to whether *Azotobacter vinelandii* MoFe protein has four identical subunits; however, recent evidence suggests that this MoFe protein also can be separated into two different components. The MoFe protein contains two atoms of molybdenum per molecule of 220,000 daltons. It has somewhere between 22 and 32 iron atoms and an equal amount of acid-labile sulfur. This is an acidic protein, with an isoelectric point of about 4.95. The only component of nitrogenase that has been crystallized to date is the MoFe protein from *A. vinelandii*. Others have been obtained in high purity, but they have refused to crystallize; the Fe protein never has been crystallized from any source. The properties of the Fe proteins

Table 1. *Properties of the MoFe and Fe proteins of nitrogenases from* C. pasteurianum *and* A. vinelandii

| | MoFe protein | | Fe protein | |
|---|---|---|---|---|
| | C. pasteurianum | A. vinelandii | C. pasteurianum | A. vinelandii |
| Molecular weight | 220,000 | 216,000 | 56,000 | 64,000 |
| Subunit composition | 2 types | 2 types | 1 type | 1 type |
| | 60,000 | 56,000 | 27,500 | 33,000 |
| | 51,000 | | | |
| Metal and sulfide content (moles/mole protein) | | | | |
| Mo | 1.95 | 1.54 | 0.0 | 0.0 |
| Fe | 22–24 | 24 | 4.05 | 3.45 |
| S | 22–24 | 20 | 4.0 | 2.85 |
| EPR spectra, *g* | 4.29 | 4.3 | 2.04 | 2.05 |
| | 3.77 | 3.65 | 1.94 | 1.94 |
| | 2.01 | 2.01 | 1.88 | 1.88 |
| Specific activities, nanomoles substrate reduced/(min × mg MoFe or Fe protein) | | | | |
| $N_2$ | 620 | 316 | 755 | 470 |
| $H_2$ | | 1488 | | |
| $C_2H_2$ | 2500 | 1638 | 3100 | 1815 |

from various sources also are shown in Table 1. The Fe protein from *C. pasteurianum* has a molecular weight of about 56,000; this protein has two equal subunits of 27,500 molecular weight. The Fe protein has four iron atoms and four acid-labile sulfur atoms. These appear to be arrayed in the molecule in the same fashion as the iron and sulfur atoms of ferredoxin, that is, there is a cubicle structure with iron and sulfur alternating at the corners of that structure. The typical bacterial ferredoxins have either four iron atoms and four acid labile sulfur atoms, or two such centers to give eight iron and eight acid-labile sulfur atoms per molecule.

The Fe protein of nitrogenase is more acidic than the MoFe protein and has an isoelectric point near 4.6. Although there is some variation in molecular weights of Fe proteins from various sources, the properties of all are similar.

The EPR signal of the iron protein in the absence of MgATP is shown in Fig. 2; note that the EPR signals always are given in the form of derivative spectra. When one adds MgATP to the Fe protein there is a marked shift in the EPR spectrum at pH 8.0. This is indicative that the MgATP binds specifically to the Fe protein. Other evidence also indicates the specificity of the binding to the Fe protein. For example, if the Fe protein plus $C^{14}$-labeled MgATP are mixed with Sephadex G25, the Fe protein binds the $C^{14}$-labeled MgATP and excludes it from the Sephadex. If one does exactly the same thing with the MoFe protein, no exclusion from the Sephadex is encountered and the $^{14}C$ concn in the supernatant is not increased. Mixing the Fe protein and the MoFe protein gives exactly the same exclusion result as the Fe protein alone. Hence, there is a specificity of binding of the MgATP to the Fe protein, and the binding is not influenced by the MoFe protein. When the Fe protein binds MgATP, there is a change in its EPR spectrum (Fig. 2), and there also is a marked shift in the potential

