References and Notes

1. J. G. Horsfall, committee chairman, *Agricultural Production Efficiency* (National Academy of Sciences, Washington, D.C., 1975).
2. G. Heichel, *Conn. Agric. Exp. Stn. Bull. (New Haven)*, No. 739 (1973).
3. I. Zelitch, *Photosynthesis, Photorespiration, and Plant Productivity* (Academic Press, New York, 1971); *Proc. Natl. Acad. Sci. U.S.A.* **70**, 579 (1973).
4. P. S. Carlson, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 598 (1973); *Science* **180**, 1366 (1973).

4a. P. S. Carlson and J. C. Polacco, *Science* **188**, 622 (1975).

5. C. S. Yocum, L. H. Allen, E. R. Lemon, *Agron. J.* **56**, 249 (1964).
6. B. Z. Schacter, M. Gibbs, M.-L. Champigny, *Plant Physiol.* **48**, 443 (1971); M. Migniac-Maslow and M.-L. Champigny, *Biochim. Biophys. Acta* **234**, 344 (1971).
7. R. E. Anderson and R. B. Musgrave, *Proceedings of the 15th Hybrid Corn Industry-Research Conference* (American Seed Trade Association, Washington, D.C., 1960), p. 97.
8. M. D. Hatch and C. R. Slack, *Annu. Rev. Plant Physiol.* **21**, 141 (1970).
9. J. A. Bassham and M. Calvin, *The Path of Carbon in Photosynthesis* (Prentice-Hall, Englewood Cliffs, N.J., 1957).
10. J. P. Decker and M. A. Tió, *J. Agr. Univ. P.R.* **43**, 50 (1959).
11. A. A. Benson and M. Calvin, *J. Exp. Bot.* **1**, 63 (1950).
12. I. Zelitch, *J. Biol. Chem.* **233**, 1299 (1958); *ibid.* **234**, 3077 (1959).
13. ———, *Annu. Rev. Biochem.* **44**, 123 (1975).
14. ———, *Arch. Biochem. Biophys.* **163**, 367 (1974).
15. ———, *Plant Physiol.* **41**, 1623 (1966).
16. W. A. Laing, W. L. Ogren, R. H. Hageman, *ibid.* **54**, 678 (1974).
17. M. R. Badger and T. J. Andrews, *Biochem. Biophys. Res. Commun.* **60**, 204 (1974).
18. I. Zelitch, *Plant Physiol.* **51**, 299 (1973).
19. G. Bowes, W. L. Ogren, R. H. Hageman, *Biochem. Biophys. Res. Commun.* **45**, 716 (1971).
20. T. J. Andrews, G. H. Lorimer, N. E. Tolbert, *Biochemistry* **12**, 11 (1973); G. H. Lorimer, T. J. Andrews, N. E. Tolbert, *ibid.*, p. 18.
21. D. D. Randall, N. E. Tolbert, D. Gremel, *Plant Physiol.* **48**, 480 (1971); M. W. Kerr and C. F. Gear, *Biochem. Soc. Trans.* **2**, 338 (1974).
22. T. J. Andrews, G. H. Lorimer, N. E. Tolbert, *Biochemistry* **10**, 4777 (1971).
23. J. A. Bassham and M. Kirk, *Plant Physiol.* **52**, 407 (1973).
24. Y. Shain and M. Gibbs, *ibid.* **48**, 325 (1971).
25. J. M. Robinson and M. Gibbs, *ibid.* **53**, 790 (1974).
26. H. R. Godavari, S. S. Badour, E. R. Waygood, *ibid.* **51**, 863 (1973).
27. I. Zelitch, *J. Biol. Chem.* **240**, 1869 (1965).
28. J. D. Eickenbusch and E. Beck, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **31**, 225 (1973).
29. J. D. Mahon, H. Fock, T. Höhler, D. T. Canvin, *Planta (Berl.)* **120**, 113 (1974); J. D. Mahon, H. Fock, D. T. Canvin, *ibid.*, p. 125; *ibid.*, p. 245.
30. A. Goldsworthy, *Phytochemistry* **5**, 1013 (1966).
31. I. Zelitch and P. R. Day, *Plant Physiol.* **43**, 1838 (1968).
32. D. Wilson, *J. Exp. Bot.* **23**, 517 (1972).
33. I. Zelitch and P. R. Day, *Plant Physiol.* **52**, 33 (1973).
34. C. C. Black, Jr., *Annu. Rev. Plant Physiol.* **24**, 253 (1973).
35. M. D. Hatch, *Biochem. J.* **125**, 425 (1971).
36. O. Björkman, *Photophysiology* **8**, 1 (1973).
37. N. E. Tolbert, *Annu. Rev. Plant Physiol.* **22**, 45 (1971).
38. I. Zelitch, *J. Biol. Chem.* **224**, 251 (1957).
39. N. E. Tolbert, in *Photosynthetic Mechanisms of Green Plants* (National Academy of Sciences–National Research Council, Washington, D.C., 1963).
40. I. Zelitch, *Arch. Biochem. Biophys.* **150**, 698 (1972); E. F. Elstner and A. Heupel, *Biochim. Biophys. Acta* **325**, 182 (1973).
41. B. Halliwell and V. S. Butt, *Biochem. J.* **138**, 217 (1974).
42. G. Heichel, *Photosynthetica* **5**, 93 (1971).
43. A. M. Lambowitz and C. W. Slayman, *J. Bacteriol.* **108**, 1087 (1971); ———, W. D. Bonner, Jr., *J. Biol. Chem.* **247**, 1536 (1972).
44. G. R. Schonbaum, W. D. Bonner, Jr., B. T. Storey, *Plant Physiol.* **47**, 124 (1971); J. T. Bahr and W. D. Bonner, Jr., *J. Biol. Chem.* **248**, 3441 (1973).
45. J. Bonner and S. G. Wildman, *Arch. Biochem.* **10**, 497 (1955).
46. R. J. Gautheret, *Annu. Rev. Plant Physiol.* **6**, 433 (1955).
47. A. D. Hanson and J. Edelman, *Planta (Berl.)* **102**, 11 (1972).
48. M. T. Chandler, N. T. deMarsac, Y. de-Kouchovsky, *Can. J. Bot.* **50**, 2265 (1972).
49. M. R. Davey, M. W. Fowler, H. E. Street, *Phytochemistry* **10**, 2559 (1971).
50. W. M. Laetsch and D. A. Stetler, *Am. J. Bot.* **52**, 798 (1965).
51. H. K. Lichtenthaler, V. Straub, K. H. Grumbach, *Plant Sci. Lett.* **4**, 61 (1975).
52. P. Koth, *Planta (Berl.)* **120**, 207 (1974).
53. J. J. Hanway and W. A. Russell, *Agron. J.* **61**, 947 (1969).
54. J. R. Quinby, *Crop Sci.* **3**, 288 (1963).
55. A. Tanaka, K. Kawano, J. Yamaguchi, *Int. Rice Res. Inst. (Los Banos) Tech. Bull. No. 7* (1966).
56. I. D. Singh and N. C. Stoskopf, *Agron. J.* **63**, 224 (1971).
57. V. A. Johnson, J. W. Schmidt, W. Mekasha, *ibid.* **58**, 438 (1966).
58. D. H. Wallace and H. M. Munger, *Crop Sci.* **6**, 503 (1966).
59. J. J. Hanway and C. R. Weber, *Agron. J.* **63**, 263 (1971).
60. Y. B. Samish, J. E. Pallas, Jr., G. M. Dornhoff, R. M. Shibles, *Plant Physiol.* **50**, 28 (1972).
61. N. R. Bulley and E. B. Tregunna, *Can. J. Bot.* **49**, 1277 (1971).
62. G. Hofstra and J. D. Hesketh, *Planta (Berl.)* **85**, 228 (1969).
63. B. Bravdo and D. T. Canvin, *Plant Physiol.* **51** (Suppl.), 42 (1973).
64. L. J. Ludwig and D. T. Canvin, *ibid.* **48**, 712 (1971).
65. N. Terry and A. Ulrich, *ibid.* **51**, 783 (1973).
66. T. Kisaki, *Plant Cell Physiol.* **14**, 505 (1973).
67. J. P. Decker, *J. Sol. Energy Sci. Engl.* **1**, 30 (1957).
68. ———, *Plant Physiol.* **34**, 100 (1959).
69. J. H. Troughton, *Planta (Berl.)* **100**, 87 (1971).
70. R. J. Volk and W. A. Jackson, *Plant Physiol.* **49**, 218 (1972).
71. G. Corduan, *Planta (Berl.)* **91**, 291 (1970).
72. W. M. Laetsch and H. P. Kortschak, *Plant Physiol.* **49**, 1021 (1972).
73. M. Berlyn and I. Zelitch, unpublished experiments.

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Nitrogen Fixation Research: A Key to World Food?

Investigators in a variety of disciplines are searching for new technologies for producing fixed nitrogen.

R. W. F. Hardy and U. D. Havelka

Population growth and changes in dietary habits accompanying economic growth will more than double the demand for agronomic crops during this quarter century. Among the many factors that could contribute to improving crop yields, increasing the availability of fixed nitrogen to crops is probably of the greatest importance. In the absence of alternative technologies for producing fixed nitrogen, about 200×10^6 metric tons of fertilizer nitrogen will be required annually by the end of this quarter century. In 1974, 40×10^6 tons of fertilizer nitrogen with an approximate value of $8 billion were used, as opposed to the 3.5×10^6 tons that were used annually 25 years ago.

The recent scarcity of nitrogen fertilizers, the high energy requirement for their manufacture, and, most significantly, their increased selling price have produced a tremendous interest in the search for alternative technologies. This interest has permeated even the popular literature, as documented by the following quotation from *Harper's Magazine*, by Horace Freeland Judson (*1*):

. . . a biologist working in Brazil, said she has found several kinds of tropical grasses that grow in symbiosis with N_2-fixing bacteria of a new kind in their roots. Could such bacteria be persuaded to live with one of the new high yield tropical climate grains by modifying the

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genetic makeup of bacteria or the grains? Cereals that could provide their own fertilizer are beyond doubt the biggest prize of all in the gift of the new biology —far bigger in terms of lives to be saved than even the conquest of cancer or a cow that could digest sawdust.

