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# INHIBITION OF NITROGENASE-CATALYZED REDUCTIONS

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## SUMMARY

Inhibition of nitrogenase-catalyzed reductions was studied under such conditions that the concentration of electron acceptors and inhibitors did not affect nitrogenase-catalyzed ATP hydrolysis, and the concentration of the electron acceptor was at least 1.8 times its Michaelis constant to reduce the effect of $H_2$ evolution. Thus, the effects were centered on the reduction site.

Based on Lineweaver–Burk plots, CO was noncompetitive with $N_2$, acetylene and $NaN_3$ and did not inhibit $H_2$ evolution; $H_2$ inhibited $N_2$ fixation competitively, but it did not inhibit reduction of azide, acetylene, cyanide, isocyanide, and $H^+$; acetylene and cyanide were noncompetitive with $N_2$; acetylene and azide acted noncompetitively with each other; cyanide and methylisocyanide were competitive with $NaN_3$. According to these observations, the following five sites or modified sites are proposed for the nitrogenase complex: (1) $N_2$ and $H_2$ site, (2) acetylene site, (3) azide, cyanide and methylisocyanide site, (4) CO site, (5) $H^+$ site.

## INTRODUCTION

Reactions catalyzed by nitrogenase require ATP, a reductant such as $Na_2S_2O_4$ or reduced ferredoxin, $Mg^{2+}$, and an electron acceptor such as $N_2$. Nitrogenase is inhibited by a variety of compounds[1], and in the current work we have examined how these inhibitors influence reductions catalyzed by a highly active nitrogenase carried by particles from *Azotobacter vinelandii* (certain points also were checked with extracts from *Clostridium pasteurianum*).

Nitrogenase has broad substrate specificity, and nitrous oxide[2], azide[3,4], acetylene[3,5,6], cyanide[7] and methylisocyanide[8] not only inhibit $N_2$ reduction but also serve as substrates for nitrogenase. The question is examined whether or not compounds which function both as substrates and inhibitors for nitrogenase all operate in the same way, and whether or not they differ in action from an inhibitor such as CO which is not reduced by nitrogenase.

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## MATERIALS AND METHODS

### *Chemicals*

$N_2$, $H_2$, argon, acetylene, CO, ATP, and creatine kinase were obtained as high purity chemicals from commercial sources and creatine phosphate[9] and methyl isocyanide[10] were prepared.

### *Preparations from A. vinelandii*

Growth of *A. vinelandii* and preparation of cell-free extracts were described previously[11]. The *A. vinelandii* nitrogenase complex was prepared as follows[11]: a crude extract obtained by breaking the cells with a French pressure cell was dialyzed anaerobically, heated under $H_2$ at 60 °C for 10 min and centrifuged. The supernatant was treated with streptomycin sulfate. The resultant supernatant was centrifuged at $144000 \times g$ for 1 h, and the dark brown precipitate obtained was resuspended in buffer and was designated as $P_{144-1}$. Suspensions of $P_{144-1}$ could fix 60–100 nmoles $N_2$ per mg of protein per min. All operations were performed anaerobically. Either freshly prepared $P_{144-1}$ or $P_{144-1}$ which had been stored in liquid $N_2$ was used; the preparations were stable for months in liquid $N_2$.

### *Preparations from C. pasteurianum*

Cultures of *C. pasteurianum* W5 were grown with $N_2$ as the sole source of $N_2$[11] in a 180-l glass-lined fermentor. The cells were dried in a rotary vacuum evaporator with the bath at 40–45 °C, and were stored at room temperature under vacuum. Cell-free extracts were prepared by autolysis of the dried cells according to the method of Carnahan *et al.*[12] in 0.025 M Tris–HCl buffer, pH 7.4. The dark brown supernatant constituted nitrogenase with specific activities ranging from 13 to 16 nmoles $N_2$ fixed per mg of protein per min. Preparations were used fresh or were stored in liquid $N_2$.

### *Analytical*

Typical reaction mixtures used in these studies contained in 1 ml: 5 μmoles ATP, 5 μmoles $MgCl_2$, 0.25 mg creatine kinase, 50 μmoles Tris–HCl buffer, pH 7.4, 15 μmoles $Na_2S_2O_4$, 0.15 ml enzyme source and 35 μmoles creatine phosphate. The gas phase is indicated in the text and legends; argon was used as a filling gas to give 1 atm pressure. The concentration of the electron acceptor used was at least 1.8 times its $K_m$ to decrease $H_2$ evolution; relatively more $H_2$ is evolved at low concentrations of the electron acceptor. The concentrations of the electron acceptor or inhibitor used did not inhibit nitrogenase-catalyzed ATP hydrolysis; thus, the effects were centered on the reduction site involved. The incubation temperature was 30 °C, and the reaction time generally was 15 min. The $P_{144-1}$ fraction of *A. vinelandii* was used, and in a few experiments a crude extract from *C. pasteurianum* was compared with the *A. vinelandii* enzyme complex. All the gases were mixed with a single measuring manometer to assure accuracy. Because they were needed in low concentration, CO and acetylene were diluted with argon before being mixed with the other desired gases.

$N_2$ fixation and azide reduction were assayed by measuring the $NH_3$ formed. Reductions of acetylene, cyanide and methyl isocyanide were determined by gas chromatographic analyses of ethylene or methane as products. Creatine formation

from creatine phosphate served as an index of ATP hydrolysis. Details of these assays were described previously[11].