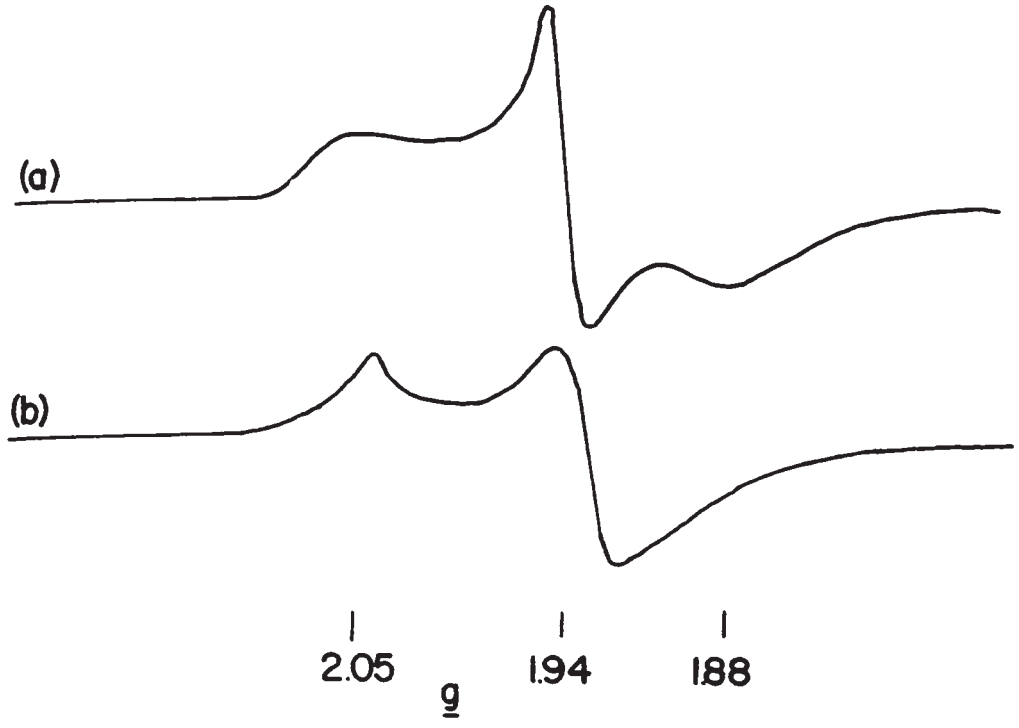


FIG. 2. EPR spectra of the Fe protein of *C. pasteurianum* nitrogenase; (a) without MgATP and (b) with MgATP. Redrawn from the data of Orme-Johnson et al. (1972); see this reference for experimental details.

of the Fe protein. The Fe protein without MgATP has a potential of about -300 mV, but with MgATP the potential is dropped to about -400 mV; i.e., it is very close to the potential of the hydrogen electrode. With this lowering of the potential to around -400 mV, the MgATP-Fe protein complex acquires the capability for reducing the MoFe protein. Apparently, this is the reason that two proteins must work together in nitrogenase; the MgATP-Fe protein complex is unique in its ability to reduce the MoFe protein. The only other way that the MoFe protein has been reduced is electrochemically, and then it is enzymatically inactive. The function of the Fe protein is to reduce the MoFe protein, which in turn can serve as the electron donor to a variety of substrates.

The EPR spectrum of the MoFe protein is shown in Fig. 3. It has a very characteristic pattern with peaks at $g = 4.29$ and 3.77. The spectrum of the Fe protein in the absence of ATP shows a high peak in the $g = 2.0$ region. If the Fe protein and the MoFe protein are mixed in the absence of ATP, the spectrum is simply additive. However, Fig. 4 shows the response in the presence of MgATP; there is a suppression of the MoFe protein EPR peak as the MoFe protein is almost completely reduced by the Fe protein-MgATP. In the steady state the MoFe protein is about 90–95% reduced, and this leaves a small residual signal. The MoFe protein can exist in three oxidation-reduction states. When the MoFe protein is isolated under anaerobic conditions, it has the characteristic EPR signals with $g$ values of 4.29 and 3.77. In the completely reduced state (sometimes called the super-reduced state), this signal is abolished. The partially reduced form can be completely oxidized by titration with ferricyanide; both the com-