In this article (*2*) we assess the need for fixed nitrogen for crop production and summarize the advances in chemical and biological research on nitrogen fixation that may, in the long run, lead to the development of alternative technologies for providing fixed nitrogen. There have been many significant research advances in the chemistry and biology of nitrogen fixation during the past 15 years (*3–8*). The breadth of the objectives of current research includes the enhancement of symbiotic nitrogen fixation by grain legumes; the domestication of associative symbioses for cereals; the development of abiological nitrogen-fixing systems with high rates of nitrogen fixation under mild conditions; the extension of rhizobial infection or the development of man-made associative symbioses for cereals; and the transfer of the genetic information for nitrogen fixation to cereals. However, no advance has produced a new technology that is suitable for direct application to high-yield crop production. Until alternative technologies are in hand, it is critical that the production of fertilizer nitrogen continue to be increased throughout the world.

Need for Fixed Nitrogen for Crops

The need for increased nitrogen input and the desire, but not the necessity, to seek alternative technologies are based on a variety of factors, for example, population growth, the limited availability of additional arable land, changing dietary habits with economic growth, cereal-grain and grain-legume production trends, the current contribution of biological and abiological nitrogen fixation to crop production, and the limitations and potentials of these nitrogen input systems (*2*).

We believe that efforts to seek alternative technologies for nitrogen fixation should emphasize the abiological or biological approaches that would be applicable to cereal grains and grain legumes. Cereal grains are the major source of food, with current annual production being about 1300×10^6 tons worldwide (*9*). Of the nonbiological inputs responsible for the increased cereal yields, the increased use of fertilizer nitrogen is probably the most important single factor. Cereal-grain yield is correlated directly to the consumption of fertilizer nitrogen divided by the area under cereal cultivation for both the more developed countries and the less developed countries during the last 20 years (Fig. 1), there being no major discontinuity between the data for the more developed and less developed countries. Current cereal-grain yield and total fertilizer nitrogen use divided by the area used for cereal cultivation in the less developed countries is almost identical to that for the more developed countries about 20 years ago. These data document the key role of additional inputs of fixed nitrogen for increasing crop production.

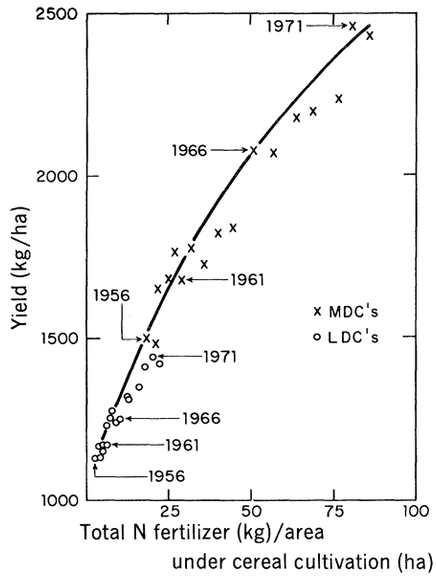

Fig. 1. The relationship between the use of nitrogenous fertilizers and the yield of cereal grains during the last 15 years for both more developed (MDC's) and less developed countries (LDC's) (*2*). Note that the total nitrogen fertilizer use is divided by the area under cultivation with cereal grain; actual rates would be about one-half since about one-half of fertilizer nitrogen is used for cereal grain production. [From Hardy (*2*), courtesy of Washington State University Press]

The grain legumes have failed to show a major yield increase in response to the application of fertilizer nitrogen. World production of grain legumes is about 115×10^6 tons, with soybeans accounting for almost one-half of this quantity, followed by peanuts, dry beans, and dry peas (*9*, *10*). The grain legumes are important sources of protein both for direct consumption by humans in several of the less developed countries and for the feeding of animals in the more developed countries. The amount of protein in the seeds of these crops ranges from 20 to 45 percent, as opposed to the cereal grains that contain 8 to 20 percent of protein. However, world production of grain legumes is only 10 percent of cereal grains and production area is about 15 percent (Fig. 2). In several of the less developed countries, the area allocated to the protein-rich, but less profitable, grain legumes has decreased with the extra area allocated to the low-protein, but more profitable, cereal grains. About one-third of the world production of grain legumes in recent years has come from soybeans grown in the United States. This crop has undergone a fivefold increase in total production in the United States in the last 25 years as a result of a fourfold increase in production area and a modest increase in yield. These data emphasize our failure to develop technology for major improvements in the yields of grain legumes. Undoubtedly, the development of a technology for increasing the nitrogen input to these crops will be a key to increasing their yields, because they require up to four times as much nitrogen per unit of yield as do cereals. For example, a corn crop yielding 100 bushels per acre consumes 150 pounds of nitrogen while a soybean crop with the same yield would consume 600 pounds of nitrogen. The ability of legumes to fix at least part of their nitrogen requirements biologically should not be ignored in seeking solutions to increased nitrogen input, and the higher protein content of nitrogen-fixing as opposed to non-nitrogen-fixing legumes may be of significance (*11*). At the same time, solutions utilizing abiological inputs should not be ignored. Economics will be the major factor that dictates the successful solution.

Potentials and Limitations of the Nitrogen Input Systems

The industrial fixation of nitrogen by the Haber-Bosch process currently provides about 40×10^6 tons of nitrogen, while the other abiological processes, lightning, combustion, and ozonization, are estimated to fix about 10, 20, and 15×10^6 tons of nitrogen, respectively, each year (*3*). The amount of nitrogen fixed biologically is difficult to estimate because of the heterogeneity of nitrogen-fixing organisms, the heterogeneity of their distribution, the heterogeneity of the environment in

which they function, and our extremely limited but improving data base. A most recent estimate of 175×10^6 tons of nitrogen fixed per annum assigns 90×10^6 tons to the amount fixed in agricultural soil (*3*). This estimate will require modification as the data base expands; it may well be too low since it was generated from a conservative approach, although it is about 75 percent greater than a previously widely quoted estimate made about a decade earlier. Regardless of the actual amount of nitrogen that is fixed biologically, the value has not changed substantially over the last 25 years and thus it is clear that biological nitrogen fixation has not contributed to the increase in crop productivity but has, at best, only maintained the unimproved base production.

The Haber-Bosch process converts nitrogen to ammonia under conditions of high temperature and pressure in highly engineered plants with capacities of 1000 tons of nitrogen per day. Both the manufacturing process and the utilization of nitrogenous fertilizers have some inherent limitations. A major part of the cost of manufacture is capital cost; a plant with a capacity of 1000 tons per day together with its associated facilities requires an investment approaching $100 million (*12*). Moreover in the less developed countries these plants operate at about one-half of their rated capacity. Transportation, storage, and application costs are high for fertilizer nitrogen in the more developed countries and there are additional problems in the less developed countries where transportation systems are often inadequate. Prior to 1973, the cost of transportation, storage, and application approached that of manufacture. On the average, crops recover and utilize only about 50 percent of applied nitrogenous fertilizer. Of the large commercial energy input used to produce a crop of corn in the United States, about one-third is required to manufacture, distribute, and apply the fertilizer nitrogen (*13*). However, the ratio of additional food energy produced by the application of 50 to 200 kg of nitrogen per hectare of corn to the energy used to produce, transport, and apply the fertilizer nitrogen is still a favorable 5 to 8 (*14*). Although it is clear that there is a need for improved alternative technologies, the need is not absolute because presumably 500 to 600 large fertilizer nitrogen plants operating at capacity could satisfy the nitrogen needs for crop production in A.D. 2000.

In contrast to the abiological process that occurs in a single type of chemical plant there is a diversity of biological nitrogen-fixing organisms and relationships (*3*). The relationships extend from asymbiotic to obligatory symbiotic, with various associative symbioses in between (Fig. 3). Asymbiotic diazotrophs (nitrogen-fixing organisms), which include some bacteria and the blue-green algae, fix nitrogen independently of other organisms. Both natural and synthetic diazotrophs are known, a strain of nitrogen-fixing *Escherichia coli* having recently been produced.

The obligatory symbiotic diazotrophs are exemplified by bacteria of the genus *Rhizobium* that occur in the root nodules of legumes and unidentified microorganisms in the nodules of some nonleguminous angiosperms. In the case of the *Rhizobium*-legume symbiosis, each of the partners is ineffective in nitrogen fixation alone in its normal environment but is effective in the symbiotic relationship. Two exceptions to this general statement were recently demonstrated: a *Rhizobium* strain was found to produce a nitrogen-fixing nodule on a nonleguminous plant, *Trema canabina* (*15*); and simple factors derived from nonleguminous as well as leguminous plant cells appeared to enable some free-living *Rhizobium* strains to express a low level of nitrogen-fixing activity (*16*). The *Rhizobium*-legume symbiosis is estimated to contribute 40×10^6 tons of nitrogen annually to grain legumes as well as a major part of the 40×10^6 tons of nitrogen fixed in permanent meadows. In the other naturally occurring obligatory symbioses, various nonleguminous angiosperms and a microorganism presumed to be an actinomycete form a symbiotic association that is important to forest crops but is not of major significance to agronomic crop production.