*Treatment of kinetic data*

The nature of the various inhibitions was determined from double reciprocal plots of activity and substrate concentration in the presence of different levels of inhibitor. Kinetic data were fitted to appropriate rate equations with computer programs[13,14] unless noted otherwise. Data were fitted to Equation 1 by the method of least squares ($v$ = velocity, $V$ = maximum velocity, $A$ = substrate concentration, $K$ = the Michaelis constant $K_m$).

$$v = \frac{VA}{K + A} \tag{1}$$

Equal variance for the velocities was assumed. Calculations by computer conformed to the Fortran IV program of Cleland[13,14] which furnishes values for $K$, $V$, $K/V$, $1/V$ and standard errors of the estimates. The slopes ($K/V$) and intercepts ($1/V$) were plotted for the various substrate concentrations. Data fitting a linear competitive inhibition were fitted to Equation 2 and for linear non-competitive inhibition to Equation 3.

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{2}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \tag{3}$$

Experimental points are recorded in the figures, and the plotted lines were calculated by the computer program which includes all data and gives greatest weight to the highest velocities and least weight to the lowest velocities (lowest velocities carry the greatest intrinsic error in measurement of initial velocities). The lines computed for the highest inhibitor concentrations (lowest velocities) in some instances have all data points on the same side of the line, because all points in the figure are considered in the computer program. In these cases, visual plots of the lines (broken lines) representing high inhibitor concentrations have been included tor comparison.

## RESULTS

Nitrogenase catalyzes reduction of a number of compounds. In the absence of electron acceptors the electrons are transferred to $H^+$ to produce $H_2$. In the presence of $N_2$, $H_2$ evolution decreased as $N_2$ fixation increased in response to an increasing $pN_2$ up to 1 atm of $N_2$ (Fig. 1). The ratio of $H_2$ evolved per 1/3 $N_2$ fixed decreased rapidly from 8.92 at a $pN_2$ of 0.032 atm to 0.90 at a $pN_2$ of 0.2 atm, and 0.60 at a $pN_2$ of 0.4 atm. This implies that $H^+$ and $N_2$ compete for a low potential compound functional in the formation of $H_2$ and $NH_3$. Since the nitrogenase-catalyzed $H_2$ evolution could not be suppressed completely under the conditions studied, the concentration of the electron acceptor furnished in inhibition studies was at least 1.8 times its $K_m$ to decrease $H_2$ evolution and thus to reduce the complications introduced by $H_2$ evolution.

Fig. 2 shows the effect of $H_2$ on $N_2$ fixation. 50% inhibition of $N_2$ fixation was observed with 0.17 atm $H_2$ in the presence of 0.10 atm $N_2$ and with 0.33 atm $H_2$ in the presence of 0.30 atm $N_2$. Nitrogenase-catalyzed ATP hydrolysis was not inhibited appreciably by $H_2$.

Fig. 3 verifies that $H_2$ inhibition of $N_2$ fixation is competitive. In this experiment, the maximal $H_2$ evolved in the absence of $N_2$ was approximately 0.2 ml in a 20-ml bottle, or only 1% of the space. 1% of $H_2$ inhibits $N_2$ fixation in the presence of 0.2 atm of $N_2$ only about 2%, and inhibition by 1% $H_2$ at higher $pN_2$ values is negligible. Therefore, the evolved $H_2$ did not constitute a product inhibitor. The observation of competitive inhibition confirmed many earlier investigations, *e.g.* Strandberg and Wilson[15].

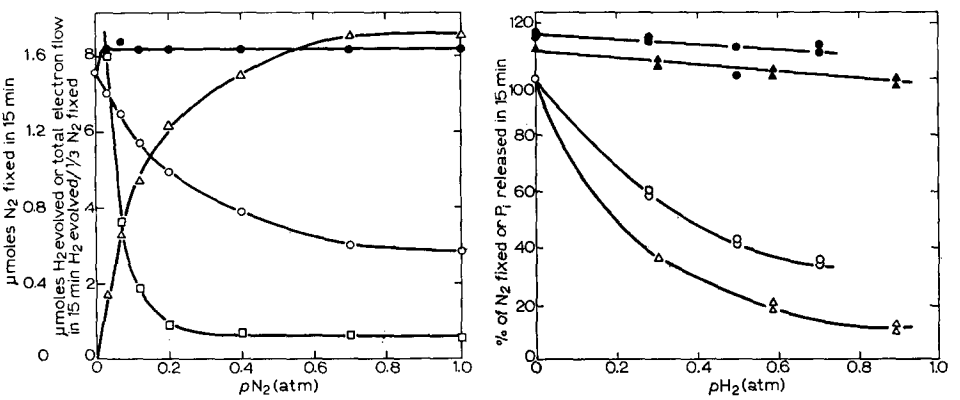


Fig. 1. Effect of $pN_2$ on $H_2$ evolution, $N_2$ reduction, total electron flow, and $H_2$ evolved/1/3 $N_2$ fixed. △—△, $N_2$ fixed; ○—○, $H_2$ evolved; ●—●, total electron flow; □—□, $H_2$ evolved/1/3 $N_2$ fixed.

Fig. 2. Effect of $H_2$ on $N_2$ fixation and ATP hydrolysis by nitrogenase. $N_2$ fixation under 0.1 or 0.3 atm $N_2$, and $P_i$ released by nitrogenase under argon were accepted as 100%. ○—○, % of $N_2$ fixed under 0.3 atm $N_2$; △—△, % of $N_2$ fixed under 0.1 atm $N_2$; ●—●, % of $P_i$ released under 0.3 atm $N_2$; ▲—▲, % of $P_i$ released under 0.1 atm $N_2$.

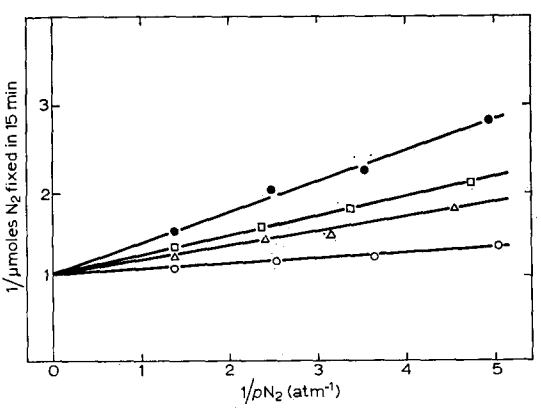


Fig. 3. $H_2$ inhibition of $N_2$ fixation in $P_{144-1}$ from *A. vinelandii*. Data fitted to Eqn 2. ○—○, control ($pH_2$ 0 atm); △—△, $pH_2$ 0.1 atm; □—□, $pH_2$ 0.18 atm; ●—●, $pH_2$ 0.29 atm.

