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# Dinitrogen ($N_2$) Fixation (with a Biochemical Emphasis)

HOWARD DALTON AND LEONARD E. MORTENSON
*Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907*



## INTRODUCTION

The importance of nitrogen as a constituent of many small and large molecular weight biologically important compounds is obvious. Its abundance is correlated with soil productivity, and whether it will be available in a form chemically suitable for plant use is generally determined by the microbial population of the soil. Nitrogen is continually lost from the soil to the atmosphere primarily by denitrification and is restored by dinitrogen ($N_2$) fixation. It has been estimated (74) that in 1970 biological and chemical $N_2$ fixation accounted for $9.1 \times 10^{10}$ and $2.2 \times 10^{10}$ kg/year, respectively. Most of the biological $N_2$ fixation is attributed to the symbiotic $N_2$-fixing systems and the $N_2$-fixing systems of photosynthetic organisms.

Free-living non-photosynthetic organisms (e.g., *Azotobacter* and *Clostridium*) seem to contribute little towards enriching the nitrogen content of soils (74, 93), although it is studies with such organisms which have been most rewarding in yielding information on the biochemistry of $N_2$ fixation.

The existence of $N_2$-fixing organisms was established in 1862 (170), but it was not until 1894 that Winogradsky isolated and characterized the anaerobe, *Clostridium pasteurianum*, as a bacterium that could use $N_2$ as its sole nitrogen source. He speculated that the product of $N_2$ fixation was ammonia. Since then a wide variety of microorganisms capable of fixing $N_2$ have been characterized, many by the use of $^{15}N_2$ tracer and, more recently, by the use of the acetylene reduction technique. Most of our understanding of the mechanism by which microbes convert $N_2$ to ammonia has come during the last 12 years.

Prior to the isolation of a cell-free $N_2$-fixing system in 1960, studies of $N_2$ fixation had been carried out exclusively with whole cells (198). Of particular importance from these studies were the findings that (i) $N_2$-fixing organisms required molybdenum for growth on $N_2$ as well as an increased Fe and Ca supply; (ii) ammonia was the product of $N_2$ fixation (141, 210); (iii) possible intermediates such as nitramide, diimide, hydrazine, hyponitrite, and hydroxylamine were not reduced or utilized as substrates in place of $N_2$ (37, 136); (iv) hydrogen, carbon monoxide, and $N_2O$ were inhibitors of $N_2$ fixation (37).

In 1960 reproducible $N_2$ fixation was attained in appropriately supplemented extracts of *C. pasteurianum* (38). Such a system required substrate amounts of sodium pyruvate, the role of which was found to be twofold. Oxidation of pyruvate led to the reduction of ferredoxin (Fd), which acted as a source of electrons for $N_2$ reduction (125), and to the production of acetyl CoA, which coupled to phosphotransacetylase and acetokinase supplied adenosine triphosphate (ATP), an absolute and specific requirement for $N_2$ fixation (77, 116, 124, 126).

In 1964 in vitro reduction of $N_2$ by an extract of the aerobe *Azotobacter vinelandii* was obtained using a ferredoxin-hydrogenase couple from *C. pasteurianum* and hydrogen as the reductant (29). Because high concentrations of ATP did not yield detectable $N_2$ fixation, an ATP-generating system consisting of creatine phosphate-creatine phosphokinase was added. ATP above 5 mM was inhibitory (29, 38) because the accumulated product, adenosine diphosphate (ADP), inhibited $N_2$ fixation (31, 134). Of great importance to further progress in understanding the mechanism of $N_2$ fixation was the finding that the electron donor system (Fd-hydrogenase) could be replaced by sodium dithionite (30, 33).

Since an ATP-generating system was used to supply ATP, and $Mg^{2+}$ was required for this system, it was difficult to establish whether $Mg^{2+}$ also was required for ATP utilization coupled to $N_2$ fixation. By the use of fluoride which at a defined level inhibited a contaminating pyrophosphatase but not reductant-dependent adenosine triphosphatase (ATPase), it appeared that $Mg^{2+}$ was required and that ATP was converted to ADP and inorganic orthophosphate ($P_i$) (80). Three years later it was shown that $N_2$ fixation could be supported directly by ATP with pure nitrogenase from *C. pasteurianum* as catalyst (103) and $Mg^{2+}$ (or to a lesser degree other divalent metals [34]) was found to be an absolute requirement. In addition it was shown that ATP bound to azoferredoxin (AzoFd) (a component of nitrogenase of *C. pasteurianum*) but only in the presence of $Mg^{2+}$ (28). The binding of the inhibitor, ADP, to AzoFd did not require $Mg^{2+}$.

Nitrogenase, the name conventionally given to the $N_2$-fixing system, when in a crude extract of *C. pasteurianum*, was soluble and oxygen sensitive (38), whereas *Azotobacter* nitrogenase sedimented (29, 80) and was stable in air. However, like the purified components of nitrogenase of *C. pasteurianum*, the purified components of *Azotobacter* nitrogenase are also $O_2$ sensitive; only the crude unpurified nitrogenase was stable. Cell-free $N_2$-fixing extracts have now been prepared from a variety of $N_2$-fixing organisms (Table 1). The presence of nitrogenase is not peculiar to any particular microbial taxonomical division but is fairly well distributed among the major groups. There is a similarity between nitrogenase preparations from different organisms, e.g., resolution into two components, one containing Fe the other Fe and Mo; their oxygen sensitivity; ability to reduce other substrates such as acetylene, cyanide, isocyanide, and $N_2O$ (see section on compounds reduced); catalysis of an ATP-dependent $H_2$ evolution and reductant-dependent ATPase activity (see section on role of reductant).

## DINITROGEN FIXATION BY WHOLE CELLS

### $N_2$-Fixing Organisms in Nature

There are many excellent reviews dealing

TABLE 1. *Examples of $N_2$ fixation by whole cells, extracts, and purified components of $N_2$-fixing organisms*

| Organism | Activity (nmoles of $N_2$ fixed per min per mg of protein) | | | | References |
|---|---|---|---|---|---|
| | Whole cells | Crude extract | Based on Mo-Fe protein | Based on Fe protein | |
| *Clostridium pasteurianum* | 20–60[a] | 4–20[a] | 318–375 | 298–770 | 47, 52, 55, 131, 135, 189 |
| *Azotobacter vinelandii* | 22 | 7.6–7.9 | 360–375 | 130–700 | 29, 32, 35, 133, 99, 180; Brill, (*unpublished data*) |
| *Mycobacterium flavum* | | 1.7–3.44 | | | 18 |
| *Klebsiella pneumoniae* | | 2.9 | | | 113 |
| *Bacillus polymyxa* | | 7 | | | 54 |
| *Chromatium* | | 4.8 | | | 201 |
| *Chloropseudomonas ethylicum* | | 7.2 | 12 | 33 | 167 |
| *Rhodospirillum rubrum* | 1.3 | 2.8–3.7 | | | 137 |
| *Rhizobium japonicum* (bacteroids)[b] | | 9.2 | 140 | 115 | 17, 104, 105 |
| *Anabaena cylindrica*[b] | | 0.6 | 4[c] | 7[c] | 154, 164, 165, 177 |

[a] Most results were converted from nanomoles of $C_2H_2$ reduced per minute per milligram of protein (assuming 1 $N_2$ fixed/3.5 acetylene reduced); the higher result of cells of *Clostridium* was obtained only under depressed conditions.

[b] For more data on symbiotic and algal systems, see reference 75.

[c] Probably much higher since the only sample taken was at 10 min.

with the ecological aspects of dinitrogen fixation (22, 23, 74, 93, 142, 171, 174, 193) and only a brief summary will be given here, mainly to familiarize readers with the importance of nitrogenase in nature.

There is little doubt that $N_2$ fixation by the symbiotic association of microorganisms with plants is the most important source of $N_2$ fixation (74, 172, 174). Such associations can be put into several groups. The most important group, the root-nodulated legumes, fix around 100 to 300 kg of N per ha per year. Because of the "tightly coupled" nature of the leguminous association, neither the rhizobial precursor of the endophyte (a "degenerate" form of a species of *Rhizobium*, called a bacteroid), nor the host plant, fixes $N_2$ independently. It has been shown that $N_2$ fixation occurs only in the bacteroids (108), but what the symbiosis does to allow $N_2$ fixation is unknown. Possibly there may be genetic or other transfer between the plant cells of the nodule and the rhizobia needed to code for nitrogenase synthesis or to derepress nitrogenase synthesis in the bacteroids.

A second group, the non-leguminous plants, is comprised of root-nodulated angiosperms and gymnosperms. The endophyte of the angiosperms has never been isolated, but is thought to be an actinomycete (9, 10, 22). This group fixes about the same amount of $N_2$ as the leguminous plants and is very important in developing areas which are low in fixed nitrogen. The gymnosperms may be further subdivided according to the nature of the endophyte. There are the nodulated cycads in which the symbiont is a blue-green alga (10, 16, 23) and *Podocarpus* in which the symbiont is a phycomycete (4, 8).

The leaf nodule bacteria represent a more casual association in that the endophyte of *Psychotria*, *Klebsiella rubiacearum*, is also a facultative anerobe capable of fixing $N_2$ when growing in laboratory culture in the absence of the plant (39, 161).

Of the free-living forms, the blue-green algae are probably the most important contributors of fixed nitrogen. Photosynthetic bacteria and algae have a distinct advantage over bacteria in that the ATP and reductant needed for $N_2$ fixation are intermediates or products of photosynthesis and an external supply of carbohydrate is not needed. As a result they are very important in colonizing areas which are unfavorable for the growth of many other $N_2$-fixing organisms. Algae found either in association with certain plants and fungi (lichens) or free-living have been estimated to fix as much as 25 to 100 kg of N per ha per year in rice paddy fields where they can be the main source of

combined nitrogen (67, 93). Blue-green algae are fairly ubiquitous, having been found in such temperature extremes as Antarctica (85) and the hot springs of Yellowstone as well as in most temperate climates. The algal blooms of tropical and subtropical oceans stretch over many square miles, and their importance in lakes is recently receiving more attention (61, 87, 173, 174, 175).

The distribution and contribution to soil fertility of other free-living microorganisms has been covered extensively elsewhere (5, 6, 7, 74, 93, 118, 174) and only a few general points will be made here. Free-living bacteria are apparently inefficient in terms of milligrams of nitrogen fixed per gram of carbohydrate consumed with values ranging from 2 to 25. When the availability of a suitable energy source is low, the contribution of fixed nitrogen by free-living bacteria is also low (74, 93). Possibly the agronomic importance of the azotobacter may have been underestimated since the efficiency of fixation can be as high as 40 in chemostat cultures grown at low $pO_2$ values (51), and cultures growing at low $pO_2$ may be more representative of the natural environment.

Because of their broad distribution in nature, the free-living microbes probably make a small but significant contribution to soil fertility (values around 5 to 20 kg per ha per year have been estimated). Measurement of $N_2$ fixation in natural environments was difficult, but has recently been simplified by use of the acetylene reduction technique (74, 78, 175, 176). The development of this technique, which is fast, sensitive, and correlates well with $N_2$ fixation, was a milestone for study of $N_2$ fixation in natural environments. With this technique $N_2$ fixation by free-living bacteria has been demonstrated in most environments. None, however, was more unexpected than a recent report of $N_2$ fixation in the intestines of humans, pigs, and guinea pigs (15). A search for the intestinal organism(s) responsible identified *Klebsiella aerogenes*, but other genera such as *Enterobacter* and *Escherichia* could also have been involved.

## Effect of Oxygen

Dinitrogen fixation by either purified nitrogenase or crude extracts from all $N_2$-fixing organisms is an anaerobic process. The aerobic $N_2$ fixers, such as *Azotobacter*, some blue-green algae, and the legume-*Rhizobium* association, fix $N_2$ in the presence of air and the ability of aerobes to catalyze this $O_2$-sensitive process is reconcilable only if the process occurs in an internal (possibly localized) anaerobic environment.

Early investigations into aerobic $N_2$ fixation revealed that the efficiency of $N_2$ fixation showed a marked dependence on oxygen (197), the efficiency of $N_2$ fixation (milligrams of N fixed per gram of carbohydrate) increased with decreasing $pO_2$ values (from 0.2 atm to around 0.04 atm) in cultures of *Azotobacter* (117, 147, 148, 153, 185). It was suggested that oxygen was a competitive inhibitor of $N_2$ fixation because it competed for electrons needed for $N_2$ reduction.