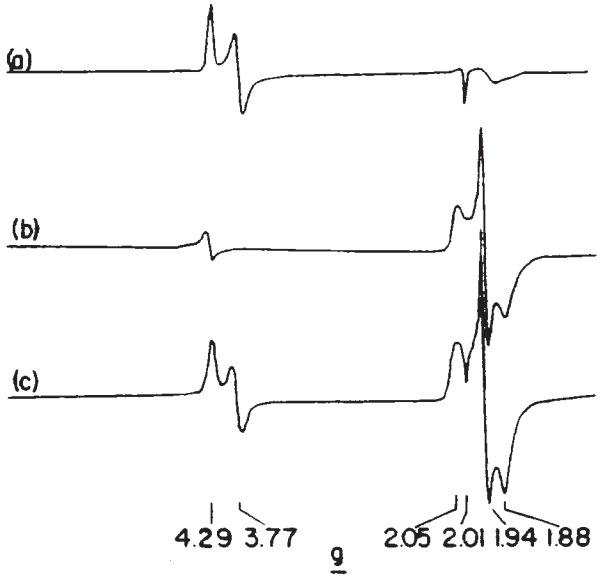


FIG. 3. EPR spectra of the components of *C. pasteurianum* nitrogenase in the absence of MgATP; (a) 10 mg MoFe protein/ml; (b) 10 mg Fe protein/ml; (c) 10 mg MoFe protein plus 10 mg Fe protein/ml. Redrawn from the data of Orme-Johnson et al. (1972); see this reference for experimental details.

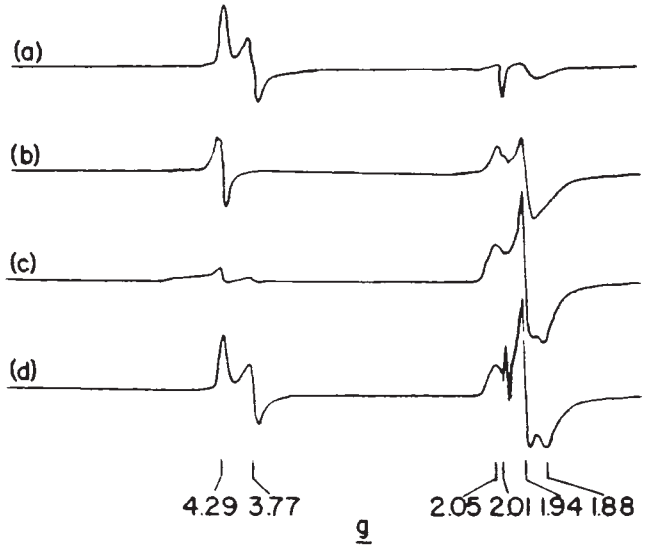


FIG. 4. EPR spectra of the components of *C. pasteurianum* nitrogenase in the presence of MgATP. An ATP-generating system was included in each sample. (a) 10 mg of MoFe protein/ml plus 5 mM $Na_2S_2O_4$; (b) 10 mg Fe protein/ml plus 5 mM $Na_2S_2O_4$; (c) 10 mg of each protein/ml plus 5 mM $Na_2S_2O_4$, frozen 45 sec after mixing; (d) same as (c) except that 0.5 mM $Na_2S_2O_4$ was initially present, together with 40 $\mu$M methylviologen, and incubation after mixing and before freezing was for 90 sec; after 90 sec the bluish cast of reduced methylviologen no longer was visible and the MoFe protein likewise had become reoxidized to its original partially reduced state. Redrawn from the data of Orme-Johnson et al. (1972); see this reference for experimental details.

pletely oxidized and the completely reduced states are EPR silent, and the partially reduced state has the characterisitc EPR signal. Physiological electron transfer involves conversions between the partially reduced and the fully reduced states, and there is no evidence that the conversion from the partially reduced to the fully oxidized state is physiologically significant. Reactions such as these are driven experimentally with sodium dithionite as the reductant; if they are allowed to run until the dithionite is exhausted, then the EPR signal of the partially reduced MoFe protein reappears in its original state. Apparently, there is a shuttle in which the Fe protein is reduced by ferredoxin or dithionite, it binds MgATP, its potential is lowered, and it transfers electrons to the MoFe protein; theMoFe protein in turn transfers the electrons to the substrate and then returns to the partially reduced state.

