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In Vivo and In Vitro Kinetics of Nitrogenase†

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We measured some of the kinetic parameters of nitrogenase in intact systems of *Clostridium pasteurianum* and *Klebsiella pneumoniae* to compare them with the kinetics of the enzyme in vitro. We found that the enzyme showed multiple apparent K_m values for acetylene reduction in vivo, as it does in vitro. Carbon monoxide was a noncompetitive inhibitor of acetylene reduction; azide was a noncompetitive inhibitor of acetylene reduction, and nitrogen was a partial inhibitor of acetylene reduction. Cyanide was a noncompetitive inhibitor of acetylene reduction in *C. pasteurianum* but it was a metabolic poison in *K. pneumoniae*, in addition to being an inhibitor of nitrogenase. The partial nature of nitrogen inhibition was apparent in assays where both nitrogen and CO were present. Nitrogen did not alter the apparent K_i for CO, nor did the presence of CO enhance the competitive effectiveness of nitrogen. By using recombined nitrogenase fractions, we found that the ability of nitrogen to inhibit hydrogen evolution or acetylene reduction varied with the ratio of protein components. The in vivo inhibition of acetylene reduction by dinitrogen was comparable to that obtained with an excess of the Fe protein in vitro. We conclude that there is an effective excess of the Fe protein available under active growth conditions in vivo.

Nitrogen fixation and the properties of nitrogenase were studied in vivo for many years before the development of active cell-free extracts of nitrogen-fixing organisms (20). The assays were difficult because nitrogen fixation had to be measured by growth of the organisms or incorporation of $^{15}N_2$ from dinitrogen gas. The ability of nitrogenase to reduce acetylene was not discovered until after the advent of active cell-free extracts, and thus this relatively sensitive and rapid assay has not been used systematically to study kinetic parameters of nitrogenase systems in vivo. Now that a large body of literature has accumulated concerning the in vitro properties of nitrogenase as a reducer of acetylene, cyanide, azide, and nitrogen, we have reexamined the in vivo kinetics of nitrogen-fixing organisms.

There are certain constraints on in vivo studies of nitrogenase activities. Nitrogen reduction may only be measured directly by use of $^{15}N_2$, which requires a significant investment in apparatus and isotopes. Hydrogen evolution is not directly accessible since most organisms have an effective uptake hydrogenase which recycles hydrogen before it is released into the culture. Some organisms such as the clostridia evolve hydrogen via a conventional hydrogenase, and this will obscure the nitrogenase hydrogen-evolving activity. Some of the substrates of interest such as azide and cyanide may be metabolic poisons to some organisms. The respiratory system(s) of other organisms may limit the manipulations which can be done with them. Keeping these constraints in mind, we have examined as many in vivo activities of nitrogenase as are accessible to us with several organisms.

Our purpose is to correlate the in vitro kinetics, which show that nitrogenase is an associating system of nonidentical protein components (11), with the observed in vivo kinetics to attempt to deduce the effective state of association in the intact organisms. This connection is important for understanding the limitations on the efficiency of nitrogen-fixing organisms. For instance, the rhizobial nitrogenase in a legume symbiosis may evolve hydrogen even at 1 atm (101.3 kPa) of nitrogen, and this loss of hydrogen may in turn limit the efficiency of nitrogen fixation by the legume symbiosis (15). In any attempt to improve the efficiency of this system we need to know whether the evolution of hydrogen is a necessary consequence of the nitrogenase mechanism per se, or if it is a result of the metabolic constraints imposed by the plant or the respiratory system of the bacterial symbiont. Our experiments may be useful in helping to distinguish between these possibilities.

MATERIALS AND METHODS

Nitrogen-fixing organisms, except *Rhizobium*, were obtained from W. J. Brill at the University of Wisconsin. *Rhizobium* 32H1 was obtained from Peter Wong at Kansas State University.

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Clostridium pasteurianum W5 is the strain previously used for in vitro studies (7, 9, 11). It was grown on the medium of Westlake and Wilson (20) to a relatively high density but still doubling logarithmically. Most cultures were used at a density of ≈250 Klett units (no. 64 filter), the density at which cultures are optimally active, and normally harvested to prepare nitrogenase (10). Acetylene reduction activities were 10 to 15 nmol/min per 100 Klett units of culture when assayed with 0.03 atm of C_2H_2.

Klebsiella pneumoniae M5a1 is the strain described by Pengra and Wilson (13). With cultures of *K. pneumoniae*, the pH in the assay was found to be important in obtaining reproducible results. Therefore the pH of a culture was routinely adjusted to 7.0 by adding 5 N NaOH about 30 min before the experiments. Nitrogenase activity was initially inhibited by this adjustment of pH, but it recovered within about 15 min. The strain of organism used produces large amounts of acid which causes a drop in pH to less than 6 in overnight cultures, yielding low and nonreproducible activities. Cultures were routinely grown in the medium described (13) with 10 μg of N per ml added as aspartic acid. Experiments were done with cultures having a density of 100 to 150 Klett units (no. 64 filter). Acetylene reduction activities were between 5 to 10 nmol/min per ml of culture at 0.05 atm of acetylene.

Extracts from *K. pneumoniae* were prepared as described by Brill et al. (1), and fractions containing the MoFe and Fe proteins were resolved by chromatography on DEAE-cellulose essentially as described for *Azotobacter vinelandii* (17). The MoFe protein was obtained in the fraction eluted with 0.25 M NaCl in pH 7.4 Tris·chloride (25 mM), whereas the Fe protein was obtained in a fraction eluted with 0.5 M NaCl in the same buffer.