Another aerobe, *Mycobacterium flavum*, showed an optimum $pO_2$ for acetylene reduction of 0.05 atm; higher or lower $pO_2$ values decreased activity (18). A similar effect was observed in cultures of the blue-green alga, *Anabaena flos-aquae*, with an optimum $pO_2$ at or below 0.2 atm (174). At very low $pO_2$, $N_2$ fixation (measured by acetylene reduction) decreased. Since ATP is required for $N_2$ fixation, this inhibition probably is a result of limiting ATP (limited oxidative phosphorylation) since respiration in *Azotobacter* decreased when the $pO_2$ decreased (56, 149). This decrease in oxidative phosphorylation would favor an increase in the ADP-ATP ratio which in turn would inhibit nitrogenase activity (see section on control of biosynthesis).

High external $O_2$ (above 0.5 atm) was sufficient to competitively inhibit $N_2$ fixation by nodules (12). It was suggested that the leghaemoglobin of nodules, which was completely reduced below an external $pO_2$ of 0.5 atm but increasingly oxygenated when the $pO_2$ increased above this value, exerted a protective effect on the bacteroids by removing excess oxygen (13). Hydrogen-deuterium exchange (catalyzed by nitrogenase) was also inhibited by high $pO_2$ values (14).

Nitrogenase in crude extracts from *C. pasteurianum* was soluble and readily inactivated by oxygen, unlike nitrogenase from *Azotobacter* that seemed to be "particulate" and stable in air. However, when azotobacter nitrogenase was made soluble by further purification, it also was readily damaged by oxygen (32, 97). The oxygen stability of the crude nitrogenase complex from *Azotobacter* is dependent upon the method of extraction from the organism. Release of the complex from the organism by the French pressure cell led to particulate, air-stable preparations; release by much gentler osmotic lysis gave preparations which resembled clostridial extracts in that they were soluble and oxygen sensitive (144). Examination with the electron microscope revealed that

osmotically lysed preparations possessed few if any membranes in the crude extract after low-speed centrifugation, whereas extracts prepared by the French pressure cell contained many membranes (144). It was suggested that in preparations from the French press the membranes of *Azotobacter* break and rejoin around nitrogenase and thus nitrogenase appears particulate. The membrane surrounding nitrogenase protects it from oxygen. The preceding model was also suggested from earlier whole-cell studies of *Azotobacter chroococcum* in which high aeration rates inhibited the growth of $N_2$-fixing but not ammonia-grown cultures (49, 50). If growth was limited by reducing the supply of carbon or phosphorus to continuous $N_2$-fixing cultures of *A. chroococcum*, there was a much greater sensitivity to oxygen than in noncarbon- or phosphate-limited cultures. Similar cultures grown in the presence of ammonia did not display this sensitivity to oxygen. Thus, *Azotobacter* must have some mechanism to allow it to fix $N_2$ (a reductive process) in the presence of oxygen. One possibility (50) is that actively growing *Azotobacter* uses its very high respiratory ability to prevent oxygen from contacting the $N_2$-fixing site. This is apparent from the increase in membranes seen when ammonia-grown cells are switched to $N_2$-dependent growth or from the oxygen damage seen with cells in a chemostat under conditions of carbon or phosphorus limitation, or both.

There also is protection of nitrogenase (by membranes) not related to activity. For example, cells grown at a high aeration rate (oxygen solution rate 22 mmoles of $O_2$ per hr) had no nitrogenase activity but activity was restored immediately on changing to a lower aeration rate (17 mmoles of $O_2$ per hr) (204). Also in lysozyme-ethylenediaminetetraacetic acid (EDTA)-treated cells, the effect of high $O_2$ treatment is reversible (60, 204). No new synthesis of nitrogenase was necessary to restore activity, only the return to the lower aeration rate, whereas in cell-free extracts nitrogenase is irreversibly inhibited by $O_2$ (97, 135).

One enzyme that could function to protect against $O_2$ is reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase since nitrogenase in extracts from *A. chroococcum* was protected against oxygen damage by its addition (203). Other proteins such as cytochrome $c_4$, ferredoxin, or azotoflavin failed to protect under the same conditions in which NADH dehydrogenase did protect.

Protective mechanisms are also necessary for nitrogenase of aerobically grown blue-green algae (64, 65). Since *Anabaena cylindrica* has heterocysts that have a high respiratory activity, it was thought possible that nitrogenase was protected by being in these structures (177). However, nitrogenase seems to exist both in the heterocyst (177), as well as in the soluble vegetative portion of the cell (110, 164). In another blue-green alga with no heterocysts, *Plectonema*, filaments do not contain nitrogenase when grown in air but do when grown in air on nitrate and then freed of nitrate and put under anaerobic conditions (178). Thus, nitrogenase of *Plectonema* acts like *Clostridia* and *Klebsiella* in which nitrogenase only exists under anaerobic conditions. Is nitrogenase only made anaerobically because of the absence of heterocysts? Perhaps the vegetative cells of *Anabaena* would also have higher nitrogenase if incubated anaerobically.

Nitrogenase from *Mycobacterium flavum* even when isolated in a "particulate" form is inactivated by oxygen. This suggests it is poorly protected from $O_2$ (18) and provides an explanation of why whole cells fix optimally at $pO_2$ values of only 0.05 atm.

## CONTROL OF NITROGENASE

### Biosynthesis

No incorporation of $^{15}N_2$ into cellular material occurred during the diauxic lag that followed exhaustion of ammonia during growth of a culture of *Klebsiella pneumoniae* genetically capable of synthesizing nitrogenase (150). The diauxic lag was eliminated if at the beginning of the lag the nitrogen-starved cells were supplied with vitamin-free casein hydrolysate under a $N_2$ atmosphere. It appeared that amino acids furnished by the casein hydrolysate were necessary for formation of nitrogenase, and that these "amino acids" were made available only at a limiting rate when no fixed nitrogen was added.

More specifically it was found that certain amino acids, particularly aspartate, stimulated formation of nitrogenase in *K. pneumoniae* after ammonia was exhausted from the medium (207). It was concluded that formation of the nitrogenase system was repressed in the presence of the ammonia and that when the ammonia supply was exhausted, nitrogenase had to be induced by $N_2$ before growth could resume. Because simple techniques for measuring nitrogenase activity were not available at that time, no direct measurement of nitrogenase activity was made. All conclusions were made by comparing growth under $N_2$ with that of another culture grown with a similar con-

centration of ammonia but under helium. Later direct measurements of nitrogenase activity were made in extracts from *Klebsiella pneumoniae* harvested after exhaustion of ammonia from the growth medium (113). No activity was detected until about 13 hr after $N_2$ was supplied. This suggested that $N_2$ was required as an inducer for the system but because of (i) the insensitive assay system, (ii) the absence of $CO_2$, a requirement for most organisms, and (iii) the low specific activities, this conclusion was questioned. In a later publication it was shown that $N_2$ was not necessary as an inducer since nitrogenase synthesis was observed after ammonia exhaustion in the "absence" of $N_2$. Under these later conditions nitrogenase also was detected sooner if amino acids were supplied during the lag phase.

An investigation of the synthesis of nitrogenase of *Azotobacter vinelandii* was undertaken using a cell-free assay system (179). Like *K. pneumoniae* a diauxic lag was observed between the time of exhaustion of the ammonia and the resumption of growth on $N_2$. When cells were grown on ammonia in a 20% oxygen-80% helium atmosphere, nitrogenase activity was detected 45 min after the exhaustion of the ammonia. Dinitrogen apparently was not required as an inducer but the inability to remove the last traces of $N_2$ (100 and 15 ppm of $N_2$ in $O_2$ and He, respectively) prevented the authors from making a definite conclusion.

Ammonia represses the synthesis of nitrogenase presumably by acting as a corepressor or precursor to a corepressor. No nitrogenase activity or nitrogenase components were detected in cells using ammonia as nitrogen source provided excess ammonia was present at the time of measurement or harvesting (128, 131, 179). The latter is an essential requirement since, during the course of an investigation on sucrose catabolism in *C. pasteurianum*, it was observed that a chemostat culture growing at a dilution rate of 0.41 per hr on a limiting concentration of ammonia in the absence of $N_2$ not only possessed nitrogenase but its concentration per cell was three times that of normal $N_2$-fixing cells (46, 47).

The increase in nitrogenase under ammonia-limited conditions has also been observed with *A. chroococcum* and *Rhodospirillum rubum* (50, 137). With limiting ammonia at a dilution of 0.2 per hr there was approximately a threefold increase in $N_2$-fixing activity (from 5 to 15 nmoles of $C_2H_2$ reduced per mg of protein per min) when the atmosphere of a chemostat culture of *A. chroococcum* was changed from air to 0.8 atm of argon plus 0.2 atm of oxygen.

The $N_2$ content of the gases, argon and oxygen, used for these experiments was in the range of $10^{-7}$ M but could be reduced to $10^{-8}$ M if the gases were treated to remove $O_2$. Therefore, for $N_2$ to be an inducer of nitrogenase it would have to act as a concentration of about $10^{-3}$ times that required for induction of $\beta$-galactosidase by the non-metabolizable substrate IPTG in *Escherichia coli* (184). Although this is not impossible, it is very unlikely that the organism would possess a system capable of responding to such a low concentration of metobolizable substrate—particularly since such an environment is most unlikely in nature and no selective pressure for such a control would exist (129). In addition, as an inducer $N_2$ would be expected to associate and dissociate from a "repressor," a highly unlikely situation, since $N_2$ is an "unreactive" molecule and known $N_2$ complexes are stable.

If control over nitrogenase synthesis is exerted by ammonia acting as a corepressor, then one can invoke a fairly simple explanation of why when ammonia is limiting, $N_2$-fixing activity in cells is present in a concentration three times that found under "normal" $N_2$-fixing conditions (46, 47, 50, 137). Organisms fixing $N_2$ possess a small intracellular pool of ammonia (repressor). This pool is continually tapped as a nitrogen source for various syntheses and is continually replaced by $N_2$ fixation. The concentration of this ammonia pool acts as a brake on nitrogenase synthesis for when there is a large excess of ammonia, as in cultures grown with excess ammonia, nitrogenase synthesis is completely repressed, and, when there is a small excess of ammonia, a condition normal to a $N_2$-fixing culture in which $N_2$ is nonlimiting, nitrogenase synthesis is only partially repressed. In a chemostat culture limited by ammonia all the ammonia in the medium is utilized by the cell and there is no ammonia pool. Under these conditions nitrogenase synthesis occurs at its maximum rate and the concentration of nitrogenase per cell is three times that of a normal $N_2$-fixing culture. Under an inert gas, the nitrogenase concentration per cell remains at this high level, but, if the atmosphere is switched to $N_2$ so that the ammonia pool increases by $N_2$ fixation, the concentration per cell drops to that of a $N_2$-fixing culture (Fig. 1).

Excess ammonia acts as a repressor of synthesis rather than an inhibitor of nitrogenase since excess ammonia when added to an actively fixing culture prevents further synthesis of nitrogenase but nitrogenase already formed is still fully active (47, 209). In *C. pasteu-*

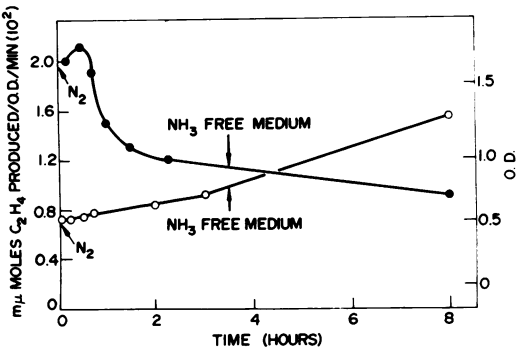



FIG. 1. *Acetylene reduction activity rate. C. pasteurianum was grown on a limited $NH_3$ medium under argon and then put under $N_2$. A chemostat culture was equilibrated on medium that was 1 mM in $(NH_4)_2SO_4$ under argon and with the flow rate of the medium such that the generation time of the culture was 3.5 hr. Samples (●) were assayed for acetylene-reducing activity and the culture was placed under an atmosphere of $N_2$. The increase in OD (○) was measured at the indicated times, and assays for acetylene reducing activity were performed. Second arrows indicate the time at which the reservoir medium was made $NH_3$-free (from Daesch and Mortenson, 47).*

*rianum* (Fig. 2) and *K. pneumoniae* the drop in nitrogenase per cell when ammonia is added is primarily a result of dilution by growth in the absence of synthesis (47, 113). This appears not to be true in *A. vinelandii* where active "destruction" of nitrogenase occurs after the first half generation (W. J. Brill, *personal communication*).