TABLE I

EFFECT OF $H_2$ ON AZIDE REDUCTION BY PREPARATIONS FROM *A. VINELANDII*

$P_{144-1}$, 0.51 mg of protein per ml of reaction mixture.

| *$pH_2$ (atm)* | *$NH_3$ formed in 15 min from* | | | |
|---|---|---|---|---|
| | *1 mM azide* | | *4 mM azide* | |
| | *μmoles* | % | *μmoles* | % |
| 0.00 | 0.615 | 100 | 1.10 | 100 |
| 0.30 | 0.610 | 99 | 1.05 | 96 |
| 0.61 | 0.620 | 100 | 1.05 | 96 |
| 1.00 | 0.610 | 99 | 1.00 | 91 |

TABLE II

EFFECT OF $H_2$ ON $N_2$ FIXATION AND AZIDE REDUCTION BY A CRUDE EXTRACT FROM *C. PASTEURIANUM*

Crude extract containing 3.66 mg of protein per ml of reaction mixture was made by the autolysis of dried cells of *C. pasteurianum* in 0.025 M Tris–HCl buffer, pH 7.4.

| *$pH_2$ (atm)* | *$NH_3$ formed in 15 min from* | | | |
|---|---|---|---|---|
| | *0.2 atm $N_2$* | | *2 mM azide* | |
| | *μmoles* | % | *μmoles* | % |
| 0 | 1.468 | 100 | 1.10 | 100 |
| 0.3 | 1.354 | 92 | 1.12 | 100 |
| 0.6 | 1.084 | 72 | 1.08 | 98 |
| 0.8 | 0.940 | 64 | | |
| 1.0 | | | 1.04 | 95 |

TABLE III

EFFECT OF $H_2$ ON ETHYLENE FORMATION FROM ACETYLENE BY A CRUDE EXTRACT OF *C. PASTEURIANUM* AND BY A PURIFIED FRACTION, $P_{144-1}$, FROM *A. VINELANDII*

*C. pasteurianum* crude extract contained 3.66 mg of protein per ml of reaction mixture. *A. vinelandii*, $P_{144-1}$, contained 0.82 mg of protein per ml of reaction mixture.

| *$pH_2$, (atm)* | *Ethylene formed in 15 min from 0.021 atm acetylene by* | | | |
|---|---|---|---|---|
| | *C. pasteurianum, crude extract* | | *A. vinelandii,* $P_{144-1}$ | |
| | *μmoles* | % | *μmoles* | % |
| 0 | 3.08 | 100 | 4.00 | 100 |
| 0.31 | 3.26 | 106 | 4.08 | 102 |
| 0.51 | 3.25 | 105 | 4.00 | 100 |
| 0.86 | 3.30 | 107 | 4.10 | 103 |

TABLE IV

EFFECT OF $H_2$ ON METHANE FORMATION FROM CYANIDE AND ON ATP HYDROLYSIS BY NITROGENASE FROM *A. VINELANDII*

$P_{144\_1}$, 0.9 mg of protein per ml of reaction mixture.

| *$pH_2$ (atm)* | *Methane formed or ATP hydrolyzed in 16 min from* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *1 mM cyanide* | | | | *3 mM cyanide* | | | |
| | *Methane formed* | | *$P_i$ released* | | *Methane formed* | | *$P_i$ released* | |
| | *nmoles* | % | *μmoles* | % | *nmoles* | % | *μmoles* | % |
| 0 | 224 | 100 | 23.2 | 100 | 320 | 100 | 25.2 | 100 |
| 0.3 | 257 | 115 | 23.4 | 101 | 325 | 102 | 24.4 | 97 |
| 0.6 | 249 | 111 | 22.2 | 96 | 343 | 107 | 27.8 | 110 |
| 1.0 | 274 | 122 | 23.3 | 101 | 360 | 113 | 25.9 | 103 |

TABLE V

EFFECT OF $H_2$ ON METHANE FORMATION FROM METHYL ISOCYANIDE AND ATP HYDROLYSIS BY NITROGENASE FROM *A. VINELANDII*

$P_{144\_1}$, 0.89 mg of protein per ml of reaction mixture.

| *$pH_2$, (atm)* | *Methane formed or ATP hydrolyzed in 15 min from 1 mM methyl isocyanide* | | | |
|---|---|---|---|---|
| | *Methane formed* | | *$P_i$ released* | |
| | *nmoles* | % | *μmoles* | % |
| 0 | 179 | 100 | 14.9 | 100 |
| 0.3 | 189 | 106 | 16.2 | 109 |
| 0.6 | 198 | 111 | 14.4 | 97 |
| 1.0 | 203 | 114 | 16.0 | 107 |

TABLE VI

EFFECT OF CO ON $N_2$ FIXATION AND ATP HYDROLYSIS BY NITROGENASE FROM *A. VINELANDII*

$P_{144\_1}$, 0.77 mg of protein per ml of reaction mixture.

| *pCO (atm)* | *% of $N_2$ fixed and ATP hydrolyzed under 0.47 atm $N_2$* | |
|---|---|---|
| | *$N_2$ fixation* | *$P_i$ released* |
| 0 | 100 | 100 |
| 0.0008 | 34 | 98 |
| 0.0012 | 23 | 101 |
| 0.0051 | 6 | 95 |
| 0.0099 | 8 | 95 |
| 0.0198 | 2 | 94 |

Although $H_2$ competitively inhibited $N_2$ fixation, there was no inhibition of azide reduction by $H_2$ (Table I). Likewise, $N_2$ fixation by an extract from *C. pasteurianum* was inhibited by $H_2$, but $H_2$ had no effect on azide reduction (Table II). Neither did $H_2$ inhibit ethylene formation from acetylene by nitrogenase from *C. pasteurianum* nor *A. vinelandii* (Table III). Finally, $H_2$ did not inhibit cyanide or methylisocyanide reduction or nitrogenase-catalyzed ATP hydrolysis (Table IV, Table V). In fact, 1.0 atm $H_2$ enhanced formation of methane by 22 and 13% from 1 and 3 mM cyanide, respectively, and by 14% from 1 mM methylisocyanide.