A. vinelandii OP is a nongummy, chromogenic strain. It was grown as described previously (17), and fractions from DEAE-cellulose were prepared as with *K. pneumoniae*. The activity of each component was determined by titrating a fixed amount of the Fe protein fraction with varying amounts of the MoFe protein fraction from the same organism. Molar concentrations were calculated by assuming that the specific activity of purified fractions is 2 μmol/min per mg.

Gas mixtures were prepared by evacuating and refilling reaction vials three times with the appropriate gas, either argon or nitrogen. Both gases were freed of oxygen by passing them over a hot copper catalyst (BASF R3-11) and then remoistened by bubbling through water. Mixtures were made by adjusting the partial pressure with a manometer attached to the vacuum system. When CO was added, it was taken by gas-tight syringe from a large reservoir containing a positive pressure of an appropriate dilution (usually 1:10) in argon. Undiluted CO was stored over alkaline pyrogallol to remove oxygen. Acetylene was generated from CaC_2 by a reaction with water previously flushed with argon and transferred in gas-tight syringes.

Azide and cyanide were prepared fresh each day in water, and appropriate amounts were added to vials to give the indicated concentrations. Water was added to give a total aqueous addition of 0.1 ml. All reactions were begun by the addition of 1 ml of active cell cultures which had been transferred to large serum bottles and maintained under argon immediately before assay. Cultures were vigorously growing at the time of assay, and no other additions were made.

Enzyme assays of recombined fractions were carried out in an atmosphere of Ar in nominal 5-ml-volume serum vials (actual volume, 6.2 to 6.3 ml) with levels of acetylene added as appropriate (5, 17). Enzyme assays to determine in vivo activity were done in Ar or Ar-plus-nitrogen atmospheres as indicated in the figure legends. Acetylene was added to the desired concentration, and then assays were initiated by adding 1 ml of the active cell suspension. Reactions were terminated by adding 0.1 ml of 30% trichloroacetic acid or saturated K_2CO_3 when we measured cyanide reduction. Product formation was measured by gas chromatography, with a Porapak R column at 60°C (3 meters by 3.18 mm) and a flame ionization detector.

Cowpea plants were grown for 5 weeks in an environmental chamber in plastic dishpans in a sterilized perlite-vermiculite mixture and watered with nutrient solution as described by Wacek and Brill (19). These plants were inoculated with *Rhizobium* 32H1 and were well nodulated. Several days before assay they were transferred to individual pint (ca. 0.5-liter) jars which could be closed with large rubber stoppers. A notch was cut so that the stem of the plant would pass through, and a small hole was fitted with a serum stopper so that gas samples could be taken. Plasticine clay was used to seal around the stem of the plant. Plants were maintained under Gro-lux lights until the time of assay. Then the stoppers were fitted into place and the indicated levels of acetylene were added through the rubber serum stoppers. We assayed each plant at five levels of acetylene by taking samples at timed intervals and increasing the level of acetylene at the same time. Rates of acetylene reductions were determined by difference after gas chromatography. Comparable results were obtained when individual plants were assayed at single levels of acetylene, but many more replicates were needed. The assay was linear for 2 h with intact Cowpea plants. Each plant was tested under both air and a mixture of 80% argon-20% oxygen either after incubation in light or after 2 days of darkness.

All chemicals were reagent grade or better. ATP and creatine kinase were from Sigma Chemical Co. Creatine phosphate was from Pierce. Acetylene was generated from calcium carbide (Union Carbide); other gases were Matheson high-purity grade.

To determine apparent K_m and K_i values for various combinations of substrate and inhibitors, we made initial estimations by Lineweaver-Burk plots or Dixon plots (16). Best-fitting values of inhibition constants and K_m's were then determined by the computer programs of Cleland (3). Whenever values are cited in the text as a mean ± standard error of the mean, they are derived from appropriate computer fits to the kinetic equations, as given by Cleland (3). Other values cited are derived by visual fitting to appropriate initial plots or replots. The definition by Cleland of noncompetitive includes both "pure noncompetitive" and mixed inhibitions and will be used throughout this paper.

RESULTS

K. pneumoniae. For each substrate and inhibitor tested, a time course was determined to establish that over the time range of interest, with the concentrations of interest, the observed inhibition of activity did not depend on the time of assay. Acetylene reduction showed an apparent lag of less than 20 s and was linear for at least 8 min. With cyanide there was a progressive inhibition which became more extensive with higher concentrations of cyanide. Thus, cyanide could not be used for inhibition studies with this organism. Reactions in the presence of 5 mM sodium azide were linear for up to 10 min. Reactions in the presence of dinitrogen gas at 1 atm or CO at less than 10 matm were linear for at least 10 min.