Addition of methylamine and 2-methylalanine to $N_2$-fixing cultures of *A. vinelandii* in some manner prevents nitrogenase synthesis (168). These non-metabolizable nitrogenase "repressors" inhibited formation of nitrogenase but had no effect on nitrogenase activity. This has been studied further (W. J. Brill, *personal communication*) and the effect occurs only when the cells are grown on glucose and maltose but not when grown on other sugars such as sucrose. No explanation of this effect is available.

Research is in progress to select mutants of *C. pasteurianum* and *A. vinelandii* that are deficient in the ability to make one or the other of three (and possibly more) different protein subunits of nitrogenase (subunits with molecular weights of about 27,500; 50,700; and 59,500 have been shown to be part of nitrogenase of *C. pasteurianum*). Mutants deficient in MoFd and AzoFd activity already have been obtained (66, 163, 169). Study of the defective components of the nitrogenase of these mutants should complement very nicely the studies on the structure of the proteins, should tell us how many structural genes are needed, and should lead to information on the genetic control of the synthesis of nitrogenase components.

Streicher et al. (181) have devised a transducing system for mapping genes required for the $N_2$-fixing system of *Klebsiella pneumoniae*. Preliminary data indicated that several gene products were required for the overall $N_2$-fixing process. Included should be the gene products not only of the structural genes for nitrogenase (at the present time this looks like three) but in addition the products of genes for permeases for Mo, $SO_4^{2-}$, and Fe. Also operator and regulatory genes and genes controlling components of the coupled electron transport system are possibly involved. They have shown that several $N_2$ fixation genes (*nif*) are located near the histidine operon (*his*). Also

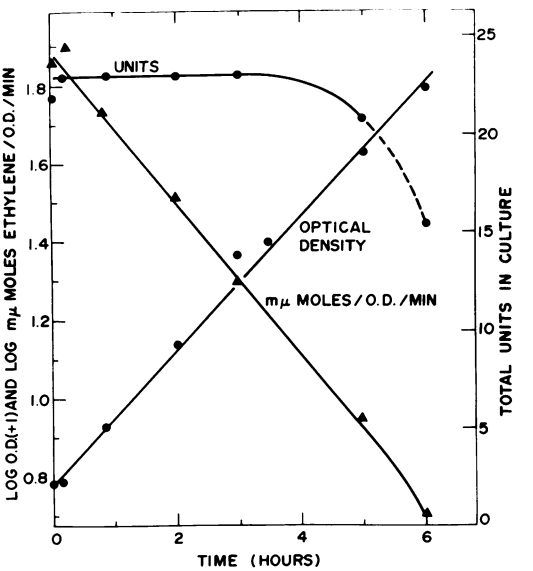



FIG. 2. *Effect of ammonia on nitrogenase in cells of C. pasteurianum. Ammonia (to make the culture 20 mM) was added to a $N_2$-fixing culture, and samples were assayed for acetylene reduction. The rate of ethylene production, optical density, and total units of acetylene-reducing activity (1 unit is 1 μmole of ethylene produced per minute per milligram of protein) were determined. Since the culture was rapidly growing, it had to be diluted occasionally to prevent acid products from accumulating, and all results were corrected for this dilution. The results for ethylene production obtained after 5 hr are questionable since the samples for measurement were so diluted that the correction factor was extremely large and the ethylene production was very small (taken from Daesch and Mortenson, 47).*

recently (59) Dixon and Postgate have demonstrated that $N_2$ fixation genes of *K. pneumoniae* can be transferred by conjugation. This has allowed them also to map the genes involved in $N_2$ fixation, and they found that *his* has a 95% linkage to one *nif* gene and an 85% linkage to another.

### Control of Nitrogenase Activity

A number of non-physiological substrates of nitrogenase either partially or totally inhibit $N_2$ fixation because they compete with $N_2$ for electrons or because they are similar in properties to $N_2$ (for example, CO), or both. In addition to these, metal complexing agents such as $\alpha,\alpha'$-dipyridyl, bathophenanthroline, and tiron also inhibit $N_2$ fixation. However, the only physiological compound demonstrated to control nitrogenase activity is ADP (31, 102, 134). At 3 and 5 mM ADP in the presence of a substrate level of ATP (18 mM), there was a 42 and 53% inhibition of acetylene reduction, respectively, when compared to activity with ATP alone (134). When the ratio of ATP to ADP was 0.5, nitrogenase activity was completely inhibited. Therefore, it is impossible for nitrogenase to consume more than 60 to 70% of the ATP supplied. Since (i) ADP and phosphate are the products of ATP utilization by nitrogenase (80, 103), (ii) phosphate only affects $N_2$ fixation when present in high concentration (38), and (iii) adenosine monophosphate (AMP) and other purine and pyrimidine derivatives are not inhibitory (103, 134), ADP probably is the only small molecule that directly regulates nitrogenase activity. The mechanism for this inhibition has not been firmly established, but evidence suggests that ADP is functioning as a mixed positive and negative modifier (102), the inverse of the ATP effect. There may be a regulatory subunit (or subunits).

In the usual assay system for nitrogenase and in whole cells, ATP is supplied by an ATP-generating system such as acetokinase and acetylphosphate, and little ADP accumulation occurs. When the precursor to ATP is exhausted and the ATP-ADP ratio reaches 0.5, further ATP utilization by nitrogenase and hence $N_2$ fixation is completely inhibited. Presumably, this control is exerted so that when the ATP supply within the cell is low, the remaining ATP can be diverted into more critical cell functions such as the completion of DNA synthesis.

Control over nitrogenase is exerted in two ways. The first is a coarse control in which synthesis of the enzyme is repressed by an excess of one of the products of its activity ($NH_3$), and the other is a fine control in which activity of existing nitrogenase is controlled by one of the products of its activity, ADP, or more specifically by the ratio of ATP to ADP.

## COMPOUNDS REDUCED BY NITROGENASE

### Substrates of Nitrogenase

Early studies concerned with $N_2$ fixation by whole cells of *C. pasteurianum* and *A. vinelandii* led several workers to the conclusion that $N_2O$, $H_2$, HCN, and $N_3^-$ were inhibitors of nitrogen fixation, the former two being competitive, the others nonspecific. However, recent investigations have revealed that with the exception of $H_2$ (180) these inhibitors of $N_2$ fixation are actually reduced by nitrogenase and are in fact "non-physiological" substrates. A more recent finding showed that acetylene, similar to $N_2$ in several of its properties (43), also was reduced by nitrogenase (155). Working independently, Dilworth (55) found that acetylene was reduced by extracts of *C. pasteurianum* and identified the reduction product as ethylene. The requirements for acetylene reduction were the same as those for $N_2$ fixation, i.e., a reductant ($S_2O_4^{2-}$), magnesium, and ATP. Extracts from ammonia-grown cells did not reduce acetylene, and acetylene reduction was inhibited by low partial pressures of carbon monoxide (0.02 atm). The product of acetylene reduction, ethylene, was not further reduced by the extracts, nor did it inhibit further reduction of acetylene or reduction of $N_2$. When the reduction of acetylene was catalyzed by a clostridial extract in $D_2O$, *cis*-$C_2H_2D_2$ was produced; no evidence for the formation of *trans*-$C_2H_2D_2$, $C_2H_2$, asymmetric $C_2H_2D_2$ or $C_2H_2D$ was found.

The reduction of acetylene is a two-electron step and $N_2$ fixation could also be considered a two-electron reduction process. Unlike acetylene, which releases ethylene after the first electron pair is accepted, $N_2$ reduction, of course, would require three two-electron steps viz.

$$RN{\equiv}N \xrightarrow[2H^+]{2e} RN{=}N \xrightarrow[2H^+]{2e} RN{-}N \xrightarrow[2H^+]{2e} 2NH_3 + R$$

dinitrogen diimide hydrazide ammonia

where R = nitrogenase or may represent a bimetal site (composed of iron and molybdenum) on nitrogenase. Since no intermediates between $N_2$ and ammonia have been isolated

or detected, the postulated diimide and hydrazide intermediates would be enzyme-bound and the final reduction product, ammonia, would be released only after complete reduction of the $N_2$ occurred.

Acetylene reduction to ethylene has been used extensively as a sensitive assay of $N_2$-fixing activity since the ethylene produced is readily and quantitatively detected by gas chromatography (55, 106). Since the reduction of acetylene occurs in either a single two-electron step or two one-electron steps, and the reduction of $N_2$ to ammonia requires either three two-electron steps or six one-electron steps, the rate of $N_2$-fixing activity should be one-third of the acetylene-reducing activity. The ratio is more in the range of 1:3.5 because there is more concomitant ATP-dependent $H_2$ evolution during $N_2$ fixation than during acetylene reduction (74).

Although nitrous oxide was demonstrated to be a competitive inhibitor of $N_2$ fixation, there was evidence that it might be metabolized by certain $N_2$-fixing organisms. Burris and coworkers (36, 86) observed uptake of $^{15}N_2O$ by whole cells of *A. vinelandii* and soybean nodules. Subsequently Lockshin and Burris (111) observed that $N_2O$ was reduced to ammonia by extracts of *C. pasteurianum* but only at about 6% of the rate of $N_2$ reduction. The products of the reduction of $N_2O$ by extracts of *C. pasteurianum* and *A. vinelandii* were shown by Hardy and Knight (81) to be $N_2$ and water. The ammonia formation observed by Lockshin and Burris (111) may have arisen from the reduction of $N_2$ released from the $N_2O$ although it was shown (81) that when $N_2O$ was reduced in extracts, the product, $N_2$, was not reduced as long as $N_2O$ was present. It may be that nodules can reduce $N_2$ in the presence of $N_2O$ because membrane restrictions make $N_2O$ less accessible. The requirements for $N_2O$ reduction were the same as those for $N_2$ fixation and the reduction was inhibited 75 to 90% by 0.1 atm of carbon monoxide.

$N_3^-$ and $CN^-$ are reduced by extracts of *A. vinelandii* to $NH_3 + N_3$ and $CH_4 + NH_3$, respectively (81, 82). *A. chroococcum* nitrogenase also was shown to reduce cyanide and isocyanide (96, 101). The main product of the reduction was methane but small amounts of ethane and ethylene also were detected, the proportions of which varied with the substrate used, e.g.,

$$MeNC \rightarrow CH_4, 100: C_2H_4, 0.28: C_2H_6, 2.1$$

$$KCN \rightarrow CH_4, 100: C_2H_4, 0.08: C_2H_6, 0.07$$

Methylamine or ethylamine were also formed from methyl isocyanide or ethyl isocyanide, respectively. It was suggested that $C_2$ compounds were formed by "limited" interaction of $C_1$ radicals formed during the reduction.

Hardy and Jackson (79) investigated the reduction of cyanides and acetylenes by nitrogenase of *Azotobacter vinelandii* and found a variety of reduction products (Table 2). The products obtained required either 2, 4, 6, 8, 10, 12, or 14 electrons for reduction and suggested that reduction by nitrogenase only occurs in two-electron steps. No products containing an odd number of electrons were found in any of the reductions (this is true also of $N_2$ fixation since $2NH_3$ are produced). Subsequently other studies gave similar results (96).

All these reductions by nitrogenase required magnesium ATP and reductant (either reduced Fd or sodium dithionite), and carbon monoxide was an inhibitor. In the absence of other substrates $H^+$ is reduced to $H_2$. Varying amounts of $H_2$ are evolved even when other substrates are added. For example, in the presence of $N_2$ approximately 75% of the electrons that could reduce $H^+$ reduce $N_2$ and the remainder reduce $H^+$.

The $K_m$ values differed (e.g., $K_m$ of $CH_3NC$ < $CH_3CN$) probably because of better end-on bonding of the electron-rich terminal carbon of $CH_3NC$; $CH_2{=}CHCN$ < $CH_3CH_2CN$ because the olefinic group of $CH_2{=}CHCN$ reacts at a second site and produces an electronic effect, and *cis*-$CH_3CH{=}CHCN$ < *trans*-$CH_3CH{=}CHCN$ because of a steric effect (68, 79).