CO is a potent inhibitor of $N_2$ fixation. A concentration of CO as low as 0.0008 atm reduced $N_2$ fixation to 34% of the control, and 0.005 atm CO completely

TABLE VII

EFFECT OF CO ON AZIDE REDUCTION AND ATP HYDROLYSIS BY NITROGENASE FROM *A. VINELANDII*

$P_{144\_1}$, 0.77 mg of protein per ml of reaction mixture.

| *pCO (atm)* | *% of $NH_3$ formed and ATP hydrolyzed in 5 mM azide* | |
|---|---|---|
| | *$NH_3$ formed* | *$P_i$ released* |
| 0 | 100 | 100 |
| 0.0133 | 5 | 87 |
| 0.0233 | 0 | 93 |
| 0.0973 | 0 | 92 |
| 0.1930 | 0 | 86 |
| 0.7130 | 0 | 88 |
| 1.0000 | 0 | 93 |

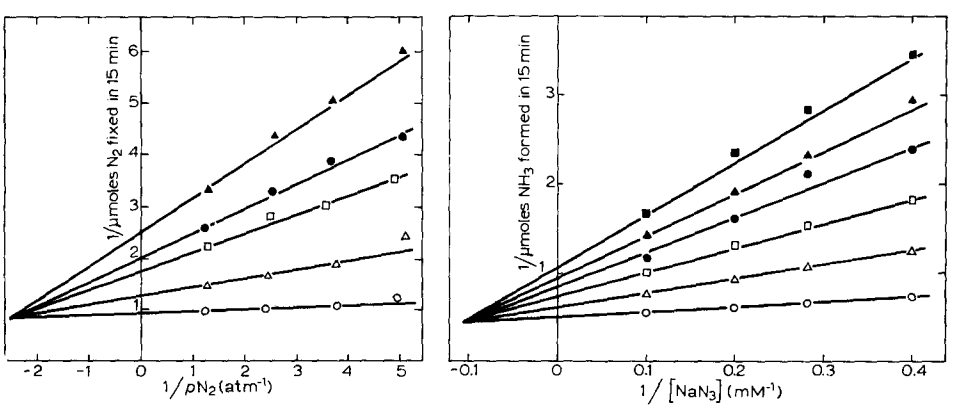


Fig. 4. CO inhibition of $N_2$ fixation in $P_{144\_1}$ from *A. vinelandii*. Data fitted to Eqn 3. ○—○, control (*p*CO 0 atm); △—△, *p*CO 0.00021 atm; □—□, *p*CO 0.00045 atm; ●—●, *p*CO 0.00058 atm; ▲—▲, *p*CO 0.00084 atm.

Fig. 5. CO inhibition of azide reduction in *A. vinelandii* $P_{144\_1}$. Data fitted to Eqn 3. ○—○, control (*p*CO 0 atm); △—△, *p*CO 0.00022 atm; □—□, *p*CO 0.00042 atm; ●—●, *p*CO 0.00064 atm; ▲—▲, *p*CO 0.00078 atm; ■—■, *p*CO 0.00099 atm.

inhibited $N_2$ fixation (Table VI). In contrast, nitrogenase-catalyzed ATP hydrolysis was not affected. Similarly, although 0.013 atm CO completely abolished the formation of $NH_3$ from azide, a full atmosphere of CO had no effect on ATP hydrolysis by nitrogenase (Table VII). Thus, CO specifically influences the reduction sites without blocking the ATP-hydrolyzing site of nitrogenase. Figs 4, 5 and 6 show noncompetitive inhibition of $N_2$ fixation, azide reduction and acetylene reduction by CO, respectively.

Acetylene competes for electrons with $N_2$ and thus inhibits $N_2$ fixation. Acetylene at 0.01 atm inhibited $N_2$ fixation by 80, 70 and 57% in the presence of 0.2, 0.4

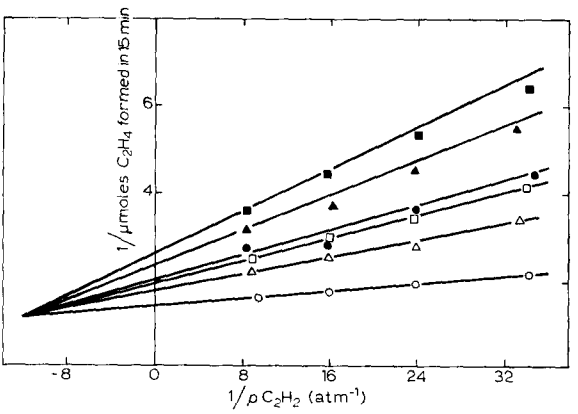


Fig. 6. CO inhibition of ethylene from acetylene by *A. vinelandii* $P_{144\_1}$. Data fitted to Eqn 3. ○—○, control ($p$CO 0 atm); △—△, $p$CO 0.00025 atm; □—□, $p$CO 0.00042 atm; ●—●, $p$CO 0.0005 atm; ▲—▲, $p$CO 0.00079 atm; □—□, $p$CO 0.00098 atm.