The apparent K_m for acetylene reduction by whole cells ought to be a particularly revealing parameter of the enzyme system because the apparent K_m for acetylene is a function of the component ratio in both *A. vinelandii* and *C. pasteurianum* (4, 18). Acetylene reduction curves were similar for whole cells and recombined fractions where the Fe protein was in excess. Different forms of the enzyme with different K_m values may have been present in the cells. The lower K_m appeared to be about 1 matm (1.05 ± 0.29 matm with substrate levels from 0.24 to 1.46 matm), whereas the higher K_m was about 5 matm (4.5 ± 1.06 matm with substrate levels from 1.88 to 32 matm). There was no substrate inhibition by acetylene levels up to 1 atm, nor was there substrate activation such as that seen in recombined *C. pasteurianum* components (4). When recombined fractions of *K. pneumoniae* nitrogenase (with an excess of the Fe protein) were assayed, we found that a best fit to a 2/1 function (3) was obtained with K_m values of 0.5 and 3.5 matm; however, the K_m values were not statistically well determined because the two K_m values were not widely different (3). By analyzing the data separately for 0.25 to 1.4 matm and 2.1 to 32 matm of acetylene we found that these values gave apparent K_m values of 0.69 ± 0.18 matm and 1.71 ± 0.37 matm. Each of these K_m values is biased toward the other since two forms of an enzyme use the same substrate and yield the same product so that the true K_m values differ more than appears from these determinations. When we used excess MoFe protein, there was a strong substrate inhibition at high concentrations of acetylene. The apparent K_i was above 0.3 atm. This behavior is similar to that observed with recombined fractions from DEAE-cellulose chromatography or with purified components of *A. vinelandii* (4, 18).

Carbon monoxide was tested as an inhibitor of acetylene reduction in intact *K. pneumoniae*. As previously reported for purified fractions of other organisms, CO was a noncompetitive inhibitor of acetylene reduction (4). The apparent K_i (from 1/velocity [v] versus inhibitior concentration [I] plots at fixed acetylene concentrations) was about 2 matm for several experiments. Upon more precise determination (Fig. 1) the K_{ii} was 4.36 ± 2.38 matm and K_{is} was 0.90 ± 0.31 matm over a range of both substrate and inhibitor concentrations.

As reported for *Azotobacter* particle nitrogenase (14), azide was clearly a noncompetitive inhibitor of acetylene reduction. These results are shown in Fig. 2 for a 1/v versus I plot. Cleland's program for fitting noncompetitive inhibition indicated that the K_{ii} was 0.63 ± 0.13 mM, whereas the K_{is} was 0.66 ± 0.22 mM for azide as

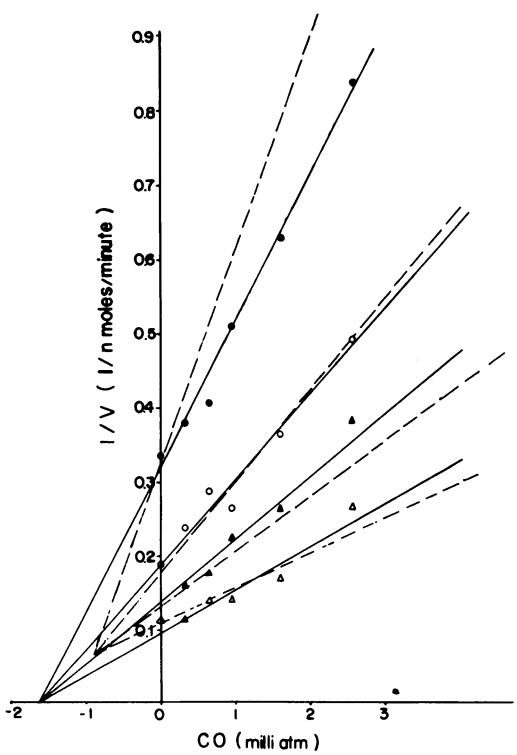

FIG. 1. *Effect of carbon monoxide on acetylene reduction in K. pneumoniae. Cells were grown as described in Materials and Methods to a final density of 142 Klett units. Assays were initiated by adding 1 ml of the culture to serum vials containing mixtures of acetylene and CO in an atmosphere of Ar. Reactions were terminated after 6 min by adding 0.1 ml of 30% trichloroacetic acid. Concentrations of CO were 0.3, 0.64, 0.95, 1.59, and 2.54 matm, whereas concentrations of acetylene were* (●) *1.5,* (○) *4.0,* (▲) *7.9, and* (△) *15.9 matm.* ——, *Visual fit;* - - -, *computer fit.*

an inhibitor of acetylene reduction.

Nitrogen, in contrast to the above-mentioned inhibitors of acetylene reduction, was a partial inhibitor as we have previously described for experiments using recombined fractions of *A. vinelandii* and *C. pasteurianum* (4, 5). In Fig. 3 we show a plot of the activity for acetylene reduction as a function of the nitrogen concentration. At the highest concentrations of acetylene used, nitrogen was only a weak inhibitor, whereas at the lowest levels of acetylene it was a potent inhibitor. At all concentrations of acetylene tested, nitrogen inhibition did not increase proportionately from 0.4 to 1.0 atm, indicating that the inhibition was only partial. Treating the same data by a plot of 1/v versus I to derive the apparent inhibition constant indicated that the apparent K_i for nitrogen as an inhibitor of acetylene reduction increased from near 0.1 atm with 0.001 atm of acetylene to several atmospheres with 0.04 atm of acetylene.