What is the stoichiometry of ATP use? The nitrogenase system reconstituted from purified components and tested under the best conditions requires 12 MgATP per $N_2$ reduced to 2 $NH_3$; that is, four ATP molecules are required per pair of electrons transferred. The reduction of $N_2$ to 2 $NH_3$ involves the transfer of six electrons. This stoichiometry is not absolute, as the amount of ATP required is highly dependent upon the ratio of the Fe protein and the MoFe protein. If there is minimal Fe protein and an excess of the MoFe protein, the system is inefficient and requires higher amounts of MgATP; apparently, the system is partially uncoupled under these circumstances.

Returning now to Fig. 1, it will be recalled that electrons were transferred from ferredoxin or dithionite to the Fe protein and thence to the MoFe protein, and the reduced MoFe protein served as an electron sink. Nitrogenase is a versatile enzyme and it not only reduces $N_2$ to ammonia but it also reduces $N_2O$ to $N_2$ and water, acetylene to ethylene, methyl isocyanide to methylamine plus methane, cyanide to methane, ammonia and methylamine, azide to $N_2$ and ammonia, cyclopropene to cyclopropane, and protons to $H_2$. So a variety of reactions are catalyzed by the system. If all these compounds get electrons from the same sink, and if one supplies more than one substrate at a time, a competition for the electrons should be anticipated. The $N_2$ always is in competition with the protons that are present in an aqueous medium, and there always will be some evolution of $H_2$ by the nitrogenase system.

There sometimes is confusion about interaction between nitrogen and hydrogen metabolism, and this is not surprising because $H_2$ can perform in a variety of ways in $N_2$-fixing organisms. Hydrogen is a specific competitive inhibitor of nitrogenase, in addition to being generated in the system from protons. Production of hydrogen by way of nitrogenase has an absolute requirement for ATP, and the reaction is not blocked by carbon monoxide. On the other hand, many of the nitrogen-fixing organisms have a hydrogenase that can release hydrogen via a reaction that does not require ATP but is sensitive to carbon monoxide. The ATP requirement and carbon monoxide sensitivity can be used to distinguish these pathways. If a hydrogenase is present together with ferredoxin, molecular hydrogen can be used to drive the nitrogenase reaction. Thus, $H_2$ can be an inhibitor of nitrogenase, or it can support nitrogenase by transferring electrons by way of hydrogenase to ferredoxin to drive the reduction of various substrates. It is not surprising that $H_2$ metabolism as observed in intact organisms was very confusing.

Recently, Ooyama (1977) demonstrated that the autotrophic hydrogen bacteria can catalyze the oxy-hydrogen reaction and they can couple the reaction to drive nitrogen fixation. So the hydrogen bacteria use a specific inhibitor of nitrogenase as the energy

source to drive $N_2$ fixation. Nitrogenase and hydrogenase can operate entirely independently, and they have different properties; their evolution of hydrogen can be distinguished on the basis of need for ATP and inhibition by carbon monoxide. Although hydrogen evolution catalyzed by nitrogenase is not blocked by carbon monoxide, all the other nitrogenase reactions are sensitive to carbon monoxide. Somehow, the reduction of protons to $H_2$ escapes the carbon monoxide block.

An intriguing question is whether it is possible to cross a nitrogenase component from one organism with the complementary component from another organism and generate an active nitrogenase. Emerich (1977) tested nonhomologous nitrogenases (Fig. 5) with components from nitrogenases purified from eight different organisms: included were *Azotobacter vinelandii, Klebsiella pneumoniae, Rhodospirillum rubrum, Spirillum lipoferum, Chromatium vinosum, Rhizobium japonicum, Bacillus polymyxa*, and *Clostridium pasteurianum*. The diagonal represents the homologous crosses, e.g., the *A. vinelandii* MoFe protein vs. the *A. vinelandii* Fe protein; obviously such homologous crosses are active. There is a potential for 56 nonhomologous crosses, and Emerich tested 55 of these. Of the 55 nonhomologous crosses tested, 80% of them gave a positive nitrogenase reaction. This indicates a tremendous homology among the components of the nitrogenases from various microbial sources. The negative crosses involved *C. pasteuranium*, which is a strictly anaerobic organism. It is interesting to note that *K. pneumoniae* was a universal crosser in both directions, i.e., everything tested crossed actively with *K. pneumoniae*. There has been a marked homology in the evolutionary development of the nitrogenases in a wide variety of microorganisms.