The reduction of azide and cyanide was reported in greater detail by Hardy and Knight (82) using crude and semipurified extracts from *A. vinelandii* and *C. pasteurianum*. These substrates had the same requirements as $N_2$ fixation, i.e., they competed with ATP-dependent $H_2$ evolution but not with reductant-

TABLE 2. *Products from reduction of cyanides and acetylenes by nitrogenase of Azotobacter vinelandii*

| Substrate | Products | Electrons | Relative rate |
|---|---|---|---|
| $N_2$ | $2NH_3$ | 6 | 1 |
| $N_3^-$ | $NH_3$, $N_2$ | 2 | 3 |
| $N_2O$ | $H_2O$, $N_2$ | 2 | 3 |
| $C_2H_2$ | $C_2H_4$ | 2 | 3-4 |
| HCN | $CH_4$, $NH_3$ | 6 | 0.6 |
| | $CH_3NH_2$ | 4 | |
| $CH_3CN$ | $C_2H_6$, $NH_3$ | 6 | 0.2 |
| $CH_3NC$ | $CH_3NH_2$, $CH_4$ | 6 | 0.8 |
| | $C_2H_4$, $C_2H_6$ | 8, 10 | |
| | $C_3H_6$, $C_3H_8$ | 12, 14 | |
| $CH_2CHCN$ | $C_3H_6$, $C_3H_8$, $NH_3$ | 6, 8 | 0.2 |

dependent ATPase (this was expected since ATP is required for their reduction) and were inhibited by CO. This suggested that the various reductions performed by nitrogenase were catalyzed at the site where $N_2$ was reduced rather than at different sites (one for each substrate).

Data based on competition studies between various nitrogenase substrates suggests, on the other hand, that there are several sites required for reduction of substrates (37). Five sites were proposed for overall interaction with substrates or inhibitors of nitrogenase; one for $H_2$ evolution, one for non-competitive inhibition of nitrogen fixation by carbon monoxide and three for the reduction of other substrates. Carbon monoxide inhibits all substrate reductions except for $H_2$ evolution; the latter was inhibited competively by all the substrates but was not affected by CO. Certain substrates inhibited reduction of other substrates, e.g., azide inhibited acetylene reduction. The only known inhibitor that is not chemically changed by nitrogenase is carbon monoxide. No site was assigned for the reduction of $N_2O$ although Hardy and Knight (81, 82) suggested that it was reduced at the same site as $N_2$.

The reduction of various substrates by nitrogenase provides some clues but unfortunately also some inconsistencies concerning the nature of the binding sites. All the substrates are longer molecules than $N_2$ and, apart from a few acetylenic analogues, are linear. The size may be important when considering the rate of reduction of the various substrates—$N_2$ is more readily reduced than acetylene which in turn is more readily reduced than nitrous oxide followed by azide and cyanide. These differences presumably reflect the ability of the site to bind or activate the molecules since the rate of reductant-dependent ATPase, a measure of maximum reducing capacity of nitrogenase, is the same for each substrate (82).

A metal probably is concerned in binding the substrate (ligand) at the active site although no rigorous proof of this now exists. Presumably, one can envisage the active site as being fairly flexible since it can accommodate such a variety of substrates. Cyanide, azide, and $N_2$, from the chemical standpoint, have a much greater tendency to bind end-on to a metal, whereas acetylene appears to complex from the side (43). If nitrogenase bound all substrates in a similar manner, i.e., from the side of the molecule, then the active site would still have to be fairly flexible to accommodate the molecules (e.g., N ≡ N bond distance in nitrogen is 1.098 A, in $N_2O$ it is 1.129 A, and azide 1.134 A). Other problems not to be discussed here arise if one considers the type of bonding involved.

### Hydrogen-Deuterium Exchange Reaction

Formation of HD from $D_2$ and "hydrogen" (the source of [H] was probably water) by excised soybean nodules was found by Hoch, Schneider, and Burris (86) to be dependent upon the presence of $N_2$. They suggested that HD was formed by exchange of $D_2$ with enzyme-bound intermediates between $N_2$ and $NH_3$. This result was later confirmed by Bergersen (14) and shown also in pea nodules by Dixon (58).

Hydrogenase from *Azotobacter* catalyzes $H_2$ uptake when suitable electron acceptors are available but will not evolve $H_2$ (30). Hydrogenase from *C. pasteurianum* will catalyze both uptake and evolution of $H_2$ (188). Because of this fact and since *Azotobacter* nitrogenase was partially purified so that hydrogenase was no longer present, an investigation of the $D_2$-H exchange related to nitrogenase was focused on this organism. Jackson et al. (88) observed $N_2$-dependent $D_2$-H exchange in extracts of *Azotobacter vinelandii*. No exchange was observed if either argon or a substrate of nitrogenase such as acetylene or cyanide replaced $N_2$. The requirements for exchange were the same as those for $N_2$ fixation, i.e., ATP and $Na_2S_2O_4$ were required and CO inhibited the exchange. HD-forming activity seemed to parallel $N_2$-fixing activity during partial purification of nitrogenase from this organism (88). It was proposed that $D_2$ exchanged with an enzyme-bound diimide or hydrazine complex. The suggestion was supported by evidence from an inorganic model system in which diimide and hydrazine complexes of platinum also could catalyze the formation of HD from $D_2$ and water.

Dinitrogen-enhanced HD formation from $D_2$ and "hydrogen" was confirmed by Kelly (95) for root nodules of *Medicago Lupulina* and *Alnus glutinosa*, but he reported that extracts of *Azotobacter chroococcum* and *A. vinelandii* catalyzed the exchange reaction in the absence of nitrogen; in fact, under his conditions $N_2$ proved to be slightly inhibitory. Like the previous study (88) the exchange required ATP and $Na_2S_2O_4$ and was inhibited by CO. No HD formation was observed when alternative substrates of nitrogenase such as acetylene or methyl isocyanide replaced nitrogen. The data suggested that HD formation occurred at the $N_2$-binding site and that other substrates, which also bound at the same site as $N_2$, would inhibit the exchange as a function of

their $K_m$ values and concentration. Kelly (95) suggested that the enhancement of exchange by $N_2$, earlier observed with nodule preparations, could be a result of a diversion of electrons of the electron transport chain from a branch that normally transferred electrons to $O_2$ to a branch that transferred to $N_2$.

To resolve the inconsistency between the results of the latter two groups, Turner and Bergersen (186) studied the $D_2$-H exchange reaction in cell-free extracts of soybean nodule bacteroids. In general they found that $N_2$, ATP, and reductant greatly stimulated $D_2$-H exchange although in the absence of $N_2$, ATP, or dithionite, a large background HD production from $D_2$ and "hydrogen" was observed. Partial purification of "nitrogenase" reduced this background to 10% (Jackson et al. [88] also observed a 10% background with their crude and partially purified preparations). Their evidence did not indicate an involvement of an electron transport system since exchange occurred with dithionite as the reductant.

In crude bacteroid suspensions but not partially purified ones (186) HD formation and $NH_3$ production depended on the $K_m$ ($N_2$). If $D_2$-H exchange did occur with an enzyme-bound diimide or hydrazide, then it would be expected to depend on the $K_m$ ($N_2$) whether purified or not. In addition they found that inhibition of $N_2$ fixation by carbon monoxide was competitive, whereas inhibition of $D_2$-H exchange by carbon monoxide was noncompetitive. One would expect $D_2$-H exchange also to be competitive with carbon monoxide if the production of HD involved intermediates of $N_2$ fixation.

Because of the above inconsistencies, the mechanism of $D_2$-H exchange remains unclear. The majority of evidence favors the requirement for $N_2$ but since all present data are from experiments with crude and partially purified preparations, a definitive statement on the exact nature of the reaction cannot be made. Experiments (Dalton and Mortenson, *unpublished data*) with purified nitrogenase from *C. pasteurianum* indicate in agreement with Jackson et al. (88) an involvement of $N_2$ for the production of HD from $D_2$ and [H].

## COMPONENTS OF THE NITROGEN-FIXING SYSTEM (NITROGENASE)—MOLYBDOFERREDOXIN (MoFe PROTEIN) AND AZOFERREDOXIN (Fe PROTEIN)

### Purification

**Clostridium pasteurianum.** A first step in the purification of nitrogenase ($N_2$ase) was demonstrated during fractionation of crude extracts of *C. pasteurianum* with protamine sulfate and calcium phosphate gel (132). It was shown that active $N_2$ fixation resulted if the protein components not removed from a crude $N_2$-fixing extract by calcium phosphate gel treatment (called the nitrogen-activating system [NAS]) were combined with the protein components remaining in solution after a second crude extract was heated to 60 C for 10 min and centrifuged (called the hydrogen-donating system [HDS]). The HDS contained an active pyruvate-metabolizing system; NAS did not. Addition of NAS from $N_2$-fixing extracts to HDS from extracts of ammonia-grown cells, cells not capable of $N_2$ fixation, resulted in a high rate of $N_2$ fixation whereas the opposite combination showed no activity. It was concluded that nitrogenase still remained in the protein solution after calcium phosphate gel treatment and that ammonia-grown cells did not contain nitrogenase. At this time pyruvate was the only supporting substrate known for $N_2$ fixation, so further purification was complicated by the need for many of the enzymes required for pyruvate metabolism (123).

The next more specific purification of nitrogenase was obtained when it was shown that Fd was required for $N_2$ fixation when pyruvate was the supporting substrate (125). It was found that if a crude extract of *C. pasteurianum* was treated batchwise with increasing amounts of diethylaminoethyl (DEAE) cellulose and the DEAE removed, $N_2$-fixing activity decreased. If Fd was added to the extract after DEAE treatment, several increments of DEAE could be added without loss of $N_2$ fixation activity; but if additional DEAE was added after the extract had been treated with 20 to 30% (w/w) of DEAE to protein, $N_2$ fixation activity now decreased even when Fd was restored. The protein components removed by DEAE and required for $N_2$ fixation were eluted from the DEAE cellulose and when the elute (which contained what is now known as azoferredoxin) was added to the unadsorbed components, it restored nitrogenase activity. The purification was further advanced when it was found that nitrogenase could be removed from a crude extract by treatment with protamine sulfate (127). At this time advantage was taken of the new knowledge that ATP was a requirement for $N_2$ fixation and that $H_2$ could serve as the electron donor (77, 124). Thus the enzymes of pyruvate metabolism were no longer needed and the system now required acetylphosphate, ATP or ADP, $Mg^{2+}$, $H_2$, $N_2$, an extract from $NH_3$-

grown cells (the source of hydrogenase, Fd, and acetokinase) and the $N_2$ase fractions. With this assay nitrogenase was shown for the first time to be made up of at least two easily separable components (127). One was removed from the crude extract by batch DEAE cellulose treatment and the second was removed from the proteins not adsorbed on DEAE cellulose by ammonium sulfate fractionation. Both fractions were shown to be absolute requirements for $N_2$ fixation when added to the above assay system.

The components adsorbed by and eluted from DEAE cellulose were further purified by gel filtration (128). Several colored bands were observed, one of which was a component of $N_2$ase called azoferredoxin (AzoFd). The ammonium sulfate fraction, which contained the other component of nitrogenase (called molybdoferredoxin [MoFd]), when subjected to gel filtration, also showed several bands, one of which was the other component required for $N_2$ fixation. A band corresponding to AzoFd was not present (128). Only when these two purified components were combined was $N_2$ fixation catalyzed.

The present method (52, 130) of purifying the two components (MoFd and AzoFd) of nitrogenase lends itself to large-scale preparation and anaerobic conditions and is a modification of previous methods (103, 128, 131, 135). The only successful method for storing AzoFd consists of freezing it as pellets in liquid $N_2$ (99) and storing the pellets in a liquid $N_2$ refrigerator. Full activity of AzoFd was maintained by this storage procedure. Molybdoferredoxin also is stored as pellets in a liquid $N_2$ refrigerator but freezing at $-20$ C is sufficient since MoFd unlike AzoFd is not labile to cold and is much less sensitive to $O_2$.

Purity of the two nitrogenase components is confirmed when one protein band is seen after disc gel electrophoresis or when the proper number of subunit bands (see section on subunits) is seen after disc gel electrophoresis in the presence of sodium dodecyl sulfate (the latter is a better criterion than the former). Use of ultracentrifugation as a criterion for purity was less adequate since preparations that looked pure by ultracentrifugation contained contaminants when examined by disc gel electrophoresis. It is also essential that the components have "full" activity since inactive components give the same bands as active ones.

Recently a method for purification of MoFd and AzoFd based on adsorption and elution from an anaerobic column of DEAE cellulose was reported (189). A crude extract of *C. pasteurianum* was passed through a DEAE cellulose column and the two components of $N_2$ase were adsorbed. The major part of the unwanted adsorbed proteins were eluted from the column with dilute buffer and the two proteins called fraction 1 (MoFd) and fraction 2 (AzoFd) were eluted with an increasing gradient of $MgCl_2$. Fraction 1 was further purified by diluting it with dilute buffer, adsorbing it on a DEAE column, and again fractionating it but with a different elution gradient from that used for the first fractionation. Fraction 2 was further purified on Sephadex G-100 similar to a method previously reported (131, 135). This procedure (189) is rapid and looks quite promising.