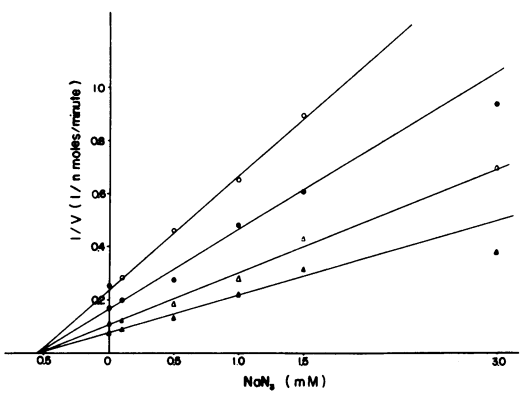

FIG. 2. *Inhibition of acetylene reduction by azide in K. pneumoniae. Assays were carried out as indicated in Materials and Methods and the legend to Fig. 1, with a culture of 134 Klett units. Levels of acetylene were* (○) *1.45,* (●) *2.8,* (△) *7.8, and* (▲) *14.8 matm.*

We tested the effect of the simultaneous presence of both nitrogen and CO on inhibition of acetylene reduction. In three experiments, the presence of nitrogen did not consistently raise or lower the amount of CO required for 50% inhibition which was 1.5 matm on the average (range 1 to 2 matm). Carbon monoxide, however, diminished the relative potency of nitrogen as an inhibitor of acetylene reduction as shown in Fig. 4. If CO and N_2 both inhibited C_2H_2 reduction independently, the Dixon plot (Fig. 4) should show intersecting lines in the left side of the plot. However, the lines are nearly parallel, indicative of mutually exclusive inhibition (16). This inhibition would be expected if CO limited the rate of electron flow and acetylene reacted with a more oxidized form of nitrogenase than nitrogen could. The apparent K_i for CO in the absence of nitrogen was 2 matm in this experiment.

C. pasteurianum. As with *K. pneumoniae*, a time course for acetylene reduction in the pres-

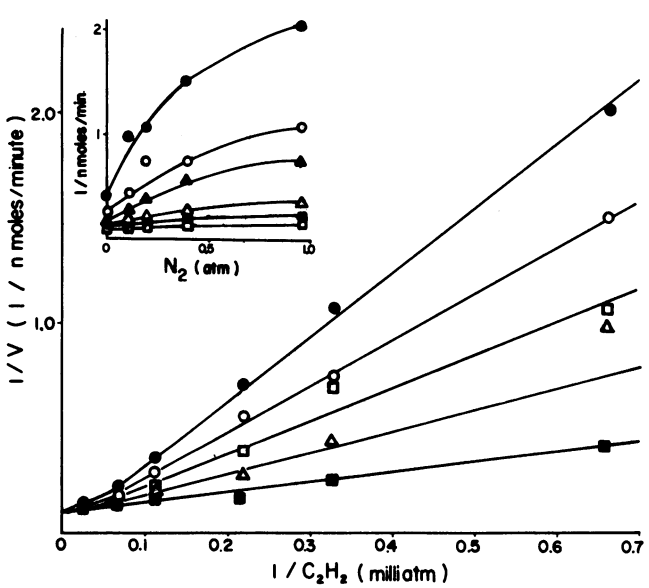

FIG. 3. *Effect of nitrogen on reduction of acetylene by K. pneumoniae. Assays were carried out as indicated in Materials and Methods and legend to Fig. 1 but with a 4-min assay time. Levels of nitrogen were* (■) *0,* (△) *0.1,* (□) *0.2,* (○) *0.4, and* (●) *1.0 atm with the balance as Ar. Inset shows a plot of 1/v versus I of the same data.* C_2H_2 *levels (in milliatmospheres) are* (□) *38,* (■) *19,* (△) *11,* (▲) *5.7,* (○) *3.8, and* (●) *1.9.*

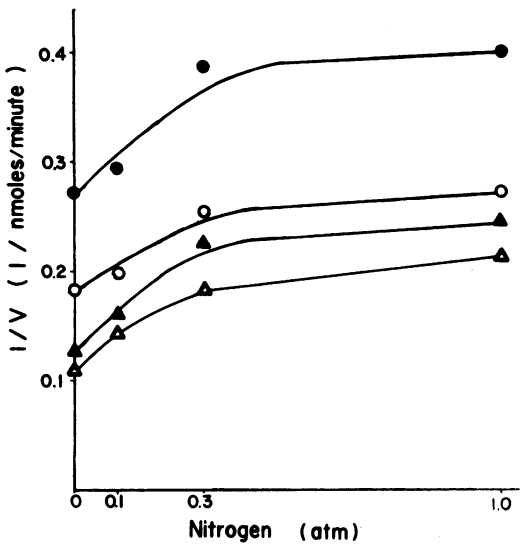

FIG. 4. *Combined effects of nitrogen and CO on the reduction of acetylene by K. pneumoniae. Assays carried out as indicated in Materials and Methods and legend to Fig. 1 with 16 matm of acetylene and nitrogen plus Ar to 1 atm. Levels of CO were* (△) *0,* (▲) *0.68,* (○) *1.36, and* (●) *2.27 matm.*

ence of each inhibitor was determined. Unlike the situation with *K. pneumoniae*, acetylene reduction in the presence of cyanide was linear for at least 6 min. Reductions in the presence of other inhibitors were also linear; with CO the response was linear for at least 10 min. There appeared to be a slight lag in acetylene reduction by this organism. It was 20 to 30 s by extrapolation of the constant rate back to the origin.

The apparent K_m for acetylene reduction, in the absence of inhibitors, was 3.2 matm (range 1.5 to 6 matm for eight experiments) when acetylene concentrations from 1.4 to 2.8 matm were used. When high levels of acetylene were used, no apparent high K_m form of the enzyme was present, unlike the situation in extracts or with purified fractions (4).