In associative symbiotic relationships, one of the two partners is an asymbiotic diazotroph. The nondiazotroph may provide an environment that is favorable for nitrogen fixation by the diazotroph. The importance of these associative symbioses is only currently being recognized and they

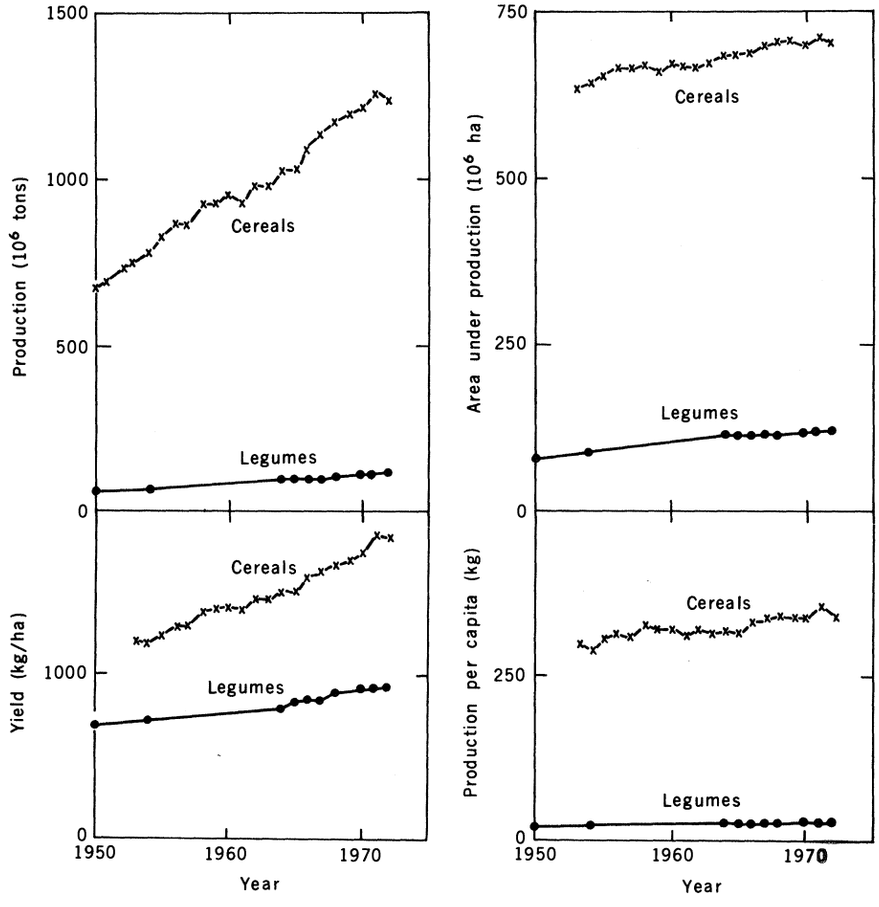

Fig. 2. Comparison of trends in world production of cereal grains and grain legumes, production area, yield, and production per capita (*2*, *9*). [From Hardy (*2*), courtesy of Washington State University Press]

may well contribute a major amount of fixed nitrogen to crop production.

Which types of nitrogen-fixing relationships should we attempt to develop for increasing nitrogen input to crops? In general, those relationships in which the site of nitrogen fixation is located on or in the plant will permit direct and thereby efficient coupling of the fixed nitrogen to the plant in amounts that parallel the changing needs of the plant throughout its complete growth cycle. Any of the obligatory and associative symbioses in which the crop plant is one of the symbiotic partners would meet these requirements, as would crop plants containing the genetic information for nitrogen fixation. Abiological systems with improved coupling of nitrogen to the plant might also be suitable for development. Free-living bacteria and blue-green algae do not meet the requirements nor do symbioses of bacteria or algae with noncrop plants.

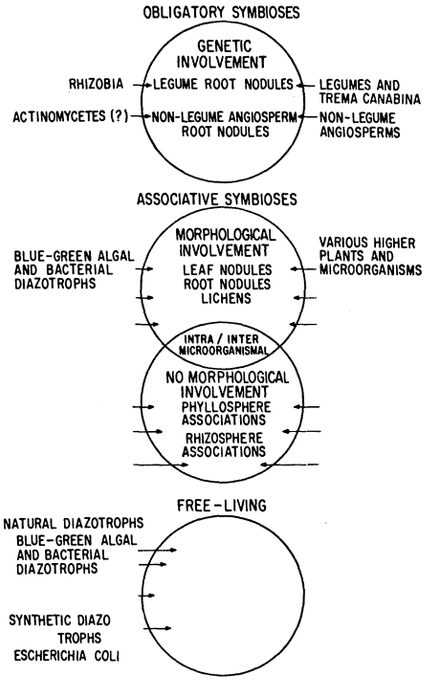

Fig. 3. Biological nitrogen-fixing relationships. [From Burns and Hardy (*3*), courtesy of Springer-Verlag, New York]

Advances in the Chemistry of Nitrogen Fixation

The molecular era of nitrogen-fixation research was initiated by three independent discoveries in three different countries, the United States, the Soviet Union, and Canada. One discovery concerned biological nitrogen fixation while the other two were purely abiological.

Nitrogen fixation by nitrogenase. In 1960 a functional nitrogen-fixing enzyme, nitrogenase, was extracted from the anaerobic bacterium, *Clostridium pasteurianum* (*17*). The addition of an inordinately large amount of pyruvate to the incubation medium was the key to this scientific achievement. Nitrogenase, which may constitute up to 5 percent of the cellular protein, has now been isolated from most other physiological classes of nitrogen-fixing organisms including aerobic bacteria, facultative anaerobic bacteria, photosynthetic bacteria, blue-green algae, and legume symbionts, but not from nonleguminous angiosperm symbionts. Nitrogenases from three or four different sources representing different physiological types have been carefully characterized (*18*). The results suggest commonality among nitrogenases with only small differences, thereby justifying a unified description of a single nitrogenase (*4*).

Nitrogenase can be fractionated into two components, one that contains molybdenum and iron and is designated Mo-Fe protein, and another that contains iron and is designated Fe protein (Fig. 4). The Mo-Fe protein has been crystallized (*19*), but the x-ray structure has not been reported. This protein is composed of four subunits arranged to form a parallelepiped as observed in electron micrographs; it contains two molybdenum and 24 to 32 iron and sulfide atoms per 220,000 daltons. A molybdenum-containing prosthetic group common to both nitrate reductase and nitrogenase has been suggested (*20*) and there are some genetic and biochemical data in support of this concept. The Fe protein is composed of two identical ellipsoidal subunits; it contains four iron and four sulfur atoms per 60,000 daltons. The chemical and physical characteristics of each protein have been determined. The electron spin resonance (ESR) spectrum of the Mo-Fe protein is unique with resonances at g values of 2.01, 3.67, and 4.3 attributed to some of the iron atoms. This spectrum has proved useful for studies of mechanism and for physiological studies (*21*). The ESR spectrum of the Fe protein is not unique but is similar to that observed for ferredoxins. Both components are essential for nitrogenase activity with a ratio of one or two Fe proteins for each Mo-Fe protein. In several but not all tested cases, the Mo-Fe protein isolated from one organism can be recombined with the Fe protein isolated from another organism to produce a functional nitrogenase, further supporting the similarity of nitrogenases from different sources.

Ammonia is the product of biological nitrogen fixation with there being no evidence for enzyme-free intermediates; ammonia is not an inhibitor of the reaction. Both adenosine triphosphate (ATP) and a suitable reductant (*22*) are essential for nitrogenase activity with the requirement of four ATP molecules for each two electrons transferred by nitrogenase (*23*). This unusually large energy requirement for nitrogen fixation is surprising because the overall reaction is energy yielding:

$$3H_2 + N_2 \rightarrow 2NH_3;\ \Delta F = -7.95 \text{ kcal/mole}$$

Unfortunately, the enzymatic nitrogen-fixing reaction is extremely inefficient and demonstrates that the evolutionary process has been no more conserving of energy than has man in his development of abiological nitrogen-fixing processes. Ferredoxins or flavodoxins that have redox potentials in the vicinity of the hydrogen electrode are the only known physiological electron-transferring agents that couple to nitrogenase, while hydrosulfite has been useful for studies in vitro (*24*); natural electron donors have been identified in only a few organisms such as *Clostridium* which utilizes pyruvate, while the donor in the agronomically important legume microsymbionts can only be suggested to be reduced nicotinamide adenine dinucleotide phosphate which supports a very low rate of nitrogen fixation by *Rhizobium* nitrogenase. Additional definition of this ancillary part of the nitrogen-fixation system is needed.

The nitrogen-fixing enzyme has an unusual versatility with respect to its ability to reduce a wide variety of substrates (*25, 26*). This versatility, which has proved quite useful with nitrogenase, is turning out to be common among enzymes that utilize small molecules and are involved in vital fixing reactions. Nitrogenase was the first example to be discovered and exhibits by far the greatest versatility. More recently, ribulose bisphosphate carboxylase was found to react with oxygen as well as carbon dioxide (*27*), and in this case the versatility appears to be an evolutionary disaster for most agronomic crops. For both of these enzymes, the versatility was probably no disadvantage at the time of their origin. For nitrogenase, the versatility may still be

of no consequence because none of the alternative substrates are present in sufficient quantities in the atmosphere to compete with nitrogen. On the other hand, the ratio of carbon dioxide to oxygen in the atmosphere has decreased over the years so that oxygen now effectively competes with CO_2 for the CO_2-fixing enzyme.

Nitrogenase can be considered as a reductase for H_3O^+ and for triple or potential triple bonds formed by NN, NO, NC, and CC functions represented by N_2, N_3^-, N_2O, RCN, RNC, and RCCH to give products representing 2, 4, 6, 8, 10, 12, and 14 electron-addition products (Fig. 5). Several of the substrate reductions catalyzed by nitrogenase were novel reactions at the time of their discovery, but subsequent work with abiological systems has duplicated in a qualitative way all of the reactions. Reduction of nitrogen is competitively inhibited by hydrogen and reduction of all substrates except that H_3O^+ is inhibited by carbon monoxide while the interaction between substrates may be more complex. Formation of HD from deuterium and water occurs during the reduction of nitrogen but not of other substrates.

Physical, metal-substitution, binding, and product studies are being used to unravel the dynamics of the nitrogenase reaction. A complex of Mg and ATP binds to the Fe protein but not the Mo-Fe protein. Changes in the ESR spectrum of the Fe and Mo-Fe protein have led to the conclusion that the Mo-Fe protein is reduced by the Fe protein with involvement of Mg • ATP (*21*). The products formed by nitrogenase reconstituted from Fe and Mo-Fe proteins from different sources are dictated by the Mo-Fe protein. Although physical methods have not been useful for following molybdenum during nitrogenase reactions, the kinetics of nitrogenase isolated from cells grown on vanadium in place of molybdenum implicate molybdenum at the active site; differences and similarities between substrates and products are used to interpret the role of molybdenum in complexation and reduction (*28*). Investigations of this type coupled with comparisons of substrate analogs and inhibitors have led to the proposal of a bimetal site of molybdenum and iron bridged by sulfur. Initial complex formation of nitrogen is suggested to occur with iron, followed by subsequent reduction involving both the molybdenum and iron to form, in sequence, metal-bound diazene, hydrazine, and ammonia, with release of the ammonia (*5, 6*). Additional studies of the nitrogenase reaction are needed to define further the active site and might include nuclear magnetic resonance studies with ^{13}C-, ^{15}N-, or ^{31}P-labeled substrates as well as infrared studies with carbon monoxide.