A third more complicated purification procedure for nitrogenase of *C. pasteurianum* was reported (183). It combines a heat-treatment step with Sephadex G-100 fractionation. The proteins from this procedure were not pure and the activity was low. These authors also reported that a third protein was required for $N_2$ fixation. A careful evaluation of the requirement for a third component was made based on the latter report and it was concluded that no "easily-separated" third component was required (91), i.e., only two proteins, MoFd and AzoFd, were required.

**Azotobacter.** The first reports of purification of $N_2$ase from *Azotobacter vinelandii* (29, 30, 32) showed that the enzyme was released by breaking with the French pressure cell and that it also could be fractionated by ultracentrifugation, protamine sulfate fractionation, and gel filtration. Although in the earlier stages of purification by ultracentrifugation *Azotobacter* nitrogenase was reasonably stable to air, after treatment with protamine sulfate it was as sensitive to air as clostridial nitrogenase (a "protective membrane" component apparently had been removed).

A clever but unexpected modification of the above method (30) was used to purify and crystallize the Mo-Fe protein (35). The protein was purified through the protamine sulfate step similar to the above procedure. This was followed by fractionation on DEAE cellulose. Finally the Mo-Fe protein was concentrated and then diluted with weak buffer. At this step it crystallized from solution. The crystals were dissolved in a stronger buffer solution and after removing a small amount of undissolved material, the crystallization procedure was repeated. The crystals were white and about 50 $\mu$m long by 3 $\mu$m at the widest point. The activity in the $H_2$ evolution system with a large excess of the Fe protein was 1,488 nmoles of $H_2$ evolved per min per mg of Mo-Fe protein.

Although this technique is a valuable one for *Azotobacter vinelandii*, it unfortunately has not worked yet for crystallizing or purifying nitrogenase from other organisms.

It was reported (94) that nitrogenase of *A. vinelandii* could be separated into at least three components. One of the components obtained in a pure state contained Fe and Zn and had a molecular weight of 93,000. Possibly subunits of the Mo-Fe protein were isolated, but no further information on this fractionation has appeared.

Nitrogenase components were also purified from *Azotobacter chroococcum* (97) by a procedure similar to a previous one (32). The major difference was the use of $MgCl_2$ in the eluting buffer (99).

The Fe protein from *Azotobacter* also has been purified (133) and is quite similar in properties to the Fe protein of *Clostridium*. It will not complement with clostridial Mo-Fe protein, however.

**Nodules.** A most significant accomplishment was the first demonstration of $N_2$ fixation in extracts of bacteroids from soybean nodules (107, 108). Phenolic compounds, released when nodules were crushed, in some way destroyed nitrogenase and prevented previous experimenters from detecting $N_2$ fixation. These authors (107) countered this by macerating the nodules in a buffered ascorbate medium containing insoluble polyvinylpyrrolidone. The phenolics were complexed to the polyvinylpyrrolidone and were removed from solution. The bacteroids obtained from this macerate by filtration and differential centrifugation were broken anaerobically in a French pressure cell, and the extract, which was obtained free from insoluble material by centrifugation, contained active $N_2$ase. The nitrogenase of nodules was further purified and fractionated into two components similar to clostridium and azotobacter (105).

**Other bacteria.** Nitrogenase has been isolated (Table 1) and partially purified from several other organisms, *Klebsiella pneumoniae* (48, 98, 113), *Bacillus polymyxa* (53, 98), and *Mycobacterium flavum* 301 (18, 20), and two fractions similar to those of *Clostridium* and *Azotobacter* were obtained. Also, purification of nitrogenase of *Chloropseudomonas ethylicum* (63) and of *Cromatium* has been reported (201).

## Cross-Reactions

All the reactions peculiar to nitrogenase are manifest only when the two components of nitrogenase are combined. Since the nitrogenase components from different organisms have similarities, it was of interest to see if the large component (MoFd or Mo-Fe protein) would function with the smaller component (AzoFd or Fe protein) from different organisms. In the first study components from four different $N_2$-fixing organisms were tested in all possible combinations for their ability to reduce $N_2$ (48, 53, 54). Although the components used were DEAE fractions of crude extracts and not pure, it was easily seen that not all combinations were active (Table 3). Later the components were investigated for their ability to reduce substrates other than $N_2$, i.e., acetylene, azide, and cyanide (48, 98). The results obtained with these more sensitive assay systems were the same as those found with $N_2$ fixation. Varying the ratio of one component to the other or increasing the incubation time did not change a negative result to a positive one.

Of particular interest from these crosses was the fact that the nitrogenase components from physiologically similar organisms complement one another. For example, components of *Bacillus polymyxa* and *Klebsiella pneumoniae* were interchangeable, and both are facultative anaerobes which fix $N_2$ only when grown anaerobically. Components from physiologically dissimilar organisms such as *Azotobacter vinelandii*, a strict aerobe, and *Clostridium pasteurianum*, a strict anaerobe, do not complement each other. Combination of components from facultative anaerobes with those of either strict aerobes or anaerobes cross-react sporadically (Table 3). For example, MoFd from *C. pasteurianum* only was active when combined with AzoFd from *C. pasteurianum*, whereas Mo-Fe protein from *Bacillus polymyxa* was active when combined with all Fe protein preparations except that of *A. vinelandii*. Recently (167) complementation between components of *Anabaena cylindrica*, a blue-green alga, and *Chloropseudomonas ethylicum*, a photosynthetic bacterium, was demonstrated. The Mo-Fe protein of the alga complemented the Fe protein of the bacterium but the reciprocal cross was negative.

A complete protein structure study of all Fe and Mo-Fe proteins would be most interesting from a phylogenic point of view. One wonders how similar in structure the components of nitrogenase are. Information on the active site of nitrogenase might be obtained if one found regions containing similar amino acids common to all nitrogenases.

## Structural Properties of Nitrogenase

Since nitrogenase from several organisms has been resolved into two easily separable metalloproteins, the next step in under-

TABLE 3. *Complementary functioning of Mo-Fe and Fe proteins of nitrogenase from different microorganisms*[a]

| Mo-Fe protein from | Fe protein from | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | A[b] | B | C | D | E | F | G | H | I |
| A. *Azotobacter vinelandii* | + | 0 | + | + | − | − | − | − | − |
| B. *Clostridium pasteurianum* | 0 | + | 0 | 0 | − | − | − | − | 0 |
| C. *Klebsiella pneumoniae* | + | 0 | + | + | + | + | − | − | + |
| D. *Bacillus polymyxa* | 0 | + | + | + | + | + | − | − | + |
| E. *Azotobacter chroococcum* | 0 | 0 | + | +0 | + | + | − | − | + |
| F. *Mycobacterium flavum* | − | − | + | + | + | + | − | − | + |
| G. *Anabaena cylindrica* | − | − | − | − | − | − | + | + | − |
| H. *Chloropseudomonas ethylicum* | − | − | − | − | − | − | 0 | + | − |
| I. *Rhodospirillus rubrum* | − | − | − | − | − | + | − | − | + |

[a] Not all combinations were tested for $N_2$-fixing activity, i.e., some were only tested for acetylene reduction, others for $CN^-$ reduction, ATPase, and various combinations of these. For details, see references 20, 48, 54, 98, 167. Symbols: +, positive activity; 0, no activity; −, not tested; +0, low activity.

[b] Headings correspond to those in first vertical column, e.g., A = *Azotobacter vinelandii*.

standing the structure of the enzyme complex was to study the structure of these two nitrogenase components.

X-ray crystallography is probably the best technique available for the study of enzyme structure, but unfortunately it requires stable crystalline proteins of fairly large dimensions. To date there has only been one report of crystallization of a protein of nitrogenase (the Mo-Fe protein from *Azotobacter*) and the crystals were too small for X-ray crystallographic studies (35). Consequently other physical (particularly EPR and Mössbauer studies) and chemical techniques, which were previously applied successfully to the study of ferredoxin structure (21, 72, 112, 114) have been used.

**Fe protein (AzoFd) from C. pasteurianum.** AzoFd has a molecular weight of 55,000 and is comprised of two subunits (Table 4) each having a molecular weight of about 27,500 (138). The dimer contains 4 atoms each of iron and acid-labile sulfide and 12 sulfhydryl groups. Based on this molecular weight and recalculation of earlier results (135), two of the iron atoms of AzoFd complex readily with $\alpha,\alpha'$-dipyridyl (a ferrous iron chelating agent), whereas the remaining two iron atoms complex with the dipyridyl only in the presence of sodium mersalyl, a mercury reagent that combines with the sulfide and sulhydryl groups. The amino acid analysis and end groups of the monomer show that the protein lacks tryptophan and the subunits are identical (130).

Azoferredoxin is oxygen, cold and salt labile. The spectral changes that occur when it is subjected to low temperatures are different from those seen after oxygen treatment (135). After oxidation for 5 min only two of the four iron atoms of AzoFd complexed with $\alpha,\alpha'$-dipyridyl (in the presence of mersalyl) compared with four when the protein was untreated and cold inactivated. Since in the absence of mersalyl, $\alpha,\alpha'$-dipyridyl reacts quickly with only half of the iron whereas it reacts rapidly with all the iron in the presence of mersalyl, the iron atoms which measure as ferrous iron seem to be in two different environments.

Oxygen treatment of AzoFd for 5 min also oxidized 2 of its 4 sulfide groups and 4 of its 12 sulfhydryl groups (based on the dimer), whereas untreated and cold-inactivated AzoFd showed no oxidation of sulfide or sulfhydryl groups. Oxidation caused an irreversible loss of activity, and, since iron, sulfide, and sulfhydryl groups are affected by oxidation, it is probable that the active site of the protein is comprised of a combination of these groups. The fact that AzoFd (i) lost its iron and sulfide when acidified, (ii) was decolorized by treatment with sodium mersalyl, (iii) lost absorption between 300 and 600 nm on reduction by sodium dithionite, and (iv) has a low-temperature ESR spectrum of the $g = 1.94$ type (*unpublished result*), suggests that it is a ferredoxin-type protein.

**Fe protein from Azotobacter.** Although the Fe protein from *Azotobacter* has been obtained in a reasonably pure state, no structural investigations have been reported. However it resembles clostridial AzoFd in that it is cold and oxygen labile (133) and the activities are comparable (2,708 for *Clostridium* and 2,128 for *Azotobacter*).

**Mo-Fe protein (MoFd) from C. pasteurianum.** The molecular weight of MoFd is about 220,000 (minimum molecular weight

would be 110,000) based on gel filtration (139) and the number of subunits (*unpublished results*). The partial specific volume was found to be 0.72 ml/g (52). Treatment of the protein with 1% sodium dodecyl sulfate (SDS) followed by disc electrophoresis on gels containing 0.1% SDS gave rise to two nonidentical "groups" of subunits (139), one with a molecular weight of about 50,700 and the other around 59,500. Densitometer tracings of SDS gels and separation of subunits by gel filtration show that there are two 59,500 units and two 50,700 units. The sulfur, metal, and amino acid composition of MoFd is given in Tables 4 and 5. The electron-spin resonance spectrum of MoFd has *g* values of 4.25, 3.78, and 2.01. Less active preparations also have a *g* value of the 1.94 type (Palmer, Multani, Zunft, Cretney, and Mortenson, *submitted for publication*).

One molybdenum atom is associated with MoFd per molecular weight of 110,000 to 120,000. Treatment of MoFd with an excess of the thiol-complexing compound, sodium mersalyl, followed by gel filtration separated the Mo from the protein. All the sulfide and most of the iron atoms originally present also were released on treatment with mersalyl.

TABLE 4. *Sulfur and metal composition of nitrogenase of Clostridium*

| Composition | Azoferredoxin | Molybdoferredoxin[a] |
|---|---|---|
| Mol wt | 55,000 | 220,000 |
| No. and mol wt of subunits | 2 at 27,500 each | 2 at 59,500 and 2 at 50,700[b] |
| Mo per molecule | None | 2 |
| Fe per molecule | 4 | 18 |
| -SH per molecule | 12 | 30 ± 1 |
| $S^{2-}$ per molecule | 4 | 18 ± 1 |

[a] Also contains some $Mg^{2+}$ and $Ca^{2+}$ but no $Co^{2+}$ $Zn^{2+}$, etc.

[b] Number of subunits of MoFd based on microdensitometer tracing of disc gel stained with Coomassie blue and separation of the subunits.