Cyanide was a noncompetitive inhibitor of acetylene reduction with an apparent K_i of 0.2 mM, but it was a poor substrate for methane reduction. For instance, 5 mM cyanide, which inhibited by 95% an acetylene reduction of 20 nmol/min at an acetylene concentration of 0.03 atm, showed only about 1 nmol of cyanide reduced to methane per min. This rate of cyanide reduction is only 1/6 that expected if it is reduced at the same site as that where it inhibits acetylene reduction, if it inhibits by simply diverting electrons, and if it is fully reduced to CH_4 rather than to methylamine (14). With cyanide concentrations varying from 0 to 3 mM and acetylene concentrations varying from 1.3 to 7.1 matm, the apparent K_{ii} was 0.22 ± 0.05 mM and the K_{is} was 0.44 ± 0.33 mM.

Azide was also a noncompetitive inhibitor of acetylene reduction with an apparent K_i derived from plots of 1/v versus I of 0.2 mM at low levels of azide and acetylene. At higher concentrations of azide, the inhibition increased in a parabolic manner, indicating that more than one combining site for azide produced inhibition of acetylene reduction (16). For an experiment with azide levels, from 0 to 3 mM and acetylene from 1.4 to 13 matm, the K_{ii} was 0.20 ± 0.066 mM and the K_{is} was 0.26 ± 0.10 mM.

As with *K. pneumoniae*, nitrogen was a partial inhibitor of acetylene reduction. The partial nature of the inhibition was more apparent with *C. pasteurianum* (Fig. 5) than with *K. pneumoniae* (see Fig. 3 inset for a comparison). An attempted computer fit to these data for linear noncompetitive kinetics (3) gave a K_{is} value of 0.18 ± 0.04 atm of N_2, whereas the K_{ii} was negative. The data would not fit a competitive inhibition equation. This is contrary to a previous report (14) but consistent with our experience (4, 5).

Inhibition of acetylene reduction by CO gave nonlinear inhibition kinetics. With 16 matm of acetylene, the inhibition was proportional to the addition of CO, with 1.6 matm of CO giving 25% inhibition and 3.2 matm of CO giving 50% inhibition. But at lower concentrations of acetylene, the inhibition increased in a parabolic manner (Fig. 6). In three experiments with a range of

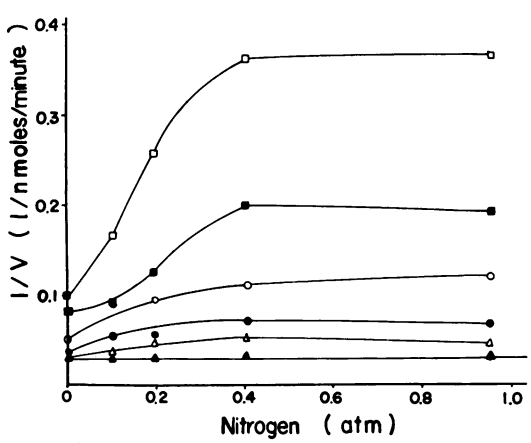

FIG. 5. *Inhibition of acetylene reduction by nitrogen in C. pasteurianum. Assays were carried out as indicated in Materials and Methods with 1 ml of culture (240 Klett units) per assay. Reactions were run for 4 min and terminated by adding 0.1 ml of 30% trichloroacetic acid. Levels of acetylene used were* (□) *1.0,* (■) *1.4,* (○) *3.0,* (●) *6.4,* (△) *10.0, and* (▲) *21.0 matm.*

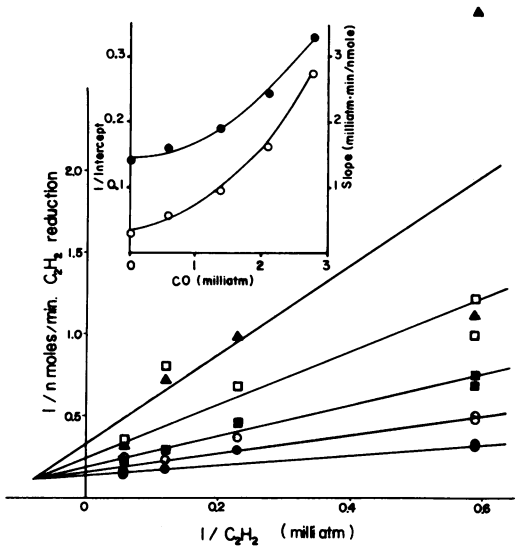

FIG. 6. *Inhibition of acetylene reduction by CO in C. pasteurianum. Assays were done as indicated in Materials and Methods with a culture of 130 Klett units. Reactions were run for 6.5 min. Levels of acetylene were varied as indicated with* (●) *0,* (○) *0.69,* (■) *1.38,* (□) *2.08, and* (▲) *2.77 matm of CO. The lines drawn are for a linear noncompetitive inhibition with* K_{ii} = 2.66 ± 1.1 *and* K_{is} = 0.64 ± 0.27 *matm. Inset shows a replot of 1/intercept versus I* (●) *and slope versus I* (○).

concentrations of CO and acetylene, we obtained a nonlinear, noncompetitive pattern. The noncompetitive nature of the inhibition is shown in the main figure. In the inset a plot of 1/v versus I makes the nonlinearity of the inhibition apparent.

In mutual competition experiments with both CO and nitrogen as inhibitors, we observed an effect nearly identical to that in *K. pneumoniae*. Nitrogen displaced upward only slightly the lines for CO inhibition in plots of 1/v versus I and 1 atm of nitrogen was no more effective than 0.4 atm. The apparent K_i for CO in these assays with a fixed concentration of acetylene (16 matm) was about 2 matm.