Although structures of nitrogenase or its component proteins based on x-ray analysis have not been obtained, x-ray structures have been reported for the electron-transferring proteins, ferredoxin, and flavodoxin (*29*). Moreover, synthetic analogs of Fe_4S_4 clusters of ferredoxin have been made (*30*) and they show remarkable chemical and physical similarity to ferredoxin, although their relative insolubility in water precluded the use of the original models in biological systems. Most of the iron and sulfur of nitrogenase is probably of the ferredoxin type but, in addition, other types of iron as well as molybdenum are present. Obviously much more nitrogenase biochemistry will have to be uncovered before we will be able to describe the enzyme and its reaction at a molecular level. Such information may be useful to the chemist and biologist as well as satisfying one's curiosity about the detailed mechanism of this vital enzyme.

The inefficiency of nitrogenase and our inability to circumvent its ATP requirement essentially eliminates any possibility of direct utilization of the unmodified enzyme as a catalyst. Nevertheless, the fundamental biochemical studies have been justified already by a nonpredictable outcome that is making a most significant impact on all investigations of nitrogen fixation. The substrate versatility of nitrogenase and, specifically, the reduction of acetylene to ethylene coupled with gas chroma-

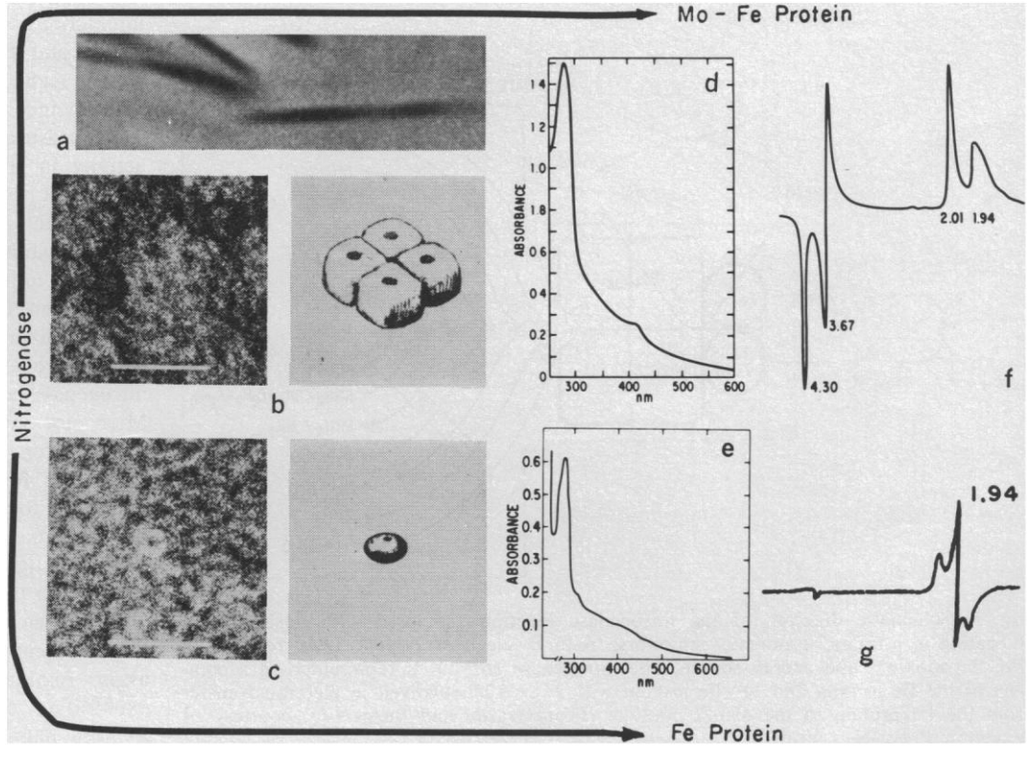

Fig. 4. Nitrogenase and the characteristics of its component proteins, Mo-Fe and Fe protein. (a) Light micrograph of crystalline *Azotobacter* Mo-Fe protein; (b and c) electron micrographs of negatively stained *Azotobacter* Mo-Fe and Fe proteins and models of each protein; (d and e) ultraviolet visible spectra of *Azotobacter* Mo-Fe protein and *Clostridium* Fe protein; and (f and g) ESR spectra of *Azotobacter* Mo-Fe protein and *Clostridium* Fe protein. [From Hardy and Burns (*4*), courtesy of Academic Press, New York]

tographic analysis was proposed (*26*), and has been broadly implemented, as a facile assay for nitrogen-fixing activity (*31*). The present thrust in genetic, physiological, and agronomic studies of nitrogen fixation would have been almost impossible without this assay.

Developments in Abiological Nitrogen Fixation

The modern abiological studies of nitrogen fixation are based on two developments in the early 1960's, each of which has led to the reduction of nitrogen to ammonia under mild conditions. One development was the serendipitous discovery of a ruthenium-nitrogen complex (*32*). This finding was expanded to include most other transition metals with the types of complexes represented by $M \cdot N_2$, $M(N_2)_2$, and $M \cdot N_2 \cdot M$. The initial hopes for reactivity of nitrogen in the isolated complexes were not fulfilled, with little or no reactivity observed, other than loss of nitrogen. Enthusiasm increased with the demonstration of the reaction of a rhenium-nitrogen complex with molybdenum to produce a ReN_2Mo species that was suggested as a model for the initial step in the nitrogenase reaction (*33*). Unfortunately, no further reaction was obtained. Finally, treatment of the bis-dinitrogen complex of either molybdenum or tungsten with hydrochloric acid produced diazene and hydrazine and, within recent months, treatments with aqueous acids such as sulfuric acid have produced ammonia (*34*). These reactions are noncatalytic and somewhat less than stoichiometric at this time.

In the other development, nitrogen was fixed to ammonia under mild conditions with the use of a soluble complex of a transition metal such as titanium, molybdenum, or iron and strong reducing agents such as alkyl metal halides, lithium aluminum hydride, or sodium naphthalide in aprotic media (*35*). Subsequently, such reactions have been demonstrated in protic media with molybdenum, vanadium, or iron complexes reduced by borohydride, hydrosulfite, or V^{2+} (*36*). One of these systems composed of molybdenum, thiol agent, and reductant duplicates all of the substrate reactions catalyzed by nitrogenase with a remarkable qualitative similarity. However, the rate of nitrogen fixation by this system is only 10^{-6} of that of nitrogenase, with the comparisons based on molybdenum content; the low turnover rate of 100 to 200 moles of nitrogen fixed per mole of nitrogenase per minute for nitrogenase emphasizes further the extremely low activity of these abiological systems. This same system has been employed in a laboratory experiment to demonstrate a method of improving the coupling of nitrogen to a crop utilizing an abiological system (*37*). Membrane fractionation of air was used to produce nitrogen-enriched air which was reduced by a membrane-enclosed catalyst, with subsequent permeation to remove the ammonia but retain the catalyst. Such a system in an irrigation stream could represent a feasible method for utilizing nitrogen-fixing catalysts that function in an aqueous environment.

A homogeneous or heterogeneous catalyst that converted molecular nitrogen to fixed nitrogen at high rates under mild conditions and in protic media might have use in the production of fixed nitrogen for agricultural crops. Such a system might improve the coupling of nitrogen to the crop; it might also help to decrease capital costs. The extremely low rates of current systems eliminate their utility until improvements of several orders of magnitude are achieved.

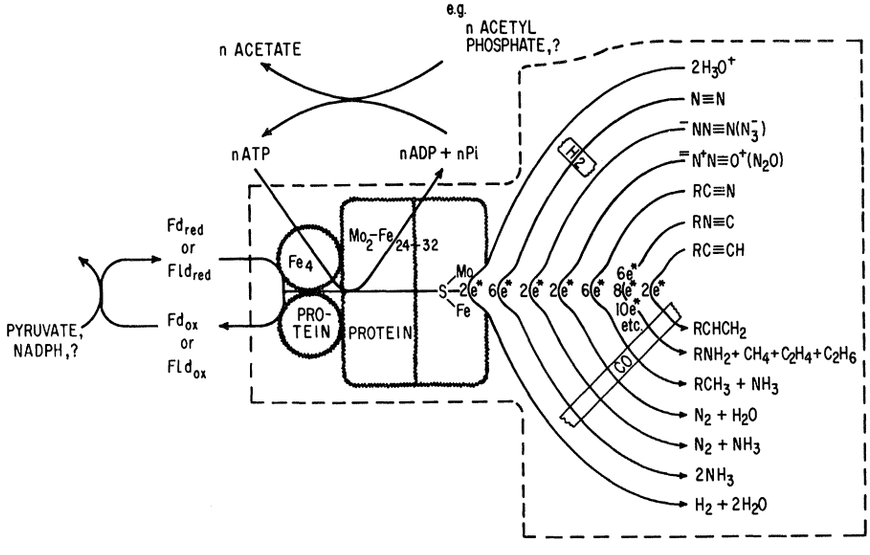

Fig. 5. Schematic diagram of the nitrogenase reaction (enclosed with dashed line) indicating substrates, products, inhibitors, natural electron donors (*Fd*, ferredoxin; *Fld*, flavodoxin), and energy source. The nitrogenase enzyme is represented as a complex of the Fe protein and Mo-Fe protein with Mg • ATP involved in electron transfer from the Fe protein to the Mo-Fe protein. [From Hardy and Burns (*4*), courtesy of Academic Press, New York]

Advances in the Biology of Nitrogen Fixation

Studies of biological nitrogen fixation prior to 1960 were restricted to the organismic level. These investigations, which provided a variety of useful information, included the identification of organisms that fix nitrogen; morphological description of infection and development of rhizobial-legume symbiosis; specificity in rhizobial-legume interactions; the discovery of leghemoglobin and the direct relationship between leghemoglobin content in the nodule and nitrogen-fixing activity; a few measurements of nitrogen-fixing activity in situ; the high ratio (about 40 : 1) of carbohydrate consumed to the amount of nitrogen fixed by free-living organisms; the indirect identification of ammonia as the first product of nitrogen fixation; the inhibition of nitrogen-fixing activity by ammonia and hydrogen; the co-occurrence of nitrogenase and hydrogenase; the low Michaelis constant (K_m) of nitrogenase for nitrogen; and the requirement for additional molybdenum and iron for nitrogen fixation as opposed to the utilization of fixed nitrogen. Investigations at the cellular level subsided in the early 1960's as attention was focused on the molecular studies. Development of the acetylene-ethylene assay reinitiated a much expanded exploration of the biological area in a more definitive manner with physi-

ological, agronomic, and genetic approaches.