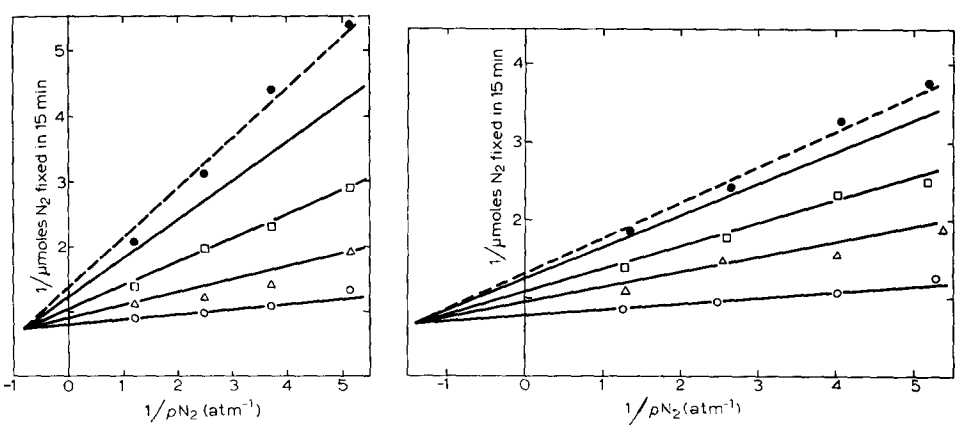


Fig. 7. Inhibition by acetylene of $N_2$ fixation by *A. vinelandii* $P_{144\_1}$. Data fitted to Eqn 3. The solid lines were derived from computer program results. The dotted line was plotted visually. ○—○, control ($pC_2H_2$ 0 atm); △—△, $pC_2H_2$ 0.0031 atm; □—□, $pC_2H_2$ 0.0067 atm; ●—●, $pC_2H_2$ 0.0122 atm.

Fig. 8. Acetylene inhibition of $N_2$ fixation by *C. pasteurianum* crude extract. Data fitted to Eqn 3. The dotted line was plotted visually. ○—○, control ($pC_2H_2$ 0 atm); △—△, $pC_2H_2$ 0.0033 atm; □—□, $pC_2H_2$ 0.0065 atm; ●—●, $pC_2H_2$ 0.0095 atm.

and 0.8 atm $N_2$, respectively. Plots of reciprocals of velocities of $N_2$ fixation *vs* the reciprocals of $N_2$ partial pressures indicated acetylene was a noncompetitive inhibitor (Fig. 7). Noncompetitive inhibition of $N_2$ fixation by acetylene also was demonstrated with a crude extract from *C. pasteurianum* (Fig. 8). 4.3 ATPs were required per two-electron transfer for $H_2$ evolution or $N_2$ reduction.

Azide, cyanide and isocyanide, like acetylene, are electron acceptors in nitrogenase-catalyzed reductions and inhibit $N_2$ fixation. Figs 9 and 10 show, respectively, that azide was a linear noncompetitive inhibitor of acetylene and that acetylene was an intersecting hyperbolic noncompetitive inhibitor of azide[16]. A similar experiment run in the presence of $pC_2H_2$ values up to 0.1 atm, which did not interrupt nitrogenase-catalyzed ATP hydrolysis, also indicated an intersecting-hyperbolic noncompetitive inhibition of azide reduction by acetylene.

A plot of reciprocals of velocities of $N_2$ fixation against reciprocals of $N_2$ con-

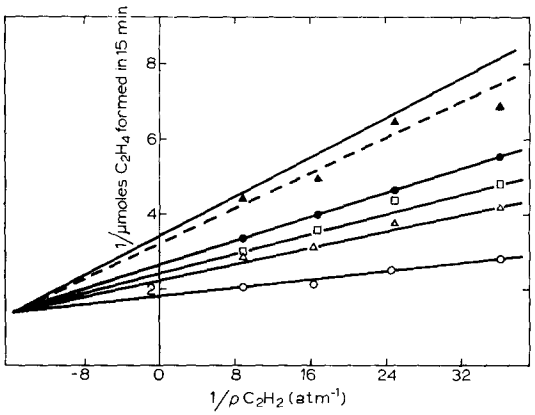


Fig. 9. Azide inhibition of acetylene reduction by *A. vinelandii* $P_{144\_1}$. Data fitted to Eqn 3 The dotted line was plotted visually. ○—○, control; △—△, 2.5 mM azide; □—□, 3.5 mM azide; ●—●, 5 mM azide; ▲—▲, 10 mM azide.

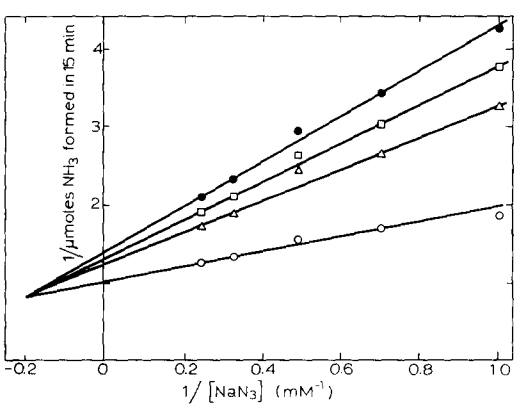


Fig. 10. Acetylene inhibition of azide reduction by *A. vinelandii* $P_{144\_1}$. The plot was made visually. ○—○, control ($pC_2H_2$ 0 atm); △—△, $pC_2H_2$ 0.039 atm; □—□, $pC_2H_2$ 0.104 atm; ●—●, $pC_2H_2$ 0.403 atm.

centration shows noncompetitive inhibition by cyanide (Fig. 11). In this and in the following experiments, the enzyme reactions were stopped with trichloroacetic acid, and the methane formed was measured by gas chromatography. Then, saturated $K_2CO_3$ was added to the reaction mixtures, and the diffused $NH_3$ was measured colorimetrically[11]. As cyanide reduction yields equimolar $NH_3$ and methane, the true $N_2$ fixation in the presence of HCN is indicated by the total ammonia formed *minus* methane formed. Both cyanide and methyl isocyanide were competitive with azide (Figs 12 and 13). In each graph the line for the highest concentration of inhibitor (minimum velocity, maximum intrinsic error) deviated from the computer-calculated