**Mo-Fe protein (MoFd) from Azotobacter.** The Mo-Fe protein from *Azotobacter* has a molecular weight of 270,000 daltons (35). It contains 2 Mo atoms, 32 to 34 Fe atoms, and 26 to 38 labile sulfide groups per molecule. It is insoluble at a concentration of sodium chloride less than 0.08 M. Mössbauer and magnetic susceptibility determinations indicated a predominance of high-spin ferric iron and a small amount of high-spin ferrous iron in the isolated protein. Electron paramagnetic resonance (EPR) signals for the reduced metalloprotein at 4 K were observed at *g* values of 4.30, 3.67, 2.01, and 1.94. The Mo-Fe protein has been obtained without the 1.94 component so this probably is not a part of active Mo-Fe protein (Orme-Johnson and Brill, *personal communication*). The visible spectrum showed a shoulder at 410 to 420 nm in an otherwise undistinguished spectrum. An investigation of Mo-Fe protein from *Azotobacter* suggested it contained subunits, but the protein used was not pure and more study is in progress (182). In addition, an analysis of the type of iron in the Mo-Fe protein of *Azotobacter* was made. Fe was removed by *o*-phenanthroline-urea treatment, but no attempt was made to correlate this with activity.

**Mo-Fe protein (MoFd) from other organisms.** The molecular weight and metal composition of the two nitrogenase components from bacteroids of soybeans had recently been

TABLE 5. *Metal composition of MoFd (Mo-Fe protein) and AzoFd (Fe protein) from various organisms*[a]

| Component | *Clostridium pasteurianum* | | *Azotobacter vinelandii* | | *Azotobacter chroococcum* | Soybean | |
|---|---|---|---|---|---|---|---|
| MoFd or Mo-Fe protein | | | | | | | |
| Fe | 89 | 94 | 63 | 126–141 | 78.5 | 44 | 47.2 |
| Mo | 7.6[b] | 6.1 | 5.1 | 7.4 | 6.5 | 2.2 | 5.1 |
| AzoFd or Fe protein | | | | | | | |
| Fe | 73 | 71.5 | 41 | | 82 | 50 | 17.7 |
| Mo | 0 | Trace | Trace | | Trace | 1.0 | 0 |
| References | 52, 131, 135 | 189 | 32 | 35 | 97 | 105 | 17 |

[a] Values expressed as nanomoles per milligram of protein. Most of the data given here was calculated by us from data submitted in publications. The data in the publication usually was not as expressed here, and, since a precise value of the molecular weight has only been determined in a few cases, the term nanomoles of metal per milligram of protein has been used to facilitate comparison.

[b] A recent unpublished result.

estimated (17). By gel filtration component I (Mo-Fe protein) had an average molecular weight of 182,000 and component II (AzoFd) a molecular weight of 51,000. Table 5 presents a comparison of the metal composition of MoFd (Mo-Fe protein) and AzoFd (Fe protein) of four different nitrogenases. It is interesting that the metal compositions agree quite well with the exception of analysis of iron in the *Azotobacter* and soybean nodule Mo-Fe proteins and the iron in one case of the soybean Fe protein.

## ROLE OF REDUCTANT AND ATP IN $N_2$ FIXATION

### Demonstration of Requirements

The first cell-free extracts that consistently fixed $N_2$ (38) required pyruvate as supporting substrate, and it was obvious that one function of pyruvate was to serve as the electron donor for $N_2$ reduction. Later it was discovered in *Clostridium pasteurianum* that the more immediate source of electrons for $N_2$ reduction was reduced Fd (125). The immediate source of electrons for $N_2$ reduction in *Azotobacter* and *Anabaena cylindrica* probably is a ferredoxin (iron sulfur protein) recently shown to be present in *Azotobacter* extracts (11, 160, 208), and the transfer of electrons to nitrogenase in bacteroids from soybean nodules also appeared to be catalyzed by an Fe protein (109, 206). NADH also can serve as an electron donor for all these systems (104, 129, 204, 205), but only indirectly, since the electrons from NADH must be transferred to a Fd before they can be used by nitrogenase (129). The production of reduced Fd needed for $N_2$ fixation can be linked to the photosynthetic electron transport chain (165, 166, 205) and, therefore, coupled to light. The latter probably is the true coupling in photosynthetic organisms.

When reduced Fd failed to serve as the sole requirement for $N_2$ fixation in extracts of *C. pasteurianum*, it was discovered that ATP was also required (77, 116, 124). It had already been established that during pyruvate metabolism ATP was produced from acetylphosphate by acetokinase and the details of these discoveries are well documented and reviewed (73, 122, 129). Now that the two components, AzoFd (Fe protein) and MoFd (Mo-Fe protein), have been purified, the precise requirements for $N_2$ fixation can be defined as ATP, $Mg^{2+}$, reductant (reduced Fd or $S_2O_4^{2-}$, an artificial donor), and the two protein components that collectively are called nitrogenase. The reaction must be run in the complete absence of oxygen and obviously $N_2$ must be supplied as substrate.

Since the products of ATP utilization by nitrogenase are ADP and inorganic phosphate and since ADP inhibits the functioning of $N_2$ase (see section on Control), ATP is best supplied to the system via an ATP-generating system, viz. ADP, $Mg^{2+}$, acetylphosphate, and ATP:acetate phosphotransferase.

### Reductant-Dependent ATPase, ATP-Dependent $H_2$ Evolution

The first report that the utilization of ATP during $N_2$ reduction was coupled to electron flow in nitrogenase stated that reduced Fd was needed for maximum ATP (acetylphosphate) utilization (124, 127). The system used was as follows: a crude extract of *C. pasteurianum*, lithium acetylphosphate, $Mg^{2+}$, and a gas phase of either $H_2$, $N_2$-$H_2$, $N_2$-argon, argon, or $N_2$. The electrons were supplied to nitrogenase from $H_2$ via the hydrogenase-Fd couple. With the first two $H_2$-containing gas atmospheres, acetylphosphate was rapidly consumed, but the rate greatly decreased (to 25% or less) under the latter three gas atmospheres. It was concluded that "the nitrogen-fixing system in the absence (and to some extent in the presence) of $N_2$, acts like a $H_2$ (reduced Fd)-requiring ATPase." In other words ATP utilization coupled to $N_2$ reduction was reductant-dependent. Shortly after this it was shown that carbon monoxide did not inhibit acetylphosphate consumption (ATP) by the nitrogenase system although it did inhibit $N_2$ fixation (57).

A valuable contribution to an understanding of the mechanism of $N_2$ fixation was the discovery that sodium dithionite ($S_2O_4^{2-}$) could serve as the reductant (30) in place of reduced Fd. *Azotobacter* extracts fixed $N_2$ if an ATP-generating system, $N_2$, and $S_2O_4^{2-}$ were supplied. When $N_2$ fixation was examined by manometric techniques using the above system, it was found that, instead of measuring $N_2$ uptake as expected, a gas was evolved which was shown to be $H_2$. Dihydrogen was evolved at an even greater rate in the control flasks under argon. The evolution of $H_2$ was completely dependent on the presence of *both* $S_2O_4^{2-}$ and ATP. Under $N_2$, $N_2$ fixation occurred which consumed reductant and hence competed with $H^+$ for the electrons. However, with $N_2$ as substrate as much as 30% of the electrons coupled to ATP utilization still reduced $H^+$ and produced $H_2$. The amount of $N_2$ fixed by this system was a function of the difference between the gas evolved under $N_2$ and under argon or helium.

These same observations were made with the clostridial system (33). However, since in crude extracts of *Clostridium* $S_2O_4^{2-}$ reduced Fd and hydrogenase oxidized the reduced Fd to yield $H_2$, hydrogenase had to be inhibited to observe ATP-dependent $H_2$ evolution in the presence of the large quantity of $H_2$ evolved by hydrogenase. Previous reports showed (57) that CO did not inhibit ATP (acetylphosphate) utilization coupled to $N_2$ fixation and that CO inhibited hydrogenase. Therefore, by addition of CO, ATP-dependent $H_2$ evolution was now readily demonstrated.

Obviously then, reductant-dependent ATPase (80, 124) and ATP-dependent $H_2$ evolution (30) represent different ways of measuring the same activity. In the previous studies (124) $H_2$ was evolved but not measured.

### ATP and Electron Requirements for Reduction of Substrates by Nitrogenase

**With pure components.** Considerable effort has been expended to determine the ratio (stoichiometry) of ATP utilized to electrons transferred to the substrates reduced by nitrogenase (27, 32, 70, 92, 107, 128, 200). The results obtained with the purest systems available, and without any corrections for ATP utilization not coupled to reduction, indicate that the lowest ATP/2e$^-$ ratios are 4.3 and 3 for *Azotobacter* and *Clostridium*, respectively. *No* ATP is hydrolyzed in the absence of either component of nitrogenase.

In the absence of reductant, clostridial nitrogenase hydrolyzes ATP at one-tenth to one-fourth the rate of reductant-dependent hydrolysis. When this reductant independent "ATPase" was examined at increasing *p*H values (27, 92), ATP consumed without reductant decreased with increasing *p*H, whereas ATP consumed with reductant remained reasonably constant. ATP-dependent $H_2$ evolution increased with increasing *p*H. If one assumes that that ATP consumed in the absence of reductant is not coupled to $N_2$ fixation even when reductant is supplied and therefore the total ATP consumed in the presence of reductant can be corrected for that ATP consumed in the absence of reductant, the ratio of ATP required for each electron transferred to nitrogenase and coupled to reduction is one. The number of ATP molecules required to reduce a molecule of $N_2$ to two of ammonia would, therefore, be six. However, it is possible that in the presence of reductant more ATP is shunted into reductant-dependent "ATPase" and it is not at all certain that these corrections are valid. Also one must consider the ratio of Fe to Mo-Fe protein present in the "nitrogenase" complex (90).

Another explanation for the higher result (4.3 ATP/2e$^-$) with *Azotobacter* is needed since under similar conditions with *Azotobacter* nitrogenase there was only a small reductant-independent ATP utilization. One suggestion was that in the functioning of *Azotobacter* nitrogenase ATP utilization is partially uncoupled after the initial required ATP-binding step (70). It was suggested that the ATP-nitrogenase complex initially formed further reacted in two pathways, one leading to ATP hydrolysis not coupled to electron transport and the other coupled. There is, of course, the possibility that more than one ATP-nitrogenase complex may be formed with different ratios of Fe and Mo-Fe proteins. These "different" nitrogenases may utilize ATP in different manners.

In spectrophotometric experiments designed to determine the mechanism of electron flow during $N_2$ fixation, it was found that reduced AzoFd gave up one electron when oxidized by certain dyes (M. Walker and L. E. Mortenson, *to be published*). Under similar conditions MoFd gave up two electrons. If reduced AzoFd (3 nmoles) and reduced MoFd (11 nmoles) were combined (in the absence of dyes), no oxidation of either component was detected. However if ATP was added, rapid "oxidation" occurred. Complete oxidation of both components occurred on addition of 36 nmoles of magnesium ATP. Since MoFd can give up two electrons and AzoFd one electron, the combined electrons that can be donated (to $H^+$?) was 25 nmoles. This suggested that one ATP was required for each electron removed. It also suggested that under these conditions there was little "reductant independent" ATP hydrolysis. The ratio of AzoFd to MoFd for this experiment was much less than the optimum for $N_2$ fixation, but apparently was high enough to allow the system to operate. Information from such experiments is valuable in suggesting a model for the catalysis of reductions by nitrogenase and substantiation of such experiments is needed.

**With whole cells.** An examination of the energy required to produce a gram of *C. pasteurianum* when cells are grown with $NH_4^+$ compared with $N_2$ as nitrogen source revealed a major difference (46). The $Y_{ATP}$ (grams of cells produced per mole of ATP consumed) for cells growing with sucrose as carbon and energy source and $NH_4^+$ as nitrogen source was about 10.3 compared to 6.3 for cells with $N_2$ as the nitrogen source. This showed that a con-

siderable amount of ATP is consumed during dinitrogen fixation. It was estimated (46) that about 13 molecules of ATP are needed for each molecule of $N_2$ fixed. This estimated result assumed certain things about the metabolism of the organism and corrected for an energy loss resulting from the slower growth rate of the $N_2$-fixing cells. The result was obtained under sucrose-limited conditions in a chemostat where presumably sucrose would be used most efficiently. Under $NH_4^+$-limiting conditions extremely high levels of nitrogenase were synthesized and the culture behaved as if it were a $N_2$-fixing culture, i.e., it had a $Y_{ATP}$ of about 6.0. In other words, if nitrogenase was present but no $N_2$ was available, energy was wasted. This indicated that even when $N_2$ was present, energy was wasted and would explain the difference between 13 ATP/$N_2$ fixed in whole cells compared to the possible 6 ATP/$N_2$ fixed in the pure system if a correction for "wasted" ATP utilization was made (92). If the correction for reductant-independent ATP utilization with pure nitrogenase was not made, then 12 ATP were required per $N_2$ fixed, a result close to the estimate for whole cells.