Physiological research advances that are relevant to the problem of nitrogen input to crops include definition of nitrogen-fixing organisms, elucidation of systems for the protection of nitrogenase from oxygen, determination of the energy cost of biological nitrogen fixation, information on the incorporation of ammonia into organic compounds, and the promiscuity of cowpea *Rhizobium* including their expression of nitrogen fixation outside of a plant cell.

The list of organisms that fix nitrogen has been redefined with deletion of some previous examples and addition of new ones. The following correlation has emerged. Nitrogenase has only been found in prokaryotic cells including the *Rhizobium* form in root nodules of legumes (*38*); the significance, if any, of the prokaryotic restriction remains to be discovered. The genetic information for nitrogenase also appears to be contained in the rhizobial component and not the leume.

Nitrogenase is an extremely oxygen-sensitive enzyme and this limitation may be one of the major barriers to the development of new nitrogen input systems utilizing a biological approach. For example, a purified Fe protein loses one-half of its activity after less than a 1-minute exposure to air. Facultative nitrogen-fixing organisms such as *Klebsiella* or *Bacillus* do not produce nitrogenase until the environment is almost anaerobic, and nitrogen-fixing photosynthetic organisms only produce nitrogenase under anaerobic conditions. Special cellular architecture or reactions occur when nitrogenase is in aerobic organisms. *Azotobacter* is proposed to utilize elevated respiratory rates to decrease its internal oxygen pressure (pO_2) and, in addition, at elevated pO_2 nitrogenase may undergo a reversible conformational change to an inactive form for protection against oxygen (*39*). It has been speculated that hydrogen produced from H_3O^+ by nitrogenase may be coupled to hydrogenase as another oxygen-protective system. Most but not all nitrogen-fixing algae contain specialized cells called heterocysts where nitrogenase is localized; heterocysts provide a more reduced environment in which the oxygen-evolving reaction of photosynthesis does not occur (*40*). The nitrogen-fixing activity of aerobic algae and bacteria is increased under subatmospheric pO_2, and an advantage of associative symbioses for these aerobic organisms may be the provision by the higher plant symbiont of an environment with a reduced pO_2. The nitrogen-fixing symbionts of legumes function in an aerobic environment, but the pO_2 within the nodule where they are located is very low; leghemoglobin which is located outside the microsymbionts can facilitate the rate of diffusion of oxygen at a low pO_2, thereby providing oxygen for respiration to form the ATP necessary for the activities of nitrogenase and other enzymes (*41*). Related to the oxygen problem is the surprising involvement of both *Rhizobium* and the legume in the production of leghemoglobin; the plant contains the genetic information for the globin while the rhizobia make the heme portion (*42*). This distribution may be a deterrent to the extension of the *Rhizobium* symbiosis to cereals, and the protection of nitrogenase from oxygen must be one of the major considerations in the extension of biological nitrogen fixation to crops where it does not occur naturally.

What is the biological cost to the organism of nitrogen fixation in view of the molecular studies that have revealed the system's inefficiency in energy use? Would it be advantageous to provide fertilizer nitrogen and enable the plant to divert the energy utilized for nitrogen fixation to the production of additional dry matter? A limited number of experiments with legumes have compared the cost of utilizing fixed nitrogen as nitrate with the cost of fixing nitrogen (*43*). In all cases, no difference was found; in the most recent measurements, 5.9 milligrams of carbon was used in the nodule per milligram of nitrogen fixed as opposed to 6.2 mg of carbon used in the root per milligram of nitrogen (applied as nitrate) reduced.

Our biochemical knowledge of the energy requirements for reduction, incorporation into an organic acid, and transportation does not enable a rigorous theoretical comparison of the costs. However, one can calculate the theoretical costs for the reduction of nitrogen to ammonia and NO_3^- to NH_3. In the case of reducing N_2 to $2NH_3$, the calculated energy equivalent is about 24 ATP molecules (9 ATP molecules being equivalent for three pairs of electrons used for reduction and 15 ATP molecules for the nitrogenase reaction based on 4 ATP molecules for two electrons and an 80 percent efficiency in coupling electrons to nitrogen); in the case of reducing $2NO_3^-$ to $2NH_3$, the total energy equivalent is about 24 ATP molecules (24 ATP molecules being equivalent for eight pairs of electrons). These calculations also suggest that there is no difference in energy costs. A corollary of these theoretical calculations is the ratio of 4 : 1 for the minimum amount of carbohydrate consumed to the amount of nitrogen fixed.

The incorporation of ammonia to produce an organonitrogen compound is not a part of the nitrogen-fixing system but is important to the operation of nitrogenase because failure to remove ammonia would lead to repression. In free-living, nitrogen-fixing bacteria and the *Rhizobium* form in soybean nodules (*44*), glutamine synthetase forms glutamine from ammonia, ATP, and glutamate, while glutamate synthetase forms two molecules of glutamate from glutamine, α-ketoglutarate, and reduced nicotinamide adenine dinucleotide. The advantage of this sequence of reactions is the relatively low K_m of glutamine synthetase for ammonia, which thereby facilitates the maintenance of a low concentration of ammonia. The occurrence of this system in other nitrogen-fixing legumes has not been established. Further transformations are necessary in legumes since asparagine (*45*) is the major form of nitrogen transported from the nodule to the aerial part of the plant. These transformations have not been described.

Laboratory and field studies are producing new understanding of the *Rhizobium*-legume symbiosis. The establishment in vitro of a nitrogen-fixing *Rhizobium*-legume symbiosis by means of cell culture techniques provided a new approach for the study of factors that control the infection and development of the symbiosis (*46*). Nitrogen-fixing activity in vitro was about 1 percent of that in the natural nodule based on measurements of both C_2H_2 reduction and ^{15}N-enrichment following incubation with $^{15}N_2$. Electron micrographs revealed rhizobia within the nonperipheral cells of the callus in an arrangement similar to that in the natural system. Subsequent studies with cowpea *Rhizobium* utilizing a solid rather than a liquid medium revealed less specificity in the system in vitro than in the nodule of the whole plant: this *Rhizobium* strain, when cultured with soybean plant cells, produced a nitrogen-fixing

soybean callus but does not, however, infect intact soybean plants (*47*).

Evidence for an even greater promiscuity of cowpea *Rhizobium* has come from studies of nitrogen-fixing nodules on a nonleguminous plant, *Trema canabina*, observed in the field. A cowpea *Rhizobium* was obtained from the nodules of this plant and was used to establish nitrogen-fixing nodules on both the normal leguminous host and the abnormal nonleguminous host (*15*). One may ask what other unusual naturally occurring nitrogen-fixing systems are waiting to be discovered. Most recently, nitrogen fixation has been expressed by cowpea *Rhizobium* externally associated with either leguminous or nonleguminous plant cells in culture (*16*). The activity is low but appears to be real and lasts for about 24 hours after removal of the plant cells. These observations suggest that a diffusible factor common to both legumes and nonlegumes will cause rhizobia to become free-living diazotrophs and improve the possibility of our extending the rhizobia symbiosis to nonleguminous crop plants such as the cereal grains. Understanding the molecular nature of specificity between plant and bacterium may be fundamental to such an extension. Lectins have been proposed as a possible basis for specificity in the *Rhizobium*-legume root nodule infection based on the observed specificity in the binding of soybean *Rhizobium* (*48*). Successful extension of the rhizobial symbiosis to cereals would be one of the most attractive alternative technologies.

Photosynthesis and Nitrogen Fixation

Rhizobium-based symbiosis and the associative symbiosis in tropical grasses appear to offer the greatest opportunity for increased input of nitrogen to grain legumes and cereal grains. Additional measurements continue to show that free-living heterotrophic organisms have a minor role in nitrogen fixation in the soil; however, a plant-algal association, *Azollo-Anabaena*, is reported to fix 60 to 140 kg of nitrogen per hectare annually (*49*).

The acetylene-ethylene assay, with its capacity for up to 200 measurements per day in the field, has enabled investigators to determine the time course of nitrogen fixation in field-grown soybeans from seedling to senescence (Fig. 6) (*50*). About 10 percent of the nitrogen fixed by field-grown soybeans occurs during vegetative growth, while about 90 percent occurs during reproductive growth—apparently an ideal timing of input to need. The nitrate utilization system in the aerial part of the plant complements nitrogen fixation in the root nodules since maximum nitrate reductase activity precedes that of nitrogenase activity. Nitrate reductase activity also is found in the nodule in high amounts and it has recently been suggested that this system may provide an important input of reduced nitrogen; however, further measurements are needed to evaluate this proposal (*51*). The nitrogen-fixation time courses revealed two major limitations of the nitrogen-fixation system. Only about 75 kg of nitrogen per hectare are biologically fixed during the complete growth cycle, and this accounts for only 25 percent of the nitrogen in the mature plant, forcing the surprising conclusion that the majority of the nitrogen required for the U.S. soybean crop comes from fixed nitrogen in the soil. The time course of nitrogen fixation also shows a loss of the exponential phase of development of the nitrogen-fixation system during the period of rapid reproductive growth, suggesting a further limitation in biological nitrogen fixation.

Both of these limitations appear to be of plant origin and to be specifically related to a less than optimum supply of photosynthate to the nodule. The products of photosynthesis support the growth of the nodules and provide ATP, reductant, and the carbon skeleton for the removal of fixed nitrogen. The large flux of carbon through the nodule has been measured in nitrogen-fixing pea plants during vegetative growth in the laboratory. For every 100 units of carbon that were fixed by photosynthesis, 32 units moved to the nodule where 12 units were lost as CO_2, 6 units were utilized for nodule growth, and 15 units were transported back to the aerial portion of the plant (*43*). Several factors that decrease the amount of photosynthate available to the nodule have been demonstrated to decrease nitrogen fixation, for example, decreased light intensity from night and shading; decreased source size by partial defoliation, high planting density, and lodging; increased demand of competitive sinks during late seed development; and cessation of translocation to nodule by girdling. Other factors that increase the amount of photosynthate available to the nodule have been demonstrated to increase nitrogen fixation, for example, increased light intensity from day and supplemental light; increased source size by grafting additional foliage and low planting density; decreased demand of competitive sinks by pod removal; and increased rate of photosynthesis by CO_2-enrichment of the foliar canopy (*52, 53*).