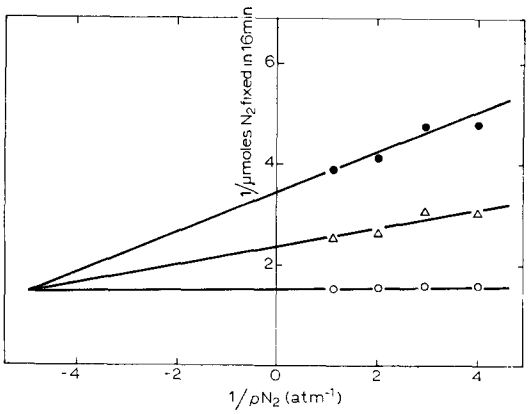


Fig. 11. Cyanide inhibition of $N_2$ fixation by *A. vinelandii* $P_{144\_1}$. Data fitted to Eqn 3. ○—○, control; △—△, 0.8 mM KCN; ●—●, 1.5 mM KCN.

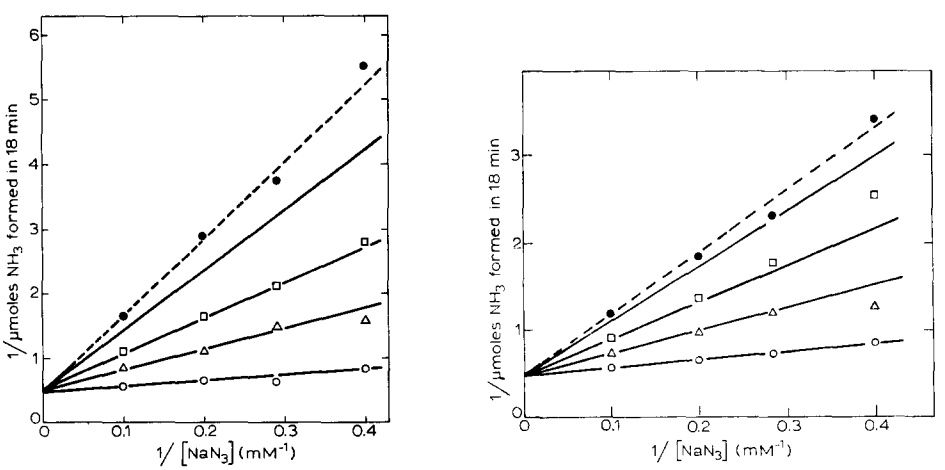


Fig. 12. Cyanide inhibition of azide reduction by *A. vinelandii* $P_{144\_1}$. Data fitted to Eqn 2. The dotted line was plotted visually. ○—○, control; △—△, 0.8 mM cyanide; □—□, 1.6 mM cyanide; ●—●, 2.4 mM cyanide.

Fig. 13. Methyl isocyanide inhibition of azide reduction by *A. vinelandii* $P_{144\_1}$. Data fitted to Eqn 2. The dotted line was plotted visually. ○—○, control; △—△, 0.8 mM methyl isocyanide; □—□, 1.6 mM methyl isocyamide; ●—●, 2.4 mM methyl isocyanide.

TABLE VIII

NATURE OF INHIBITION AND KINETIC CONSTANTS OF ELECTRON ACCEPTORS AND INHIBITORS

For meaning of $K_{is}$, $K_{ii}$ see equations in Materials and Methods cited from ref. 28.

| *Substrate* | *Inhibitor*: | | | | | | |
|---|---|---|---|---|---|---|---|
| | $N_2$ (*atm*) | $N_3^-$ (*mM*) | *Acetylene* (*atm*) | *HCN* (*mM*) | *Methyl isocyanide* (*mM*) | $H_2$ (*atm*) | *CO* ($\times 10^{-4}$ *atm*) |
| $N_2$ | $K_m$: 0.122* 0.10** | | Linear noncompetitive $K_{is}$: 0.0021 ± 0.0007 $K_{ii}$: 0.019 ± 0.009 | Linear noncompetitive $K_{is}$: 0.15 ± 0.03 $K_{ii}$: 0.61 ± 0.08 | | Linear competitive $K_{is}$: 0.112 ± 0.003 | Linear noncompetitive $K_{is}$: 1.14 ± 0.24 $K_{ii}$: 4.7 ± 0.6 |
| $N_3^-$ | | $K_m$: 1.15* 1.43** | Intersecting hyperbolic noncompetitive $K_{id}$: 0.03 (intercepts) $K_{id}$: 0.059 (slopes) | Linear competitive $K_{is}$: 0.24 ± 0.05 | Linear competitive $K_{is}$: 0.41 ± 0.08 | No inhibition | Linear noncompetitive $K_{is}$: 1.14 ± 0.24 $K_{ii}$: 4.4 ± 0.6 |
| Acetylene | | Linear noncompetitive $K_{is}$: 2.7 ± 0.8 $K_{ii}$: 1.1 ± 0.3 | $K_m$: 0.015* 0.0155 ± 0.003 | | | No inhibition | Linear noncompetitive $K_{is}$: 2.1 ± 0.5 $K_{ii}$: 12.9 ± 0.4 |
| HCN | | | | $K_m$: 1.28* | | No inhibition | |
| Methyl isocyanide | | | | | $K_m$: 2.0* | No inhibition | |
| Nitrogenase-catalyzed ATP hydrolysis | Enhanced by $N_2$ | No inhibition up to 20 mM; enhanced by $N_3^-$ | Inhibition by acetylene above 0.1 atm | Enhanced by HCN; HCN above 3 mM decreased enhancement | Enhanced by isocyanide; above 10 mM decreased enhancement | No inhibition | No inhibition |
| Nitrogenase-catalyzed $H_2$ evolution | Inhibited | Inhibited | Inhibited | Inhibited | Inhibited | No inhibition | No inhibition |

* $K_m$ values marked with an asterisk were obtained from a visual plot by the method of Lineweaver and Burk; all the other $K_m$, $K_{is}$ and $K_{ii}$

line. The results show that azide, cyanide and methyl isocyanide bind to the same site on the nitrogenase.