A. vinelandii. Although Kleiner (8) and Hardy et al. (6) have reported some studies of intact *Azotobacter*, we had difficulties with precisely controlling relative oxygen tension when taking samples from large cultures and transferring them to small vials. This problem led to a large variability in the results of assays, making impractical the extensive sorts of studies done with *C. pasteurianum* and *K. pneumoniae*. The apparent K_m for acetylene reduction by intact *A. vinelandii* was 40 matm when tested with acetylene concentrations ranging from 6.7 to 67 matm (four experiments).

We have used nitrogenase fractions from *A. vinelandii* for many experiments on the relative effectiveness of nitrogen in inhibiting acetylene reduction (4, 5; unpublished data). Fractions were prepared and then recombined to give three different ratios of protein components with a fixed concentration of the Fe protein (Table 1). By comparing the pattern of inhibition, we found that the whole cells have a component ratio with an effective excess of Fe protein. In the cells, the relative extent of complex formation is greater because the enzyme is more concentrated than in in vitro assays (17), which may explain the greater competitive effectiveness of nitrogen in vivo. When hydrogen evolution in vitro was measured (Table 2), we found that a 1:5.5 ratio of MoFe protein to Fe protein showed 73% inhibition under 1 atm of nitrogen compared with the ratio under argon, whereas a 1.7:1 ratio of MoFe protein to Fe protein showed only 48% inhibition. The assay conditions were as shown for Table 1, except that acetylene was omitted and varying levels of nitrogen in argon were used as the atmosphere. A 0.1-atm level of nitrogen was nearly as effective as a 1-atm level in suppressing H_2 evolution.

The relative effectiveness of nitrogen as an inhibitor of acetylene reduction was tested with intact cowpea plants (Table 3). When plants were kept in darkness for 2 days the maximal activity declined 70%, and N_2 was a less effective inhibitor of acetylene reduction, although not significantly so. In comparison to the in vitro experiments of Table 1, the cowpea plants appear to have a functional excess of Fe protein, even after 2 days in darkness.

TABLE 1. *Relative effectiveness of* N_2 *as an inhibitor of* C_2H_2 *reduction in vivo or in vitro with varying component ratio*

C_2H_2 (matm)	% Effectiveness of:				
	K. pneumoniae in vivo	*C. pasteurianum* in vivo	*A. vinelandii*[a] in vitro component ratio		
			1:5.5	1:2.8	1.7:1
1.6	80	73	77 ± 2	62 ± 12	51 ± 3
3.2	76	58	63 ± 5	53 ± 5	18 ± 15
4.8	77	59	58 ± 5	47 ± 7	20 ± 10
8.0			22 ± 10	13 ± 13	10 ± 5
9.5	61	45			
15.8	49	28	28 ± 5		3 ± 10
31.6	39	6	−2 + 5		2 ± 15

[a] Mean ± standard deviation of three experiments except that for 1:2.8 the mean and range of two experiments is shown. Concentrations of components I and II (MoFe protein and Fe protein) were 0.1 and 0.55 μM, 0.18 and 0.5 μM, and 0.51 and 0.3 μM for the three series.

TABLE 2. *Relative suppression of H_2 evolution by N_2*

N_2 (atm)	% Inhibition relative to velocity under argon at molar ratio of:[a]		
	1:5.5[b]	1:2.8[c]	1.7:1[d]
0.1	65	62	40
0.3	65	65	40
1.0	73	70	48

[a] Average of duplicate samples. Hydrogen was measured by gas chromatography on a modified Carle 8501 thermal conductivity chromatograph with a Porapak S column (3.18 mm by 1.83 m) at 30°C with nitrogen carrier gas at a flow rate of 13 ml/min.

[b] Activity under argon, 64 nmol/min per assay containing 0.1:0.55 μM MoFe and Fe proteins (components I and II), respectively.

[c] Activity under argon, 64 nmol/min per assay containing 0.18:0.5 μM as in footnote *b*.

[d] Activity under argon, 40 nmol/min per assay containing 0.5:0.3 μM as in footnote *b*.

TABLE 3. *Relative effectiveness of nitrogen as an inhibitor of acetylene reduction by intact cowpea plants*

Acetylene (matm)	% Inhibition by 0.8 atom of N vs. 0.8 atm of Ar	
	Maintained in light	Maintained for 2 days in dark
2.2	38 ± 16[a]	54 ± 16
3.3	51 ± 9	48 ± 13
4.45	44 ± 10	41 ± 13
11.1	36 ± 11	27 ± 18
22.2	24 ± 11	11 ± 15

[a] Mean ± standard deviation of 10 or 11 replicates.

DISCUSSION

Attempts to study the kinetics of nitrogenase in vivo are subject to some of the same constraints now as they were 30 years ago, except that the acetylene reduction and cyanide reduction assays allow one to do rapid assays of at least some enzyme reactions. With organisms, the apparent K_m values and the overall pattern of acetylene reduction as a function of substrate concentration suggest that in intact *K. pneumoniae* there is an effective excess of the Fe protein (4, 5). If there were limiting Fe protein, one would expect to see the same sort of substrate inhibition at high acetylene as is observed with recombined fractions when Fe protein is limiting. A more sensitive measure of the relative availability of the Fe protein may be the ability of nitrogen to compete with acetylene as a substrate of the enzyme, because when the enzyme is dilute or Fe protein is limiting, nitrogen is a relatively poor inhibitor of acetylene reduction (4; L. C. Davis, H.-Y. Chang, and T. J. Socolofsky, manuscript submitted for publication).