The most dramatic demonstration that photosynthate is a major limiting factor for nitrogen fixation in field-grown soybeans was obtained from a threefold CO_2 enrichment of the soybean canopy during the period of reproductive growth (Fig. 6). The amount of nitrogen fixed was increased from 75 to 425 kg per hectare as a result of CO_2 enrichment, while the amount of nitrogen obtained from the soil was decreased from 220 to 85 kg per hectare, showing that the CO_2-enriched soybeans fixed 85 percent of their nitrogen requirement, whereas the unenriched plants fixed only 25 percent. In addition, the total nitrogen input was increased from 295 to 510 kg per hectare, representing the first example of a major increase in input of nitrogen to a grain legume. This major effect of CO_2 enrichment has been attributed to an increased net production of photosynthate made possible by the decrease in photorespiration brought about by the elevated CO_2 to O_2 ratio.

The increase in nitrogen fixation by the CO_2-enriched plants is a product of three effects. There is an almost immediate doubling in the nitrogen-fixing activity per mass of nodules, suggesting that there is excess nitrogenase in the nodule but that its activity is limited because of an inadequate supply of photosynthate. As a result of CO_2 enrichment the number of nodules approximately doubles. In addition, there is a delay in the loss of the exponential phase of nitrogen fixation, which thereby extends the period of nitrogen fixation to meet the needs of the later stages of seed development.

A limited amount of similar information has been obtained for peanuts and peas, and there is a need for more information of this type on the other grain legumes so that we can establish the limiting factors of biological nitrogen fixation in these different crops. For the results to be relevant to the field production of crops, the measurements must be made on plants grown under natural field conditions. It is clear in the case of the soybean that, in order to achieve a major increase in nitrogen fixation, attention must be focused on

practical approaches that will lead to improvement in the amounts of photosynthate available to the nodule. The same approach may also be found to be the key to other grain legumes since they are also photosynthetically inefficient. The distribution of associative symbioses described in the next section suggests that the availability of photosynthate may also be a key factor in these systems. It may be suggested that improved photosynthate production by cereals such as wheat and rice may be a prerequisite for the useful extension of any biological nitrogen-fixing system to these crops.

Associate Symbioses in Tropical Grasses

In the early 1960's a nitrogen-fixing associative symbiosis was observed in the rhizosphere (the root zone) between a tropical grass, *Paspalum notatum*, and a free-living, nitrogen-fixing bacterium, *Azotobacter paspalum* (*54*). The bacteria were located underneath a mucilagenous sheath on root surfaces of the plant. This observation has been extended to several additional photosynthetically efficient tropical grasses including sugarcane and maize (*55*). Associations of nitrogen-fixing bacteria with rice roots are also suggested. Most recently an associative symbiosis between *Digitaria decumbens* and *Spirillum lipoferrum* has been identified (*56*). In this association the bacteria infect the root cortex cells beneath the epidermis. The amount of nitrogen fixed by these associations is suggested to be as much as 100 kg per hectare per year, but data obtained from measurements in situ over a substantial period of time are not yet available. Most of the measurements on these systems have been made after the roots of plants have been incubated under reduced pO_2 for extended periods of time so that the reported measurements may have little relationship to the nitrogen-fixing activity in situ. Attempts to reconstitute a nitrogen-fixing associative symbiosis have in general not been successful, although a recent preliminary report suggests successful use of cultures of *Spirillum* as an inoculum for forage grasses (*57*).

These new discoveries of naturally occurring nitrogen-fixing associations are receiving increased attention. Attempts must be made to measure in situ the amounts of nitrogen fixed as a result of these associations, to search for additional associations, and to domesticate these associations to our major cereal grains. Another approach is the development of forced associative symbioses involving a diazotroph mutant with desirable nitrogen-fixing properties and a dependency on the cereal grain for a vital factor (*58*). Success of these systems depends on the intimacy of the partners. The energy contribution of the higher plant must be readily available without loss to the microsymbiont, while the fixed nitrogen must also be taken up effectively by the higher plant.

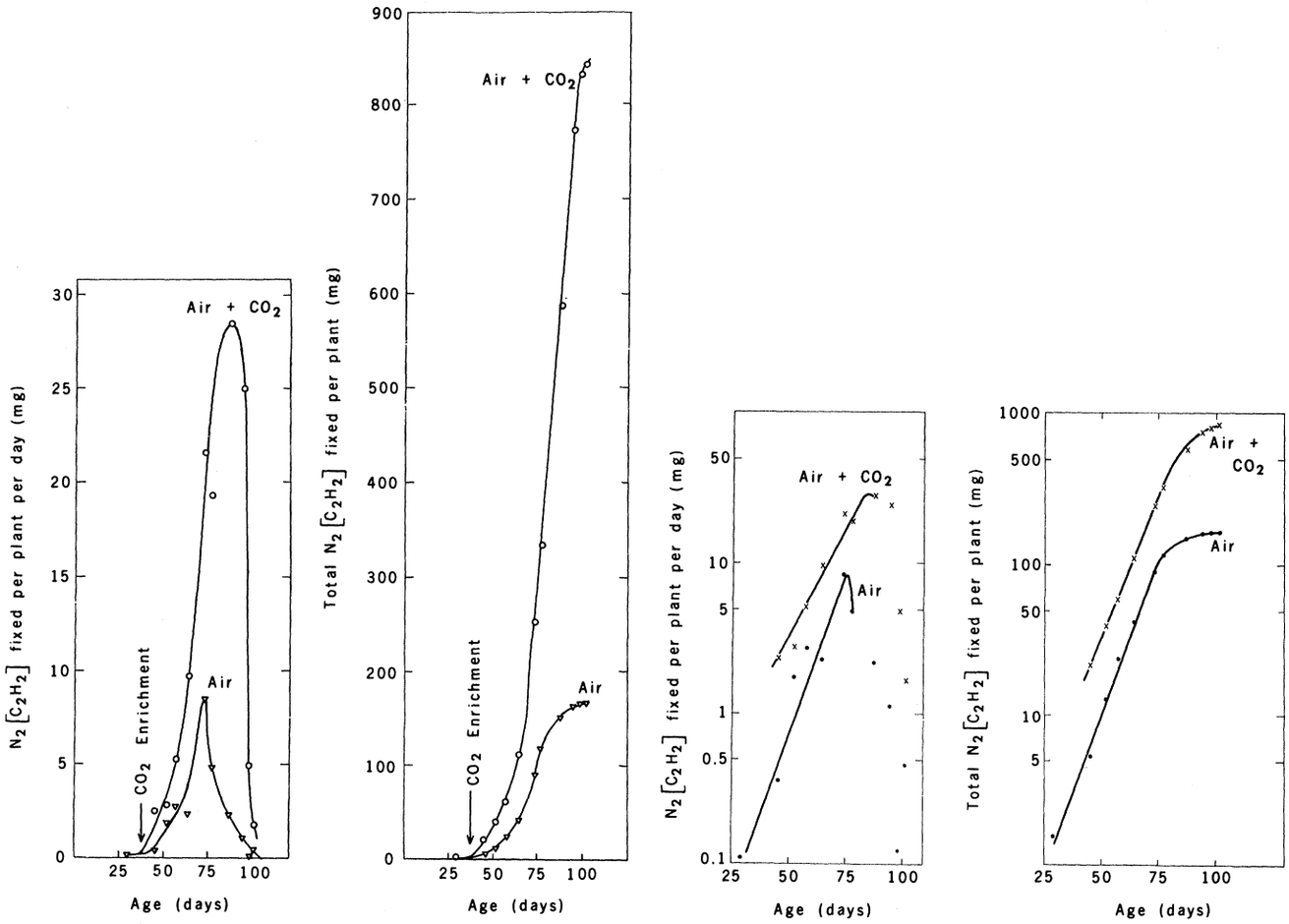

Fig. 6. Time course of nitrogen-fixing activity per plant per day and the total amount of nitrogen fixed per plant for control and CO_2-enriched field-grown soybeans. The CO_2-enrichment occurred from 40 days of age to maturity during the day and produced a CO_2 concentration of 800 to 1000 parts per million in the canopy. [From Hardy and Havelka (*52*), courtesy of Cambridge University Press, England]

The Application of Genetics to Nitrogen Fixation

At present, many investigators are focusing on the control, transfer, and mapping of the genetic information for nitrogenase. Ammonia is well established as a repressor of nitrogenase synthesis, while no evidence has been obtained to suggest that nitrogen is required as an inducer (*59*). Recently, glutamine synthetase acting as a positive control agent has been proposed as the more immediate regulator of nitrogenase synthesis (*60*). Evidence to support this proposal includes mutants with constitutive glutamine synthetase that synthesize nitrogenase in the presence of NH_4^+; mutants that are unable to produce glutamine synthetase and that are also unable to synthesize nitrogenase; simultaneous restoration of glutamine synthetase and nitrogenase activities; and nitrogenase synthesis in the presence of excess NH_4^+ and methionine sulfone and sulfoxime (glutamate analogs and inhibitors of glutamine and glutamate synthetase). The studies providing this evidence were conducted with *Klebsiella pneumoniae*, and empirical approaches have yielded *Azotobacter* mutants that produce nitrogenase in the presence of ammonia. A rhizobial mutant that is constitutive in nitrogenase is being sought and could be useful in eliminating the inhibitory effect of fixed nitrogen on legume symbionts; however, nitrate, which is not a repressor of nitrogenase synthesis, is usually a more potent inhibitor of nitrogen fixation than NH_4^+, suggesting additional complexities in this system. Mutants constitutive for nitrogen fixation may find more utility in the associative symbioses such as those with tropical grasses or in animal rumens. Mutants that excrete ammonia and are constitutive for nitrogenase have been prepared but the high ratio of the amount of carbohydrate consumed to the amount of nitrogen fixed probably eliminates the utility of these organisms as microbiological fertilizer nitrogen factories (*61*).