The control lines in the figures ($N_2$ without inhibitor) should define the $K_m$ for $N_2$. The $K_m$ values from these lines are variable, because the $N_2$ concentrations used for studies of inhibitors are higher than optimal for establishing the $K_m$. The $K_m$ values for $N_2$ cited in Table VIII are more reliable.

## DISCUSSION

$H_2$ is a competitive inhibitor of $N_2$ fixation in $N_2$ fixing cells[17] and in cell-free extracts[1,15]. However, the inhibition of $N_2$ fixation by $H_2$ was described as non-competitive in *A. vinelandii* cells[18]. Extracts from *C. pasteurianum* in unusually concentrated phosphate buffer (0.05 M) exhibited uncompetitive inhibition of $N_2$ fixation by $H_2$ when pyruvate was provided as substrate[1]. Inhibition of $N_2$ fixation by the *A. vinelandii* $P_{144-1}$ fraction supplied $Na_2S_2O_4$ and an ATP-generating system was competitive. As reported by Hardy and Knight[19], we observed no inhibition by $H_2$ of nitrogenase-catalyzed ATP hydrolysis. Although $H_2$ competitively inhibits $N_2$ fixation, it did not inhibit reductions of azide, acetylene, cyanide and isocyanide. $N_2O$, another electron acceptor as well as an inhibitor, was not tested; it had been reported that $H_2$ did not inhibit reduction of $N_2O$ by soybean root nodules[20]. Apparently the site for $N_2$ binding also is the site for attachment of $H_2$. As $H_2$ does not inhibit the reduction of other electron acceptors, it follows that the sites for other electron acceptors are distinct (by modification or physical separation) from the $N_2$ and $H_2$ binding site.

CO inhibition of $N_2$ fixation has been reported to be noncompetitive (red clover plants[21] and *A. vinelandii* cells[22]) and in other instances competitive (extracts of *C. pasteurianum*[1] and intact *Nostoc muscorum*[23]). CO, like $H_2$, has no effect on reductant-dependent ATP hydrolysis and ATP-dependent $H_2$ evolution[24,25]. Our experiments with the *A. vinelandii* $P_{144-1}$ fraction showed that $p$CO values of 0.0051 atm and 0.0133 atm completely inhibited $N_2$ fixation (Gallon *et al.*[26] have found virtually complete inhibition of nitrogenase from the blue-green alga *Gloeocapsa* sp by 0.01 atm CO) and azide reduction, but a concentration of CO as high as 1 atm did not change the rates of nitrogenase-catalyzed ATP hydrolysis. This indicates that CO specifically influences the substrate reduction sites without blocking the ATP hydrolysis site or the $H_2$ evolution site of nitrogenase. Our experiments recorded in Figs 4, 5, and 6, show CO to be a noncompetitive inhibitor of $N_2$ fixation, azide reduction and acetylene reduction. The basis for the discrepancies in reports on the nature of CO inhibition remains to be defined.

Inhibition of $N_2$ fixation by acetylene in *C. pasteurianum* or *A. vinelandii* extracts was reported to be competitive[5], but M. J. Dilworth, (unpublished) found acetylene to be a noncompetitive inhibitor of $N_2$ fixation by *C. pasteurianum* extracts. In our current work with the *A. vinelandii* $P_{144-1}$ fraction and with crude extracts from *C. pasteurianum* supplied $Na_2S_2O_4$ and an ATP generating system, acetylene proved to be a noncompetitive inhibitor of $N_2$ fixation (Figs 7 and 8). As other individuals in our laboratory also have found acetylene to be a noncompetitive inhibitor, our earlier statement[5] that acetylene is competitive with $N_2$ apparently was incorrect. Since $H_2$ affected $N_2$ fixation, but did not have any effect on acetylene

reduction, the site for acetylene and the site for $N_2$ and $H_2$ binding appear to be separate. The noncompetitive inhibition of $N_2$ fixation by acetylene and the difference in ATP requirement per two electrons transferred for $N_2$ and acetylene reduction also support the conclusion that the $N_2$ and acetylene binding sites are not the same.

Azide and acetylene were noncompetitive with each other. Whereas inhibition by azide of acetylene reduction was linear noncompetitive, the inhibition by acetylene of azide reduction appeared to be slope hyperbolic intercept hyperbolic noncompetitive (plotting either the slopes or intercepts from the Lineweaver–Burk plots against concentrations of acetylene gave a hyperbolic curve). Because acetylene inhibition of azide reduction was the only nonlinear noncompetitive inhibition observed, it will require detailed examination to establish that the effect is real rather than arising from experimental artifacts. If real, it will suggest that the reduction of azide can follow alternative pathways exhibiting differential inhibition by acetylene.