When *Azotobacter chroococcum* was grown in a chemostat with limiting carbon source and its growth with $N_2$ as nitrogen source compared with $NH_4^+$, a distinct difference also was seen (51). Assuming a $Y_{ATP}$ of 10.5 and that at maximum growth the respiration required for "respiratory protection" was at a minimum, these authors estimated that 4 to 5 moles of ATP were available for each mole of $N_2$ fixed. This estimated result is about half that estimated for *C. pasteurianum* (46) and might suggest that in *A. chroococcum* ATP utilization for $N_2$ fixation is more tightly coupled than in *Clostridium*.

## FUNCTION OF MOLYBDOFERREDOXIN (MoFe PROTEIN) AND AZOFERREDOXIN (Fe PROTEIN)

Neither of the two metalloprotein components of nitrogenase has activity alone. Thus it can be concluded that sites on both are required for the reductions catalyzed. Since ATP and ADP affect dinitrogen fixation, binding of either of these two components specifically to either or both MoFd and AzoFd might suggest a role for the individual proteins in $N_2$ fixation. In addition, if it could be shown that only one of the two proteins is reduced *specifically* with sodium dithionite, one might argue that the one reduced accepts electrons from either reduced Fd or dithionite and transfers the electrons to the substrate to be reduced ($N_2$). Finally, since the two components are absolutely necessary for reduction with reduced Fd or dithionite as electron donors, one must know the optimum ratio of the two components required for activity (ratio in the functioning unit).

### ATP Binding

The first problem, binding of the supporting substrate ATP and its inhibitory product, ADP, was investigated with pure components of *C. pasteurianum* (28). It was found that AzoFd bound ADP and ATP but that $Mg^{2+}$ was required only for ATP binding. Furthermore, it was found that MoFd bound only traces of these components. This suggested that in the functioning of nitrogenase AzoFd bound the supporting substrate, magnesium ATP, and brought it into a complex with MoFd where it was utilized in the reduction process (for electron activation). Less than 100% of this ATP utilization is coupled to reduction of substrates since it was shown in studies with pure nitrogenase that some of it (various between 10 to 25%) was hydrolyzed in the absence of reductant. ADP also bound to AzoFd and since its role seems to be to decrease the functioning of nitrogenase as the ATP concentration decreases, the facts that it and ATP (as the $Mg^{2+}$ complex) bound to AzoFd and that the kinetics indicated three sites for ADP action (134) suggested that ADP controls by a combination of competitive inhibition and a negative modifier action. Other nucleoside diphosphates such as GDP and CDP did not displace ADP from AzoFd nor did they inhibit so the binding was specific for ADP.

ATP binding was investigated also with partially purified components of *Klebsiella pneumoniae* (19). In these studies it was found that both the Fe protein and the Mo-Fe protein bound ATP. An hypothesis for why the previous studies (28) showed specific binding to AzoFd whereas these studies showed nonspecific binding was given but was not adequate. No demonstration of ADP binding was presented nor was an explanation of the specificity for ADP binding given. One possible explanation could be the differences in the two proteins, i.e., the proteins from the two species do not cross-react (37, 54).

### $CN^-$ Binding

A substrate of nitrogenase, $CN^-$, binds to both nitrogenase components so its binding can not be used to speculate on the site where

$CN^-$ is reduced. Early studies with clostridial components (28) suggested $CN^-$ bound mainly to MoFd but the binding was not specific enough to make this claim. Mossbauer studies have shown that $CN^-$ in the presence of $^{57}Fe$ protein and Mo-$^{56}Fe$ protein affects the Mossbauer signal, whereas with the opposite labeling no effect was seen (101). This suggested that the Fe protein was required for $CN^-$ to be bound and reduced. These studies are still speculative since they could not be repeated with purer preparations.

### Reduction by Dithionite

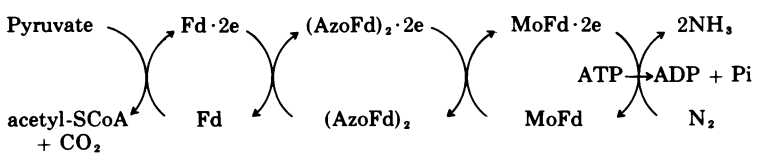
Since reduced Fd and dithionite act as electron donors for $N_2$ fixation, a demonstration that they reduce either of the two components of nitrogenase in a specific manner would be indicative of the mechanism of electron flow from reduced Fd to $N_2$. Oxidized and reduced spectra of partially purified *Azotobacter* Fe and Mo-Fe proteins indicated a change in spectrum of both proteins when dithionite was added (32). With pure components of *C. pasteurianum* there is a change in spectrum on addition of dithionite to AzoFd (135), but there is very little change in the spectrum of MoFd. This is a result of how the proteins are prepared. If, during purification, they are kept under completely reducing conditions, a requirement necessary for full activity, then there is little effect of dithionite addition. Oxidation (in air) of either protein led to distinct changes in the spectrum of both proteins (32, 76, 121, 135). Oxidation of MoFd and AzoFd also resulted in the appearance of a large signal in the $g = 2$ region when examined by electron-spin resonance techniques. The signal could result from high-spin ferric iron but also could result from "super oxide" formation (145). The "$g = 2$" signal with AzoFd disappeared on longer air treatment, whereas the signal with MoFd was much more stable (52). Attempts to eliminate the $O_2$-produced signal of MoFd by reduction with dithionite were unsuccessful. Addition of AzoFd (active form with no "$g = 2$" signal) to MoFd that had a large $O_2$-produced signal also showed no loss of signal, but, if both AzoFd and dithionite were added, the signal decreased. This suggested that electrons for $N_2$ reduction flowed from reduced Fd to AzoFd to MoFd to $N_2$:

Since AzoFd (55,000 molecular weight) appears to accept one electron (*unpublished data*), this suggested system would need to generate six reduced AzoFd molecules to completely reduce $N_2$ to two $NH_3$ and, of course, lesser numbers for other reductions (two to reduce $2H^+$ to $H_2$). A precise mechanism must await further data from the clostridial system as well as the purified systems from other organisms.

### Ratio of Fe Protein to Mo-Fe Protein

The final structure of nitrogenase must take into account the number of each of the subunits of AzoFd (Fe protein) and MoFd (Mo-Fe protein) required for the functioning unit. Kinetic studies with pure components should allow one to make reasonable estimates of the optimum ratio of subunits. For example, if one assayed the activity of nitrogenase by maintaining either of the components at a constant level and varying the other, one readily would see, from the shape of the curves of a plot of activity versus the concentration of the varied species, whether or not the ratio of AzoFd to MoFd was greater than one to one. Results with a partially purified clostridial system (37,189) suggested that two "40,000"-dalton Fe protein units were required for each "160,000"-dalton Mo-Fe protein. Since the molecular weight of AzoFd as isolated in solution is actually 55,000 daltons (two identical subunits of 27,500 each) and since MoFd has a minimum molecular weight of 110,000 (this is the molecular weight when the number of subunits are at the lowest common denominator, i.e., one 59,500 unit to one 50,700 unit; a ratio that has been firmly established), the above results were only suggestive. In addition, pure proteins must be used to know how much of each protein is present.

This ratio was recently estimated (90) with pure components of nitrogenase and knowledge of the molecular weights (138,139). Two assays were used, ATP-dependent $H_2$ evolution and $N_2$ fixation. From the kinetics obtained by varying each of the components of nitrogenase individually and from plots of activity versus AzoFd/AzoFd + MoFd and MoFd/MoFd + AzoFd, the lowest common ratio of the subunits of AzoFd and MoFd that was required for the functioning unit was two 59,500- to two 50,700-dalton subunits of MoFd to four (this has not been rigorously estab-



lished) 27,000-dalton subunits of AzoFd (90 and unpublished recent results). A computer program was set up based on this data and the following equations: MoFd (220,000) + (AzoFd dimer) (55,000) $\rightleftarrows$ MoFd·AzoFd dimer; MoFd·AzoFd dimer + AzoFd dimer $\rightleftarrows$ MoFd·(AzoFd dimer)$_2$. Plots of activity versus either MoFd or AzoFd (with the other component constant) were close to the experimental plots provided the equilibrium constants for the two reactions were similar and close to one. When MoFd was kept constant and AzoFd varied, the plot of activity versus AzoFd exhibited sigmoidal kinetics as would be expected from the above equation. That is, since the active complex appears to be MoFd (AzoFd dimer)$_2$, the major complex at low AzoFd concentrations would be the inactive 1:1 adduct and one would expect sigmoidal kinetics. When AzoFd was kept constant and MoFd varied, the plot of activity versus MoFd exhibited initial hyperbolic kinetics followed by a decrease in activity. Again this would be expected since initially most MoFd would be in the active form MoFd·(AzoFd dimer)$_2$, whereas at high MoFd concentration a greater portion of the AzoFd would be in the inactive form, MoFd·AzoFd dimer.

## CHEMICAL $N_2$-FIXATION

To supplement biological $N_2$ fixation, the fertilizer industry has relied upon a number of chemical processes to obtain ammonia. Probably the most important is the Haber-Bosch process (69) in which dinitrogen and dihydrogen react to form ammonia. The Haber-Bosch process requires temperatures as high as 300 C and pressures between 200 and 1,000 atm, whereas the biological $N_2$-fixing process performs with maximum efficiency at about 30 C and 0.1 atm of $N_2$. In both industrial and biochemical $N_2$ fixation, metals are involved; Fe and Mo are part of nitrogenase and Fe and other metals are part of the industrial catalysts. Before one can understand how these metals are involved in $N_2$ fixation, it is necessary to study the structure of the nitrogen molecule itself and to inquire how such a molecule can be attacked and subsequently reduced.

Nitrogen is extremely inert; it has a high dissociation energy (224.5 kcal) and high ionization potential (15.5 ev). It is isoelectronic with carbon monoxide (dissociation energy, 256.2 kcal; ionization potential, 14.0 ev). However, carbon monoxide is much more reactive than $N_2$, even though it has a higher dissociation energy, because of a lone pair of electrons on the carbon atom which allows bond formation with various metals (89). The energy levels in the nitrogen molecule are given in reference (43). The lone pairs of electrons found in the 2 s orbitals of low energy, are very tightly held, and are therefore unsuitable for bond formation with metal.

How then can $N_2$ react? It is very difficult to oxidize since an electron must come from the highest filled orbital, the 3 $\sigma_g$ orbital at $-15$ ev (43). It is also very difficult to reduce $N_2$ since electrons must be transferred into the vacant orbital of lowest energy, 1 $\pi_g$ at $-7$ ev. Any molecule capable of putting electrons in this high orbital, and the very electropositive elements such as lithium will do so, is also capable of effecting the reduction of $H^+$.

Since it appears that neither oxidation nor reduction solely was involved in biological $N_2$ fixation, Chatt and Leigh (43) proposed that the nitrogen molecule behaved as a concerted electron donor and acceptor with respect to the metal species. As a donor, electrons from the filled orbital of highest energy (the 3 $\sigma_g$ orbital) are donated to a vacant orbital on the metal to form a sigma bond. This bonding would allow electrons to move from the orbitals of the metal to the vacant orbitals of lowest energy in the nitrogen molecule (1 $\pi_u$) to form two $\pi$ bonds. Such coordination would weaken the N $\equiv$N bond and cause the molecule to become electrically assymetric. A transition metal ion of group VIII of the periodic table should have electrons of suitable energy in its d orbital in normal oxidation states to allow bond formation with $N_2$.

A recent series of discoveries have led to the isolation of various transition metal complexes in which $N_2$ is present as a ligand (Table 6). In addition to these complexes there have been two reports of the binding of $N_2$ by organic sulfur compounds (62, 146).