Nitrogen inhibition of acetylene reduction by *C. pasteurianum* appeared to be similar to that observed in *K. pneumoniae*, except that the partial nature of the competition was more apparent, as shown in the figures. Even at the lowest levels of acetylene used, nitrogen was unable to fully suppress the acetylene reduction activity. In the experiments shown for whole cells, the inhibition reached a limit of about 75%. This observation is similar to the observation that the ability of nitrogen to limit hydrogen evolution is about 75% under many conditions in vitro (14). This may be a simple coincidence but it could mean that there is a redox state of the enzyme which is able to reduce protons (and, incidentally, acetylene) but which is unable to react with nitrogen to reduce it (5). We have observed that with *C. pasteurianum*-purified components, there are two forms of the enzyme which vary in their apparent K_m values for acetylene (4). The high K_m form appears to be present when the Fe protein is limiting. This may correspond to the redox state of *A. vinelandii* and *K. pneumoniae*, which shows substrate inhibition by acetylene at high levels and probably represents the enzyme reduced by only two electrons (4, 18). The extent to which acetylene is reduced in a high concentration of nitrogen may thus reflect the concentrations of those states of the enzyme reduced by only two or four electrons, which are inaccessible to nitrogen as a reducible substrate (5, 12).

We may be able to deduce the available concentration of the Fe protein in legume systems or other intact systems by examining the effectiveness of nitrogen in suppressing the reduction of acetylene in the intact system. This can be compared with the effectiveness of nitrogen in suppressing acetylene reduction by recombined fractions of the purified enzyme to estimate the relative concentration of the reduced Fe protein present at steady state. An example of this method of estimation is shown in Tables 1 and 3. With a ratio of 1 part MoFe protein to 5.5 parts Fe protein at the indicated concentrations, we found that the pattern of inhibition of acetylene reduction by nitrogen was similar to that observed in vivo with free-living microorganisms. We have previously shown that the enzyme kinetic data can be fitted by assuming a complex of 1 mol of the MoFe protein and 2 mol of the Fe protein with apparent association constants of 3×10^6 and 7×10^6 for two rounds of association required to form the active enzyme complex (11; Davis et al., submitted for publication). The relative fraction of active complex

(assumed to be the 1:2 molar complex) is sensitive to the ratio of components in the range of ratios near 1:2, and thus the relative efficiency of formation of active complex depends strongly on the availability of the Fe protein. If the energy supply to keep the Fe protein reduced and the ATP saturated is limiting, the nitrogenase may be able to reduce protons and acetylene relatively more effectively than it can reduce nitrogen (5). Although it is not easy to quantitate the absolute concentrations of enzyme in vivo, it is possible to develop an efficiency assay for nitrogen-fixing organisms based on the relative ability of nitrogen to inhibit acetylene reduction, as shown in Tables 1 and 3.

Our results for the inhibition of acetylene reduction by CO differ somewhat from those of Hwang et al. (7) but are similar to our results obtained with purified fractions of *A. vinelandii* and *C. pasteurianum* when the Fe protein was in relative excess (4). The inhibition apparently is noncompetitive in *K. pneumoniae*. We have found no reports in the literature for CO inhibition of nitrogenase from *K. pneumoniae* in vivo or in vitro. The value which we have determined, about 1 matm in vivo, agrees with that of reports for other organisms in vitro (4, 6).

With *C. pasteurianum*, it was somewhat harder to get good measurements of the inhibition constant for CO since there seemed to be a weaker inhibition than we expected at low levels of CO. This could have been due to binding of the CO to components of the cell other than nitrogenase. In six experiments using the acetylene levels of 10 matm or more, the apparent K_i derived from plots of 1/v versus I was about 2 matm of CO. When lower levels of acetylene were used, the results were similar to those shown in Fig. 6; that is, the inhibition appeared parabolic. This might suggest apparent competitive inhibition when few C_2H_2 and CO levels are used for the determination (9). On the other hand, there are known to be multiple binding sites for CO on nitrogenase (4) and, at different concentrations of substrate and inhibitor, the inhibition patterns may in fact differ.

We find no previous reports of azide as an inhibitor of reduction of substrates other than nitrogen in *K. pneumoniae* or *C. pasteurianum* (9) either in vivo or in vitro. The apparent K_i for inhibition of acetylene reduction by azide in *K. pneumoniae* (0.6 mM) agrees reasonably well with the apparent K_m for azide reduction (1.5 mM in a crude extract and 0.8 mM in recombined fractions) reported by Parejko and Wilson (12).

Because the reduction of azide is a two-electron reaction, it might be competitive with acetylene or noncompetitive if it is able to combine with a different form of the enzyme than that which is able to bind acetylene effectively. The experiments shown here are consistent with previous reports of *Azotobacter* particle nitrogenase (7) that the inhibition is noncompetitive. The effectiveness of azide in inhibiting acetylene reduction is similar in intact organisms to that observed with fractionated and recombined components of *A. vinelandii* (Davis et al., manuscript in preparation) or with partially purified preparations of the particulate *A. vinelandii* nitrogenase (7). We found that azide was a somewhat more potent inhibitor of acetylene reduction by *C. pasteurianum* and *K. pneumoniae* than Hwang et al. (7) reported for *Azotobacter* particle nitrogenase (K_i = 0.2 to 0.6 mM for whole cells, compared with 2.5 mM for the particle nitrogenase). Particulate preparations of nitrogenase have about 1:1 molar components (2), whereas whole cells probably have a greater excess of Fe protein as discussed above. This may affect the inhibition constants observed in the different systems (17).