If a fixed amount of *C. pasteurianum* MoFe protein is titrated with increasing amounts of the *C. pasteurianum* Fe protein and nitrogenase activity is measured, one gets a characteristic titration curve activity that reaches a constant high value. If a fixed amount of the Fe protein is titrated with increasing amounts of the MoFe protein, activity increases to a peak value and then decreases as more MoFe protein is added.

Source of MoFe Protein

| Source of Fe Protein | Av | Kp | Rr | S1 | Cv | Rj | Bp | Cp |
|---|---|---|---|---|---|---|---|---|
| Av | | + | + | + | + | + | + | − |
| Kp | + | | + | + | + | + | + | + |
| Rr | + | + | | + | + | + | + | − |
| S1 | + | + | + | | + | + | + | − |
| Cv | + | + | + | + | | | + | − |
| Rj | + | + | + | + | + | | + | − |
| Bp | + | + | + | + | + | + | | + |
| Cp | − | + | − | − | − | − | + | |

FIG. 5. Nitrogenase activity generated by crossing nonhomologous nitrogenase proteins. The diagonal represents homologous crosses. Data are from Emerich (1977). Av = *Azotobacter vinelandii*, Kp = *Klebsiella pneumoniae*, Rr = *Rhodospirillum rubrum*, S1 = *Spirillum lipoferum*, Cv = *Chromatium vinosum*, Rj = *Rhizobium japonicum* (bacteroids), Bp = *Bacillus polymyxa*, Cp = *Clostridium pasteurianum*.

In this region of inhibition by high levels of MoFe protein, there is an abnormally high requirement for MgATP (Ljones and Burris 1972).

Nonhomologous crosses may be examined further with complete nitrogenase from *A. vinelandii* plus one of the proteins from *C. pasteurianum* (components of *C. pasteurianum* and *A. vinelandii* when crossed do not form active nitrogenases). For example, adding the MoFe protein from *C. pasteurianum* to *A. vinelandii* nitrogenase is not inhibitory, but the Fe protein from *C. pasteurianum* is strongly inhibitory (Fig. 6). As increasing amounts of the Fe protein from *C. pasteurianum* are added, there is a precipitious drop in nitrogenase activity. Analysis shows that the first molecule of the Fe protein added does not inhibit drastically, but the second molecule is more strongly inhibitory and leaves very little residual nitrogenase activity. A small amount of the active complex always is formed, so the reaction rate never drops to zero. What occurs is that the Fe protein from *C. pasteurianum* forms an intensely tight-binding complex with the MoFe protein from *A. vinelandii*. As one might predict, an active nitrogenase from *C. pasteurianum* is inhibited by the MoFe protein from *A. vinelandii*, whereas it is not inhibited by the Fe protein from *A. vinelandii*. The tight binding between the