What does the existence of such complexes tell us about $N_2$ fixation? To begin with, it is now obvious that dinitrogen can be chemically "complexed" by various metals at ordinary temperature and pressure in an aqueous environment. In addition, it appears that coordination of dinitrogen to metals reduces its bond strength as evidenced by the appearance of a strong infrared absorbance around 2,100 per cm. (The stretching vibration of the nitrogen molecule occurs at 2,331 per cm and is active only in the Raman spectrum and inactive in the infrared.) Although in one of the complexes (Rhenium) the N $\equiv$N stretching frequency was around 1,920 per cm, an indication that the

TABLE 6. *Isolation of transition metal complexes in which $N_2$ is present as a ligand*

| Complex | N≡N stretching frequency | Year | Reference |
|---|---|---|---|
| $[Ru(NH_3)_5N_2]X_2$[a] | 2,105–2,167 | 1965 | 1 |
| $(Ph_3P)_2Ir(N_2)Cl$ | 2,095 | 1966 | 44 |
| $(Ph_3P)_3Co(N_2)H$ | 2,082 | 1967 | 152 |
| $(Ph_3P)_3Co(N_2)$ | 2,088 | 1967 | 119, 202 |
| $[Os(NH_3)_5N_2]L_2$[b] | 2,010–2,061 | 1967 | 3 |
| $(Ph_3P)_2Rh(N_2)Cl$ | 2,152 | 1967 | 187 |
| $Fe\ H_2\ N_2\ L_3$[c] | 2,055–2,060 | 1968 | 151 |
| *trans* $Fe\ H(N_2)$-$depe_2^+\ BPh_4^-$[d] | 2,090 | 1969 | 3 |
| [Re Cl ($N_2$) L][e] | 1,920–2,035 | 1969 | 41 |
| [Re Cl ($N_2$) (P Me Ph)$_4$] | 1,920 | 1969 | 41 |
| [Re (CO)$_3$ $NH_2$ ($N_2$) L][e] | 2,220 | 1969 | 120 |
| [Re (CO)$_2$ $NH_2$ ($N_2$) L][f] | 2,225 | 1969 | 120 |
| *trans*-[Mo(diphos)$_2$ ($N_2$)] | 2,020 Weak<br>1,970 Strong | 1969 | 84 |

[a] X = $PF_6$, $BF_4$, I, Br, Cl.
[b] L = $BPh_4$, $BF_4$, $ClO_4$, I, Br, Cl.
[c] L = $PEtPh_2$, $PBu\ Ph_2$.
[d] depe = $Et_2\ PH_2\ CCH_2\ PEt_2$.
[e] L = $(Ph_2\ PCH_2 \cdot CH_2 \cdot PPh_2)_2$; $(PMe_2Ph)_4$; $(Ph_2\ PCH_2 \cdot PPh_2)_2$; $(Ph_2\ PCH = CHPPh_2)_2$; $(PMe\ Ph_2)_4$; $[(PPh_3)_2(CH_3C[OCH_2]_3P)_2(Ph_2\ PCH_2 \cdot PPh_2)_2]Cl$.
[f] L = $P\ Me_2Ph$.

nitrogen molecule may have been made more reactive by being complexed, none of the complexes have produced ammonia upon reduction (41).

We also know that, if a metal ion is to combine with $N_2$ in end-on bonding as predicted (43), it must have a sufficient number of electrons in its d orbitals to permit effective $\pi$ bonding with $N_2$. Metals from group VIII of the periodic table can generally satisfy this condition and, in fact, Fe, Co, Ru, Rh, Re, and Mn, all group VIII elements, will form $N_2$ complexes. This might rule out Mo (a constituent of nitrogenase) as a possible candidate for complexing $N_2$ since it would have to adopt an unnatural oxidation state of about 2, but molybdenum complexes containing the $N_2$ molecule have now been found in which the oxidation state of the molybdenum atom is 4 (42). Iron, the other metal component of nitrogenase, satisfies the above condition in either of its valency states (40). Obviously the ligands of the metals in nitrogenase are extremely important in facilitating coordination of $N_2$ to the metal. At present we have only tentative evidence concerning such ligands but data (Dalton and Mortenson, *unpublished data*) indicated that two of the ligands are R-SH and sulfide (see section on components of nitrogenase).

The preparation of these synthetic $N_2$ complexes have been helpful in clarifying the possible mode of attachment of $N_2$ to a metal. However, unlike the $N_2$ complex in the biological system, no method for reducing the coordinated dinitrogen to ammonia has been found. There have been, however, reports of systems in which ammonia has been formed from $N_2$. Characteristic of these reducible systems is the *lack* of stable $N_2$ complexes. This indicates that a stable $N_2$ complex should not be proposed as part of a nitrogenase model but rather the model should invoke an unstable $N_2$ complex.

The first of the systems where $N_2$ fixation occurred but no $N_2$ complex was isolated was published in 1964. Haight and Scott (71) showed that $N_2$ could be electrolytically reduced to ammonia in the presence of molybdate or tungstate ions. They suggested that $N_2$ interacted with either Mo IV or Mo V.

In their search for activation of molecular nitrogen by transition metal complexes, Vol'pin and Shur (194, 195) studied the behavior of $N_2$ in systems capable of forming complexes with olefins, carbon monoxide, acetylene, and various other unsaturated compounds. The system they studied most completely was a solution of dicyclopentadienyltitanium dichloride in ether with ethyl magnesium halide (196). At room temperature and a pressure of 1 atm of $N_2$, 0.7 mole of ammonia was produced for each titanium species. Like the biological system this $N_2$ fixation was completely inhibited by carbon monoxide. The mechanism of this reaction has been elucidated further by Brintzinger (24–26) and Maskill and Pratt (115).

Certain metals (Cr, V, Ti) complexed to $\sigma$ and $\pi$ naphthalene residues also reacted with nitrogen gas and the complex liberated ammonia on hydrolysis (83). In the absence of $N_2$, the reduced species liberated dihydrogen on hydrolysis. The amount of dihydrogen released was proportional to the number of electrons available. In this respect the system is similar to nitrogenase and to the system of Vol'pin and Shur (194) which releases more $H_2$ in the absence of $N_2$ than in its presence. This similarity with nitrogenase should be noted (see section on ATP dependent $H_2$ evolution).

Van Tamelan et al. (190–192) extended this work to develop a $N_2$-fixing process which was the first report of a regenerative (catalytic) as opposed to a stoichiometric conversion of $N_2$ to ammonia at normal temperature and pressure. In this system a proton source (isopropyl alcohol) was added to titanium tetraisopropoxide in the presence of an electron donor

(the anion of naphthalene) and $N_2$. The ammonia produced was removed, and, after regeneration of the electron source and the titanium species, the cycle was repeated. Over a period of five cycles a 340% yield of ammonia was obtained, during which approximately 2 moles of ammonia were produced for each mole of $N_2$ consumed. The nonprotonated $N_2$ intermediate was unstable and could not be characterized. This appears to be a property similar to nitrogenase.

Recently Schrauzer and Schlesinger (157–159) investigated the ability of various thiol complexes of transition metals (which may approximate the active site of nitrogenase) to reduce acetylene (a substrate of nitrogenase) to ethylene in aqueous solutions in the presence of sodium dithionite (an electron donor for nitrogenase). Of 25 metals tested, molybdenum was the most active followed by iridium which converted acetylene to ethylene at 15% of the rate of the molybdenum system. Little or no activity was observed with their other metal thiol systems including iron, although iron stimulated the reduction. Substrates of nitrogenase such as sodium azide, nitrous oxide, and methyl acetylene were reduced to the same products as observed with nitrogenase. It was also reported that $N_2$ was reduced to ammonia (159), but the rate was $10^5$ to $10^6$ less than nitrogenase even when the pressure was 2,000 psi.

This group is presently trying to increase the rates of these reactions. Since iron did not function in their system whereas Mo did and since the products of the reductions catalyzed by the Mo thiol are the same as nitrogenase, these authors have concluded that iron is not involved in $N_2$ reduction but only in the transfer of electrons of the $N_2$-Mo-thiol complex. Newton et al. (140) have taken exception to this conclusion and, in fact, have shown that certain iron complexes in a system including either sodium borohydride or sodium dithionite and substrate (acetylene, $N_2$, etc.) do catalyze the reduction of nitrogenase substrates.

### Conclusions

We now have some model systems consisting of metal complexes that will mimic nitrogenase, albeit at *much* reduced rates. It seems likely that studies on nitrogenase and the metal complexes will complement each other and should in the future give us a reasonable and workable model for the enzyme.

## SUMMARY OF PURIFICATION, PROPERTIES AND MECHANISM SECTIONS

As it stands now nitrogenase consists of two *easily* separable major metalloprotein components; one is called azoferredoxin (AzoFd) or Fe protein and the other molybdoferredoxin (MoFd) or Mo-Fe protein. AzoFd from all organisms examined seems to have a molecular weight in the 50,000 range, and purified AzoFd when isolated from *Clostridium pasteurianum* is a dimer of two identical subunits of molecular weight of 27,500. On the basis of 55,000 daltons, it contains 4 ferrous iron atoms (all measure as $Fe^{2+}$), 4 acid-labile sulfide groups, and 12 free sulfhydryl groups which together appear to comprise the chromophore(s). MoFd has a molecular weight of about 220,000 when from *Clostridium*, about 180,000 when from soybean nodules, and 270,000 when from *Azotobacter vinelandii*. Based on a molecular weight of 220,000, MoFd from *Clostridium* contains 2 Mo atoms, 18 iron atoms, 18 sulfide groups, and 30 "free" sulfhydryl groups all of which may complex to form the chromophore(s). MoFd from *Clostridium* contains subunits of 59,500 and 50,700 daltons. Data have suggested there are two 59,500 and two 50,700 subunits in each MoFd, but this has not been firmly established. No data are published yet on the subunit structure of the Mo-Fe proteins from other organisms although *Azobacter* Mo-Fe does seem to have subunits.

Nitrogenase reduces $N_2$, $CN^-$, $N_3^-$, $N_2O$, and isocyanides as well as $H^+$. For these reductions magnesium ATP, reduced Fd (or $S_2O_4^{2-}$), and the Mo-Fe and Fe proteins are required. The products of ATP utilization are ADP and Pi. ADP, if allowed to accumulate, inhibits further nitrogenase activity.

In the clostridial system, the requirement for a unit of nitrogenase appears to be two moles of AzoFd (55,000 molecular weight) to each mole of MoFd that contains two 59,500- and two 50,700-dalton subunits of MoFd.

Almost every laboratory working in the area of $N_2$ fixation has proposed a general mechanism for the process (25, 28, 37, 70, 73, 88, 124, 129). Based on these and from present knowledge, a mechanism can be suggested (Fig. 3). All mechanisms must take into account the requirements listed above but it is premature to decide how each of the subunits of nitrogenase functions in $N_2$ fixation. For example, it is possible that the Fe protein (AzoFd) initially binds the [MgATP] required for $N_2$ reduction and brings it into a complex with Mo-Fe pro-

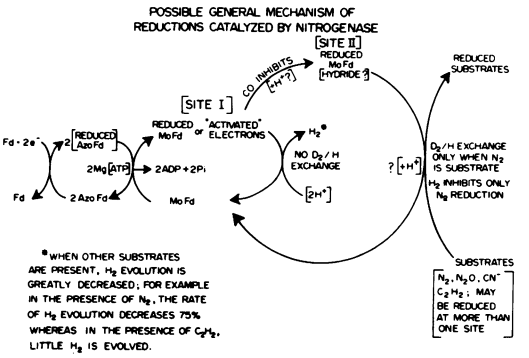



FIG. 3. *Proposed general scheme for reductions catalyzed by nitrogenase.*

tein (MoFd) where it is consumed in activating the electrons needed for $N_2$ reduction. Data also suggested that AzoFd is the component reduced by dithionite and that electrons travel from the donor, ferredoxin, to AzoFd and then to MoFd.

Since MoFd is absolutely necessary for $N_2$ fixation and ATP utilization and since electrons can possibly flow from either ferredoxin (Fd binds to MoFd) or AzoFd to MoFd, MoFd may function in an "electron activation" step and act as a pool of electrons for $N_2$ reduction. It may also play a role in transferring electrons to the substrate reduction site(s). This is suggested from the observations that when iron was removed from MoFd in a stepwise manner, the first activities lost were ATP-dependent $H_2$ evolution, acetylene reduction, and $N_2$ fixation; ATP utilization remained after as much as 50% of the other activities or iron was lost. That molybdenum may play a role in reductant-dependent ATP utilization is suggested by the result that, when up to 50% of the iron has been removed from MoFd by $\alpha,\alpha'$-dipyridyl, little or no Mo was released.

There is no doubt that in the next few years a more detailed and accurate model for the structure and mode of action of nitrogenase will be available. At the present time, however, there are many conflicting results and inconsistencies, and any model now proposed will be subject to considerable error. Quantitative data necessary for designing a detailed model only now is becoming available, mainly because pure nitrogenase and the techniques for handling pure nitrogenase only now are available.

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