Cyanide reduction by *C. pasteurianum* was weak and was not studied extensively. Somewhat surprisingly, cyanide was reduced nearly as effectively at 0.1 mM as at 3 mM although its effectiveness as an inhibitor of acetylene reduction showed a K_i of 0.2 mM. As reported by Rivera-Ortiz and Burris (14), acetylene stimulates the reduction of cyanide. Our conclusions on the K_i for cyanide inhibition of acetylene reduction agree with theirs although again we find a somewhat lower value (0.2 mM rather than 0.5 mM).

We can draw the overall conclusion that for those reactions accessible to study in the intact organism, the apparent kinetic constants are similar to those reported previously in vitro but agree best with studies of recombined components in which an excess of the Fe protein was present. A similar effect on kinetic constants is obtained by increasing the concentration of the enzyme in the assay system (Davis et al., submitted for publication), and the cells themselves insure that the enzyme is maintained at a high concentration.

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LITERATURE CITED

1. **Brill, W. J., A. L. Steiner, and V. K. Shah.** 1974. Effect of molybdenum starvation and tungsten on the synthesis of nitrogenase components in *Klebsiella pneumoniae*. J. Bacteriol. **118**:986-989.
2. **Bulen, W. A., and J. R. LeCompte.** 1972. Nitrogenase

complex and its components. Methods Enzymol. **24**: 456–469.
3. **Cleland, W. W.** 1967. The statistical analysis of enzyme kinetic data. Advan. Enzymol. **29**:1–32.
4. **Davis, L. C., M. T. Henzl. R. H. Burris, and W. H. Orme-Johnson.** 1979. Iron-sulfur clusters in the molydenum-iron protein component of nitrogenase. Electron paramagnetic resonance of the carbon monoxide inhibited state. Biochemistry **18**:4860–4869.
5. **Davis, L. C., V. K. Shah, and W. J. Brill.** 1975. Nitrogenase VII. Effect of component ratio, ATP and H_2 on the distribution of electrons to alternate substrates. Biochim. Biophys. Acta **403**:67–78.
6. **Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns.** 1968. Acetylene-ethylene assay for nitrogen fixation: laboratory and field evaluation. Plant Physiol. **43**:1185–1220.
7. **Hwang, J. C., C. H. Chen, and R. H. Burris.** 1973. Inhibition of nitrogenase catalyzed reactions. Biochim. Biophys. Acta **292**:256–270.
8. **Kleiner, D.** 1974. Quantitative relations for the repression of nitrogenase synthesis in *Azotobacter vinelandii* by ammonia. Arch. Mikrobiol. **101**:153–159.
9. **Lockshin, A., and R. H. Burris.** 1965. Inhibitors of nitrogen fixation in extracts from *Clostridium pasteurianum*. Biochim. Biophys. Acta **111**:1–10.
10. **Orme-Johnson, W. H., and L. C. Davis.** 1977. Current topics and problems in enzymology of nitrogenase, p. 15–60. *In* W. Lovenberg (ed.), Iron sulfur proteins, vol. 3. Academic Press Inc., New York.
11. **Orme-Johnson, W. H., L. C. Davis, M. T. Henzl, B. A. Averill, N. R. Orme-Johnson, E. Munck, and R. Zimmerman.** 1977. Components and pathways in biological nitrogen fixation, p. 131–178. *In* W. Newton et al. (ed.), Recent developments in nitrogen fixation. Academic Press Inc., New York.
12. **Parejko, R. A., and P. W. Wilson.** 1971. Kinetic studies on *Klebsiella pneumoniae* nitrogenase. Proc. Natl. Acad. Sci. U.S.A. **68**:2016–2018.
13. **Pengra, R. M., and P. W. Wilson.** 1958. Physiology of nitrogen fixation by *Aerobacter aerogenes*. J. Bacteriol. **75**:21–25.
14. **Rivera-Ortiz, J. M., and R. H. Burris.** 1975. Interactions among substrates and inhibitors of nitrogenase. J. Bacteriol. **123**:537–545.
15. **Schubert, K. R., and H. J. Evans.** 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc. Natl. Acad. Sci. U.S.A. **73**:1207–1211.
16. **Segel, I. H.** 1975. Enzyme kinetics. John Wiley & Sons, Inc., New York.
17. **Shah, V. K., L. C. Davis, and W. J. Brill.** 1973. Nitrogenase I. Repression and derepression of the iron-molybdenum and iron proteins in *Azotobacter vinelandii*. Biochim. Biophys. Acta **256**:498–511.
18. **Shah, V. K., L. C. Davis, and W. J. Brill.** 1975. Nitrogenase VI. Acetylene reduction assay: dependence of nitrogen fixation estimates on component ratio and acetylene concentration. Biochim. Biophys. Acta **384**: 353–359.
19. **Wacek, T. J., and W. J. Brill.** 1976. Simple, rapid assay for screening nitorgen-fixing ability in soybean. Crop Sci. **16**:519–522.
20. **Westlake, D. W. S., and P. W. Wilson.** 1959. Molecular hydrogen and nitrogen fixation by *Clostridium pasteurianum*. Can. J. Microbiol. **5**:617–620.
21. **Wilson, P. W.** 1958. Asymbiotic nitrogen fixation, p. 9–47. *In* W. Ruhland (ed.), Encyclopedia of plant physiology, vol. 8. Springer-Verlag, Berlin.