Another area that might have a major impact on nitrogen input to crops concerns the possibility of moving the genetic information for the control and structure of nitrogenase, that is, the nitrogen operon, from plant species that fix nitrogen to others that do not. Already this operon has been transferred by transduction or conjugation in heterotrophic bacteria and blue-green algae, and in one case a new strain of *E. coli* that can fix nitrogen has been produced (*62*). The possible transfer of a plasmid containing *nif*, or nitrogen-fixing, genes from *Rhizobium* to *Klebsiella aerogenes*, which does not naturally fix nitrogen, has been reported. These experiments have been used to locate the *nif* gene near the *his* and *shi* genes. Transfer of the nitrogen operon to legume or cereal plants is another possible approach for increasing nitrogen input to crops. There are reports of the transfer of the lactose and galactose operons from *E. coli* to higher plants. Plasmids containing the nitrogen operon could be introduced into protoplasts of the desired crop. These protoplasts would be grown in culture and redifferentiated to produce mature plants with the genetic information carried with the seed (Fig. 7). Several laboratories are working on this possibility. This approach might provide the most ideal solution with respect to the production of alternative technologies for nitrogen fixation but is perhaps the most speculative.

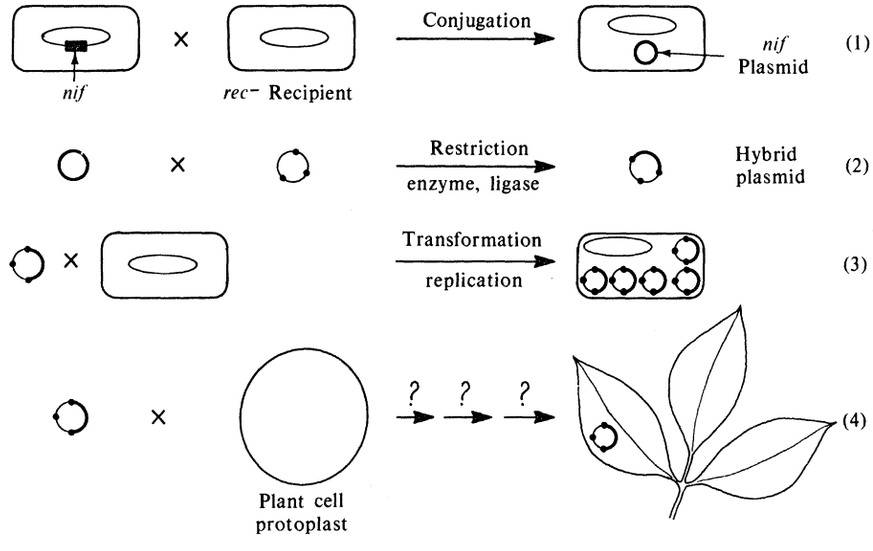

Fig. 7. Hypothetical steps in the production and transfer of plasmids containing *nif* genes to higher plants. [From Shanmugam and Valentine (*7*)]

References and Notes

1. H. F. Judson, *Harper's Magazine* (March 1975), p. 32.
2. Condensed in part from R. W. F. Hardy, in *Symposium on Dinitrogen Fixation*, W. E. Newton and D. J. Nyman, Eds. (Washington State Univ. Press, Pullman, in press).
3. R. C. Burns and R. W. F. Hardy, *Nitrogen Fixation in Bacteria and Higher Plants* (Springer-Verlag, New York, 1975).
4. R. W. F. Hardy and R. C. Burns, in *Iron-Sulfur Proteins*, W. Lovenberg, Ed. (Academic Press, New York, 1973), vol. 1, p. 65.
5. ———, G. W. Parshall, *Adv. Chem. Ser.* **100**, 219 (1971).
6. ———, in *Inorganic Biochemistry*, G. Eichhorn, Ed. (Elsevier, Amsterdam, 1973), p. 745.
7. K. T. Shanmugam and R. C. Valentine, *Science* **187**, 919 (1975).
8. A. D. Allen, R. O. Harris, B. R. Loescher, J. R. Stevens, R. N. Whiteley, *Chem. Rev.* **73**, 11 (1973); F. J. Bergersen, *Annu. Rev. Plant Physiol.* **22**, 121 (1971); F. Bottomley, R. C. Burns, R. W. F. Hardy, Eds., *Treatise on Dinitrogen Fixation: Inorganic and Physical Chemistry and Biochemistry* (Wiley, New York, in press), vol. 1; J. Chatt and G. J. Leigh, *Chem. Soc. Rev.* **1**, 121 (1972); H. Dalton and L. E. Mortenson, *Bacteriol. Rev.* **36**, 231 (1972); M. J. Dilworth, *Annu. Rev. Plant Physiol.* **25**, 81 (1974); R. R. Eady and J. R. Postgate, *Nature (Lond.)* **249**, 805 (1974); A. Gibson, R. W. F. Hardy, W. Silver, Eds., *Treatise on Dinitrogen Fixation: Biology and Agronomy and Ecology* (Wiley, New York, in press), vol. 2; R. W. F. Hardy and R. C. Burns, *Annu. Rev. Biochem.* **37**, 331 (1968); T. A. Lie and E. G. Mulder, Eds., *Biological Nitrogen Fixation in Natural and Agricultural Habitats*, in *Plant Soil*, special volume, 1971; W. E. Newton and D. J. Nyman, Eds., *Symposium on Dinitrogen Fixation* (Washington State Univ. Press, Pullman, in press); P. Nutman, Ed., *Symbiotic Nitrogen Fixation in Plants*, International Biological Programme Series, vol. 7 (Cambridge Univ. Press, London, 1975); J. R. Postgate, Ed., *The Chemistry and Biochemistry of Nitrogen Fixation* (Plenum, London, 1971); A. Quispel, *The Biology of Nitrogen Fixation* (North-Holland, Amsterdam, 1974); A. E. Shilov, *Uspekhi Khim.* **48** (5), 863 (1974); W. D. P. Stewart, Ed., *Nitrogen Fixation by Free-Living Microorganisms*, International Biological Programme Series, vol. 6 (Cambridge Univ. Press, London, 197➡ S. L. Streicher and R. C. Valentine, *Annu. Rev. Biochem.* **42**, 279 (1973).
9. *FAO Production Yearbook*, vol. 26 and prior volumes (FAO, Rome, Italy, 1972 and prior years); R. Ewell, in *Hearings before the Select Committee of the United States Senate on Nutrition and Human Needs*, 93rd Congress, 2nd session, part 2—"Nutrition and the international situation," 19 June 1974 (U.S. Government Printing Office, Washington, D.C., 1974), p. 354.
10. Grain legumes include soybeans, peanuts, dry beans, dry peas, chick-peas, broad beans, vetches, pigeon peas, cowpeas, lentils, and lupines.
11. U. D. Havelka and R. W. F. Hardy, *Agron. Abstr.* (1974), p. 133; in *Symposium on Dinitrogen Fixation*, W. E. Newton and D. J. Nyman, Eds. (Washington State Univ. Press, Pullman, in press).
12. J. R. Douglas and E. A. Harre, in *Symposium on Dinitrogen Fixation*, W. E. Newton and D. J. Nyman, Eds. (Washington State Univ. Press, Pullman, in press); R. Ewell, *Chemtech.* (September 1972), p. 570; E. A. Harre, O. W. Livingston, J. T. Shields, *Bulletin Y70* (National Fertilizer Development Center, Tennessee Valley Authority,