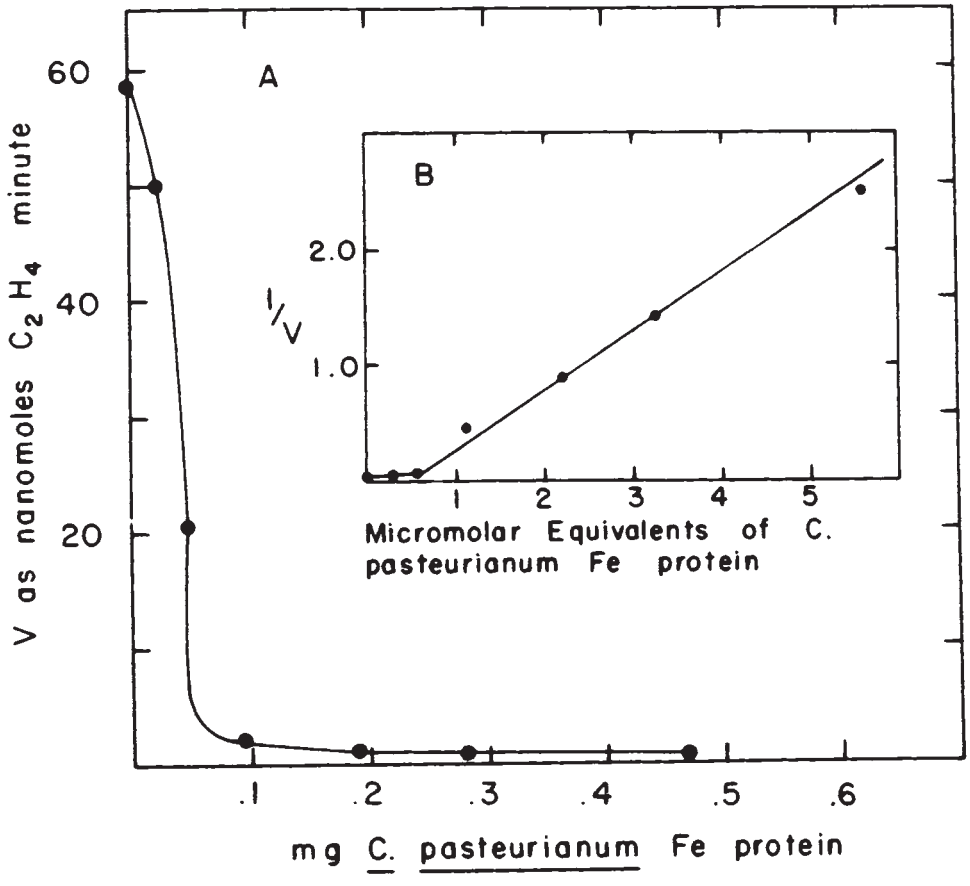


FIG. 6. (A) Activity of *A. vinelandii* nitrogenase as inhibited by *C. pasteurianum* Fe protein. At each inhibitor concn the amount of ethylene produced was measured at 5, 10, and 15 min. (B) Reciprocal of velocity of *A. vinelandii* enzymatic activity vs. concn of *C. pasteurianum* Fe protein (as inhibitor); data are replotted from (A). Data are from Emerich and Burris (1976).

Fe protein of *C. pasteurianum* and the MoFe protein of the *A. vinelandii* gives the inhibitory response in each case. The affinity is about 10–100 times as great as the affinity between the homologous Fe and MoFe proteins. It becomes apparent that the mere ability to form a complex between Fe and MoFe proteins is not adequate to confer enzymatic activity.

Do these inhibited three-way crosses still bind MgATP? It turns out that MgATP is bound in normal fashion. Titration indicates that one molecule of the MoFe protein binds two molecules of the Fe protein. A unit of the tightly bound complex binds four MgATP. As one Fe protein binds two MgATP molecules and two Fe proteins form the complex, one would anticipate that a unit of the complex should bind four MgATP.

The response of the Fe protein to the addition of MgATP is shown in Fig. 7. Initially, the added chelating agent, bathophenanthroline disulfonate, reacts with the small amount of contaminating free iron, and this gives a small increase in absorbance at 535 nm as the colored Fe chelate forms. Then when MgATP is added to the system, there is a very rapid rise in the color. When the MgATP is bound to the Fe protein, the $Fe_4S_4$ center of the Fe protein is exposed so that the chelating agent can react with the Fe. This occurs simultaneously with the production of the low oxidation-reduction potential of the Fe protein-MgATP complex. Apparently, there is a drastic change in the protein conformation to make the $Fe_4S_4$ center accessible to the chelating agent. The complex between the Fe protein of *C. pasteurianum* and the MoFe protein from