Cyanide was noncompetitive with $N_2$. Both cyanide and methylisocyanide were competitive with azide. These results show that azide, cyanide and methylisocyanide bind to the same site on the nitrogenase. Gogoleva and Ivanov[27] observed that a variety of inhibitors, including $NaN_3$ and KCN, had different effects on the respiratory chain in the presence of succinate or ethanol, and they concluded that there may be two distinct pathways of electron transfer involved in $N_2$ reduction by *A. vinelandii.*

Table VIII records kinetic constants and summarizes information on the nature of inhibitors[11]. The concentrations of the electron acceptors or inhibitors used did not inhibit nitrogenase-catalyzed ATP hydrolysis, so observations focus on reduction sites and are not complicated by interactions between the ATP-hydrolyzing site and electron acceptors or inhibitors. The $K_m$ for $N_2$ obtained from computer analysis of five experiments averaged 0.10 atm $N_2$ ($0.131 \pm 0.016$; $0.059 \pm 0.008$; $0.102 \pm 0.027$; $0.09 \pm 0.02$; $0.12 \pm 0.03$), whereas an average of the $K_m$ values obtained by the method of Lineweaver–Burk with visual plots from two other experiments gave a $K_m$ of 0.122 atm $N_2$ (0.13; 0.114). The average $K_m$ for azide from computer analysis of the experimental results was 1.43 mM ($1.12 \pm 0.2$; $1.7 \pm 0.4$; $1.48 \pm 0.33$); the $K_m$ obtained from visual Lineweaver–Burk plots of two other experiments was 1.15 mM azide (1.15, 1.15). The $K_m$ values for acetylene from the computer program ($0.016 \pm 0.003$ atm; $0.015 \pm 0.003$ atm) and from Lineweaver–Burk visual plots of two other experiments (0.015 atm; 0.015 atm) were 0.0155 and 0.015, respectively.

The inhibition constant, $K_{is}$, for $H_2$ was $0.112 \pm 0.003$ atm $H_2$; this value was much lower than 0.3 atm, 0.32 atm and 0.55 atm $H_2$ reported, respectively, by Hadfield and Bulen[29], Dilworth *et al.*[30], and Lockshin and Burris[1]. An inhibition constant for $H_2$ of 0.016 atm was reported for cell-free extracts of bacteroids from soybean root nodules[31].

Under the conditions described (Table VIII), CO was noncompetitive with $N_2$, acetylene and azide; it did not inhibit nitrogenase-catalyzed $H_2$ evolution and ATP hydrolysis. $H_2$ inhibited $N_2$ fixation only, and its action was competitive. Acetylene and cyanide were noncompetitive with $N_2$, and acetylene showed hyperbolic noncompetitive inhibition of azide reduction. Cyanide and methyl isocyanide were competitive with azide. The site for $H^+$ binding or $H_2$ evolution could not be demonstrated, because $H^+$ is not limiting in the system. However, since CO does not inhibit nitrogenase-catalyzed ATP hydrolysis or $H_2$ evolution, apparently the CO and $H^+$ binding sites are separate. Based on these observations, one can propose five sites

or modified sites for binding electron acceptors or inhibitors: (1) $N_2$ and $H_2$ site; (2) azide, cyanide and methyl isocyanide site; (3) acetylene site; (4) CO site; (5) $H_2$ evolution site ($H^+$ site).

Based on thermodynamic considerations and the inhibition of $H_2$ evolution and ATP hydrolysis by ADP, Linde *et al.*[32] proposed that there are separate enzyme sites for electron activation, $H_2$ evolution and $N_2$ reduction in $N_2$-fixing organisms.

Nitrogenase from all sources contains transition metals. The triple bond or potential triple bond characteristic of nitrogenase substrates may allow their side-on-bonding to active sites; their lone electron pairs may permit end-on-bonding[7,25]. Brintzinger[33] proposed double side-on-bonding by insertion of $N_2$ into metal hydride bonds. These and other models proposed for the mechanism of nitrogenase function[34,35] suggest that the enzyme has multiple binding sites.

The reduction of azide and the reduction of $N_2O$ serve as helpful examples in discussing the nature of the multiple sites of nitrogenase. Schöllhorn and Burris[4] found a stoichiometric 1:1 relationship between $NH_3$ formed and $N_2$ evolved from azide. In two of their experiments there were 128 $\mu$moles $NH_3$ per 125 $\mu$moles $N_2$ and 147 $\mu$moles $NH_3$ per 138 $\mu$moles $N_2$ formed from azide. If azide and $N_2$ bind at the same site, then a portion of the $N_2$ formed from azide should be reduced promptly to $NH_3$, because the affinity of the enzyme for $N_2$ is 16 times that for azide[11]. $N_2$ formed from azide should have close proximity to the active site and its relatively low molecular weight should give it ready access to the site. It is unlikely that a stoichiometric ratio of 1:1 for $NH_3$:$N_2$ formed would exist, if azide and $N_2$ bind at the same site; apparently the sites for $N_2$ and azide are separate. Hardy and Knight[7] concluded from their experiments on the stoichiometry of the products from azide reduction that "the $N_2$ formed from azide does not appear to be in the correct position for further reaction, although it must be within 1–2 Å of the binding site". Our data show that $H_2$ inhibits $N_2$ reduction but not azide reduction, again indicating the existence of multiple binding sites.

Similar reasoning also may be applied to the formation of $N_2$ from $N_2O$. Hoch *et al.*[20] reported that $H_2$ did not inhibit $N_2O$ reduction by soybean root nodules. Lockshin and Burris[1] showed that $N_2O$ was reduced to $NH_3$ at about 6% the rate of $N_2$ fixation by extracts of *C. pasteurianum*. Hardy and Knight[36] reported that 26 $\mu$moles of $N_2$ and only 0.8 $\mu$moles of $NH_3$ were formed from 0.55 atm of $N_2O$ by an extract from *A. vinelandii*. Assuming that $N_2O$ and $N_2$ bind at the same site, the $N_2$ formed from $N_2O$ would be expected to be trapped and reduced when it is formed. Contrary to this assumption and the observed competitive inhibition of $N_2$ fixation by $N_2O$, the stoichiometric data for the products of $N_2O$ reduction suggest that there are different binding sites for $N_2O$ and $N_2$.

It is not clear whether five specific catalytic sites exist on nitrogenase or whether sites are modified in the presence of specific substrates to alter the properties of the sites. However, it is clear that the multiple reactions of nitrogenase are not catalyzed at a single, unmodified active site.

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