Muscle Shoals, Ala., 1974); G. C. Sweeney, in *Symposium on Dinitrogen Fixation*, W. E. Newton and D. J. Nyman, Eds. (Washington State Univ. Press, Pullman, in press).
13. D. Pimentel, L. E. Hurd, A. C. Bellotti, M. J. Forster, I. N. Oka, O. D. Sholes, R. J. Whitman, *Science* **182**, 443 (1973).
14. R. G. Hoeft and J. C. Siemens, *Illinois Research* (Univ. of Illinois, Urbana-Champaign, 1975); **17** (No. 1), 10 (1975).
15. M. J. Trinick, *Nature (Lond.)* **244**, 459 (1973).
16. J. J. Child, *ibid.* **253**, 350 (1975); W. R. Scowcroft and A. H. Gibson, *ibid.*, p. 351.
17. J. E. Carnahan, L. E. Mortenson, H. F. Mower, J. E. Castle, *Biochim. Biophys. Acta* **44**, 520 (1960).
18. R. C. Burns and R. W. F. Hardy, *Methods Enzymol.* **24B**, 480 (1972); R. R. Eady, B. E. Smith, K. A. Cook, J. R. Postgate, *Biochem. J.* **128**, 655 (1972); D. W. Israel, R. L. Howard, H. J. Evans, S. A. Russell, *J. Biol. Chem.* **249**, 500 (1974); E. Moustafa and L. E. Mortenson, *Biochim. Biophys. Acta* **172**, 106 (1969); W. G. Zumft, W. C. Cretney, T. C. Huang, L. E. Mortenson, *Biochem. Biophys. Res. Commun.* **48**, 1525 (1972).
19. R. C. Burns, R. D. Holsten, R. W. F. Hardy, *Biochem. Biophys. Res. Commun.* **39**, 90 (1970).
20. G. M. Cheniae and H. J. Evans, *Plant Physiol.* **35**, 454 (1960); A. Kondorosi, I. Barabas, Z. Svab, L. Orosz, T. Sik, R. D. Hotchkiss, *Nat. New Biol.* **246**, 153 (1973); H. H. Nagatani, N. K. Shah, W. J. Brill, *J. Bacteriol.* **120**, 697 (1974); A. Nason, K. Y. Lee, S. S. Pan, P. A. Ketchum, A. Lamberti, J. DeVries, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 3242 (1971).
21. L. E. Mortenson, W. G. Zumft, G. Palmer, *Biochim. Biophys. Acta* **222**, 422 (1973); W. H. Orme-Johnson, W. D. Hamilton, T. Ljones, M.-Y. Tso, R. H. Burris, V. K. Shah, W. J. Brill, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3142 (1972); V. K. Shah, L. C. Davis, J. K. Gordon, W. H. Orme-Johnson, W. J. Brill, *Biochim. Biophys. Acta* **292**, 246 (1973); B. E. Smith, D. J. Lowe, R. C. Bray, *Biochem. J.* **135**, 331 (1973).
22. R. W. F. Hardy and A. J. D'Eustachio, *Biochem. Biophys. Res. Commun.* **15**, 314 (1964); L. E. Mortenson, *Proc. Natl. Acad. Sci. U.S.A.* **52**, 272 (1964).
23. H. C. Winter and R. H. Burris, *J. Biol. Chem.* **243**, 940 (1968); W. A. Bulen and J. R. LeComte, *Proc. Natl. Acad. Sci. U.S.A.* **56**, 979 (1966).
24. W. A. Bulen, R. C. Burns, J. R. LeComte, *Proc. Natl. Acad. Sci. U.S.A.* **53**, 532 (1965); E. Knight, Jr., A. J. D'Eustachio, R. W. F. Hardy, *Biochim. Biophys. Acta* **113**, 626 (1966); L. E. Mortenson, R. C. Valentine, J. E. Carnahan, *Biochem. Biophys. Res. Commun.* **7**, 448 (1962); P. Wong, H. J. Evans, R. Klucas, S. Russell, *Plant Soil*, special volume (1971), p. 361.
25. M. J. Dilworth, *Biochim. Biophys. Acta* **127**, 285 (1966); R. W. F. Hardy and E. Knight, Jr., *Biochem. Biophys. Res. Commun.* **23**, 409 (1966); M. Kelly, J. R. Postgate, R. L. Richards, *Biochem. J.* **102**, 1c (1967); R. Schöllhorn and R. H. Burris, *Fed. Proc.* **24**, 710 (1967).
26. R. W. F. Hardy and E. Knight, Jr., *Biochim. Biophys. Acta* **139**, 69 (1967).
27. W. L. Ogren and G. Bowes, *Nature (Lond.)* **230**, 159 (1971).
28. R. C. Burns, W. H. Fuchsman, R. W. F. Hardy, *Biochem. Biophys. Res. Commun.* **42**, 353 (1971); R. C. Burns, R. W. F. Hardy, in *Symposium on Dinitrogen Fixation*, W. E. Newton and D. J. Nyman, Eds. (Washington Univ. Press, Pullman, in press); J. R. Benemann, C. E. McKenna, R. F. Lie, T. G. Traylor, M. D. Kamen, *Biochim. Biophys. Acta* **264**, 25 (1972); C. E. McKenna, J. R. Benemann, T. G. Traylor, *Biochem. Biophys. Res. Commun.* **41**, 1501 (1970).
29. E. T. Adman, T. C. Sieker, L. H. Jensen, *J. Biol. Chem.* **248**, 3987 (1972); K. D. Watenpaugh, T. C. Sieker, T. H. Jensen, J. LeGall, M. Dubourdieu, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3185 (1972).
30. T. Herskovitz, B. A. Averill, R. H. Holm, J. A. Ibers, W. D. Phillips, J. T. Weiher, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2437 (1972).
31. R. W. F. Hardy, R. C. Burns, R. D. Holsten, *Soil Biol. Biochem.* **5**, 47 (1973).
32. A. D. Allen and C. V. Senoff, *Chem. Commun.* (1965), p. 621.
33. J. Chatt, J. R. Dilworth, R. L. Richards, J. R. Sanders, *Nature (Lond.)* **224**, 1201 (1969).
34. C. R. Brulet and E. E. van Tamelen, *J. Am. Chem. Soc.* **97**, 911 (1975); J. Chatt, A. J. Pearman, R. L. Richards, *Nature (Lond.)* **253**, 39 (1975).
35. M. E. Vol'pin and V. B. Shur, *Nature (Lond.)* **209**, 1236 (1966).
36. G. N. Schrauzer, G. Schlesinger, P. A. Doemeny, *J. Am. Chem. Soc.* **95**, 5582 (1973); A. Shilov, N. Denisov, O. Efimov, N. Shuvalov, N. Shuvalova, A. Shilova, *Nature (Lond.)* **231**, 460 (1971).
37. R. W. F. Hardy, R. C. Burns, J. T. Stasny, G. W. Parshall, in *Nitrogen Fixation by Free-Living Microorganisms*, W. D. P. Stewart, Ed. (Cambridge Univ. Press, London, 1975).
38. S. Hill and J. R. Postgate, *J. Gen. Microbiol.* **58**, 277 (1969); B. Koch, H. J. Evans, S. Russell, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1343 (1967); J. W. Millbank, *Arch. Mikrobiol.* **68**, 32 (1969).
39. J. R. Postgate, in *Microbes and Biological Productivity*, D. E. Hughes and A. H. Rose, Eds. (Cambridge Univ. Press, London, 1971), p. 287.
40. P. Fay, in *The Biology of Blue-Green Algae*, N. G. Caw and B. A. Whitton, Eds. (Blackwell, Oxford, 1973), p. 238.
41. F. J. Bergersen, G. L. Turner, C. A. Appleby, *Biochim. Biophys. Acta* **292**, 271 (1973); J. D. Tjepkema, thesis, University of Michigan, Ann Arbor (1971); B. A. Wittenberg, J. B. Wittenberg, C. A. Appleby, *J. Biol. Chem.* **248**, 3178 (1973).
42. M. J. Dilworth, *Biochim. Biophys. Acta* **184**, 432 (1969); J. A. Cutting and H. M. Schulman, *ibid.* **192**, 486 (1969).
43. G. Bond, *Ann. Bot. (Lond.)* **5**, 313 (1941); A. H. Gibson, *Aust. J. Biol. Sci.* **19**, 499 (1966); F. R. Minchin and J. S. Pate, *J. Exp. Biol.* **24**, 259 (1973).
44. M. J. Dilworth, in *Symposium on Dinitrogen Fixation*, W. E. Newton and D. J. Nyman, Eds. (Washington State Univ. Press, Pullman, in press); H. Nagatani, M. Skimizu, R. C. Valentine, *Arch. Microbiol.* **79**, 164 (1971); E. Ryan and P. F. Fottrell, *Phytochemistry* **13**, 2646 (1974).
45. J. G. Streeter, *Agron. J.* **64**, 311, 315 (1972).
46. R. D. Holsten, R. C. Burns, R. W. F. Hardy, R. R. Hebert, *Nature (Lond.)* **232**, 173 (1971); R. W. F. Hardy and R. D. Holsten, U.S. Patent 3,704,546 (1972).
47. D. A. Phillips, *Plant Physiol.* **53**, 67 (1974).
48. B. B. Bohlool and E. L. Schmidt, *Science* **185**, 269 (1974).
49. J. H. Becking, in *Symposium on Dinitrogen Fixation*, W. E. Newton and D. J. Nyman, Eds. (Washington State Univ. Press, Pullman, in press); in *Nitrogen Fixation by Free-Living Organisms*, W. P. D. Stewart, Ed., International Biological Programme Series, vol. 6 (Cambridge Univ. Press, London, 1975).
50. R. W. F. Hardy, R. D. Holsten, E. K. Jackson, R. C. Burns, *Plant Physiol.* **43**, 1185 (1968); R. W. F. Hardy, R. C. Burns, R. R. Herbert, R. D. Holsten, E. K. Jackson, *Plant Soil*, special volume (1971), p. 561; D. F. Weber, B. E. Caldwell, C. Sloger, H. G. Vest, *ibid.*, p. 293.
51. J. E. Harper and R. H. Hageman, *Plant Physiol.* **49**, 146 (1972); J. E. Harper, *Crop Sci.* **14**, 255 (1974); W. J. Russell, D. R. Johnson, D. D. Randall, *Agron. Abstr.* (1974), p. 76.
52. R. W. F. Hardy and U. D. Havelka, in *Symbiotic Nitrogen Fixation in Plants*, P. Nutman, Ed., International Biological Programme Series, vol. 7 (Cambridge Univ. Press, London, 1975).
53. U. D. Havelka and R. W. F. Hardy, in *Symposium on Dinitrogen Fixation*, W. E. Newton and D. J. Nyman, Eds. (Washington State Univ. Press, Pullman, in press); T. A. LaRue, personal communication; R. J. Lawn and W. A. Brun, *Crop Sci.* **14**, 11 (1974); C. Sloger, D. Bezdicek, R. Milberg, N. Boonkerd, in *Symbiotic Nitrogen Fixation in Plants*, P. Nutman, Ed., International Biological Programme Series, vol. 7 (Cambridge Univ. Press, London, 1975); J. G. Streeter, *J. Exp. Bot.* **25**, 189 (1974).
54. J. Döbereiner, *Pesqui. Agropecu. Bras.* **1**, 357 (1966).
55. ———, J. M. Day, P. J. Dart, *Plant Soil* **37**, 191 (1972); J. Döbereiner and J. M. Day, in *Nitrogen Fixation by Free-Living Microorganisms*, W. P. D. Stewart, Ed., International Biological Programme Series, vol. 6 (Cambridge Univ. Press, London, 1975); Y. Dommergues, J. Balandreau, G. Rinaudo, P. Weinhard, *Soil Biol. Biochem.* **5**, 83 (1973); D. G. Patriquin and R. Knowles, *Mar. Biol. (N.Y.)* **16**, 49 (1972); P. N. Raju, H. J. Evans, R. J. Seidler, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3474 (1973).
56. J. Döbereiner and J. M. Day, in *Symposium on Dinitrogen Fixation*, W. E. Newton and D. J. Nyman, Eds. (Washington State Univ. Press, Pullman, in press).
57. R. L. Smith, M. H. Gaskins, S. C. Schank, D. H. Hubbell, 72nd Annual Meeting, Southern Association of Agricultural Scientists, New Orleans, February 1975.
58. P. S. Carlson and R. S. Chaleff, *Nature (Lond.)* **252**, 393 (1974).
59. R. A. Parejko and P. W. Wilson, *Can. J. Microbiol.* **16**, 681 (1970); G. W. Strandberg and P. W. Wilson, *ibid.* **14**, 25 (1968).
60. J. K. Gordon and W. J. Brill, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3501 (1972); ———, *Biochem. Biophys. Res. Commun.* **59**, 967 (1974); S. L. Streicher, K. T. Shanmugam, F. Qusubel, G. Morandi, R. B. Goldberg, *J. Bacteriol.* **120**, 815 (1974); R. S. Tubb, *Nature (Lond.)* **251**, 481 (1974).
61. K. T. Shanmugam and R. C. Valentine, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 136 (1975).
62. F. C. Cannon, R. A. Dixon, J. R. Postgate, S. B. Primrose, *J. Gen. Microbiol.* **80**, 227 (197→ R. A. Dixon and J. R. Postgate, *Nature (Lond.)* **234**, 47 (1971); →——, *ibid.* **237**, 102 (197→ L. K. Dunican and A. B. Tierney, *Biochem. Biophys. Res. Commun.* **57**, 62 (1974); W. D. P. Stewart and H. N. Singh, *Biochem. Biophys. Res. Commun.* **62**, 62 (1975); S. L. Streicher, E. Gurney, R. C. Valentine, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1174 (1971); —→, *Nature (Lond.)* **239**, 495 (1972).