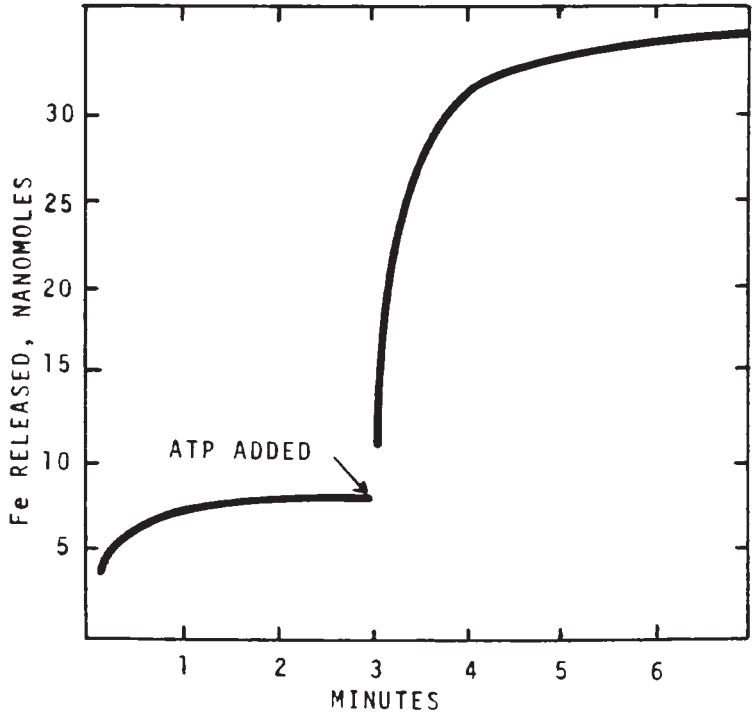


FIG. 7. Record from a strip-chart recorder of the change in absorbance at 535 nm as bathophenanthroline disulfonate chelates the Fe from the Fe protein of *C. pasteurianum* when MgATP is added to expose the $Fe_4S_4$ center of the protein. Figure has been redrawn from data presented by Ljones and Burris (*Biochem. Biophys. Res. Comm.*, in press).

*A. vinelandii* blocks the Fe protein incompletely, as it still can bind MgATP, but the $Fe_4S_4$ center is not exposed as with normal nitrogenase.

The hydrogenase reaction and hydrogen metabolism in the nitrogen-fixing system were described earlier, and the question arises whether or not hydrogen metabolism can be coupled to utilize some of the hydrogen to drive nitrogen fixation. There always is a loss of hydrogen from the nitrogenase reaction. To recapitulate, hydrogen can be evolved by nitrogenase in a reaction that is driven by MgATP and is insensitive to carbon monoxide. Nitrogenase-bearing organisms frequently carry a hydrogenase that can generate hydrogen without the mediation of MgATP; this system is sensitive to carbon monoxide. The presence of hydrogenase opens the possibility that the system can use hydrogen evolved by nitrogenase and couple the energy derived from it to support ATP formation.

Peterson (1976) has been working with the hydrogen uptake system in isolated heterocysts from blue-green algae. The heterocysts contain nitrogenase, and so they will release hydrogen through the nitrogenase reaction. In addition, they have a hydrogen uptake reaction; they can mediate the oxy-hydrogen reaction, with oxygen serving as a highly active oxidant. Not only do isolated heterocysts oxidize hydrogen to water in the oxy-hydrogen reaction, but Peterson found that they can couple the reaction to ATP formation.

Schubert and Evans (1976) observed marked variations in the amount of hydrogen evolved from different $N_2$-fixing root nodule systems. This could not be attributed to any inherent difference in the nitrogenases, but it was associated with the presence or absence of hydrogenase to incorporate the hydrogen. All nitrogenase systems release hydrogen; in some systems it is dissipated as the gas, but in other systems it is recycled. It is reassuring that it has been possible to demonstrate ATP formation in some systems. This could explain why nodules that release a minimum of hydrogen appear to be more efficient than others because they can recycle some of the hydrogen. Evans et al. (1977) have presented evidence for the recycling of $H_2$ in nodules and this property appears to be correlated with plant efficiency.

We have worked little on the control of nitrogenase systems, but Ludden (1977) has uncovered an interesting mechanism that may have potential for control in *Rhodospirillum rubrum*. There has been great difficulty in obtaining active nitrogenase preparations from *R. rubrum*, a photosynthetic bacterium. Ludden found that the Fe protein isolated in these preparations is in an inactive form. However, elution of *R. rubrum* chromatophores with 0.5 mM NaCl yields a protein fraction that will activate the Fe protein. When the Fe protein is incubated with the activating factor, the Fe protein is converted to an active form. It remains active after separation from the activating factor. Clearly, the activating factor is a catalytic agent, and Ludden has evidence that during the activation process an adenine-like unit is removed from the Fe protein. In a variety of nitrogenases we have examined, only the Fe proteins from *R. rubrum* and from *S. lipoferum* show a requirement for activation; the activating factors can be interchanged successfully between these two organisms.

## Conclusion

There has been considerable progress in elucidating the mechanism of biological nitrogen fixation, but many challenging problems remain. These inlude establishing the rate-limiting step in the nitrogen fixation process; the total function of MgATP in the overall

system; and establishing why the hydrogen evolution system escapes the carbon monoxide block whereas all the other nitrogenase-catalyzed reductions are blocked by carbon monoxide. Another puzzling thing concerning the interaction of various substrates on nitrogenase is why acetylene is a noncompetitive inhibitor of nitrogen reduction whereas $N_2$ is a competitive inhibitor of acetylene reduction? Although these and many other important problems remain to be resolved, I think that there has been real progress in the last decade, and as many additional people are moving into the area I would anticipate that the progress in the next decade will accelerate.

## Acknowledgments

I would like to acknowledge the participation in the research reported here of my current group of research colleagues: Stephan Albrecht, Daniel Arp, David Emerich, Robert Hageman, Jeffrey Houchins, Torbjørn Ljones, Paul Ludden, Richard Peterson, A. William Ruscoe, and David Stumpf. Our research is supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, by Public Health Service grant AI-00848 from the National Institute of Allergy and Infectious Diseases, by National Science Foundation grant PCM-74-17604, and by a grant from the University of Wisconsin Graduate School Research Committee.

## Questions

*Dr. E. O. Stapley:* Would you care to speculate on the practical applications for increasing nitrogen supplies for agricultural uses?

*Dr. Burris:* This is a matter of research priorities, and in my opinion the legume/*Rhizobium* system is still the most important as far as practical applications are concerned. It is disturbing that in recent years the practice in almost all countries, except the United States, Brazil, and England, has been to decrease the acreage of legumes and to increase the acreage of nonlegumes. This is a response to the "Green revolution." I think we must reverse this trend and utilize legumes more effectively. It may be feasible to introduce useful new legumes as well as improving the use of established legumes. In range areas it may be helpful to exploit $N_2$ fixation by the angiospermous nonlegumes. These have great potential. There also is a great deal of interest in the association between free-living organisms and nonleguminous plants. So far, the practical increases which have been demonstrated in the field have been small, but this should not discourage us, because people have been working seriously on these associations for just a few years. The genetic engineers have exciting things to promise, but actual progress in this area probably is way down the road. Nature has had a few millions of years to develop our $N_2$-fixing systems, and I think we ought to take full advantage of them.

*Dr. R. F. Acker:* Do your remarks cover symbiotic relationships in cereal crops?

*Dr. Burris:* There is much interest in this, and the organism that has aroused the greatest interest is *S. lipoferum* (now *Azospirillum lipoferum* or *Azospirillum brazilense*.) This organism will invade roots and is a good nitrogen fixer. The big problem is that the number of bacteria found in the roots is not very large. On the other hand, there are considerably larger numbers on the surface of the root. This is a microaerophilic or-

ganism, and it fixes $N_2$ only at low partial pressures of oxygen. A serious search for the proper association of bacterium and plant cultivar may reveal an association that will work well. We have just scratched the surface of this problem. The limiting factor in the field is not the presence of free-living, $N_2$-fixing organisms, but rather an adequate energy source to support their growth. Energy is commonly the limiting factor, and this is why the legumes have a great advantage. In the leguminous root nodule the bacteria have direct access to the photosynthate translocated to the nodule. Although the energy is derived from sunlight, you do not get it free but only at the expense of overall plant growth processes. $N_2$ fixation is an extremely energy-demanding process and much of the photosynthate is expended in driving it. If a system is developed that will fix nitrogen in association with a nonleguminous plant, that plant must expend energy to fix $N_2$, and the plant's productivity may go down rather than up compared to productivity with added fertilizer nitrogen. The energy requirement must always be kept in mind; there is no way to escape it.

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