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ATP-DEPENDENT REDUCTION OF AZIDE AND HCN BY N_2-FIXING ENZYMES OF *AZOTOBACTER VINELANDII* AND *CLOSTRIDIUM PASTEURIANUM**

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SUMMARY

1. The novel reductions of HCN to CH_4, NH_3, and tentatively, small amounts of CH_3NH_2, and of N_3^- to NH_3 and N_2 are demonstrated with cell-free extracts of *Azotobacter vinelandii* and *Clostridium pasteurianum*.

2. Requirements for the reductions are: (i) extracts of cells grown on N_2 (those grown on urea or NH_3 are ineffective); (ii) ATP-generator; and (iii) reductant ($Na_2S_2O_4$ or in some cases H_2 or KBH_4) and characteristics of the reductions are: (i) inhibition by either substrate of ATP-dependent H_2 evolution from $Na_2S_2O_4$, but no effect on reductant-dependent ATPase; and (ii) complete inhibition by CO.

3. The rate of electron consumption for reduction of N_3^- is similar to the rate for reduction of N_2 or N_2O, while the rate for reduction of HCN is about 30% of the others. The observed K_m values are: N_2, 0.1 mM; C_2H_2, N_2O, and N_3^-, 1 mM; and HCN, 4 mM with heated extract of *A. vinelandii*.

4. It is proposed that the reductions of HCN and N_3^- are catalyzed by the same enzymes that reduce N_2 to NH_3, and N_2O to N_2, since the requirements, distribution of enzyme activity, and characteristics are all remarkably identical. Thus, it is suggested that the N_2-fixing system is composed of a substrate-binding and/or -activating site of unmatched versatility and this site is coupled to the previously proposed electron-activating site.

5. The 2, 4, and 6 electron products of N_3^-, N_2O and HCN reduction provide, indirectly, the first evidence for HN=NH and H_2N–NH_2 as enzyme-bound intermediates in N_2 fixation.

6. Utilization of $H^{14}CN$ and radioactive determination of $^{14}CH_3NH_2$ or hydrogen-flame gaschromatographic determination of CH_4 from HCN or C_2H_4 from C_2H_2 provide new sensitive assays for N_2-fixing activity.

* A preliminary report of this research was presented at the Annual Meeting of the American Society for Microbiology, May 5, 1966 (ref. 2).

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INTRODUCTION

The reduction of N_2O to N_2 and H_2O is catalyzed by cell-free extracts of *Azotobacter vinelandii* or *Clostridium pasteurianum*[1-3]. It has been proposed that this reduction is catalyzed by the same enzyme(s) that catalyzes the reduction of N_2 to NH_3 (ref. 1). This proposal was based on the following observations: (1) N_2O and N_2 reduction have identical requirements—ATP, reductant, extract of cells grown on N_2 but not those grown on urea or ammonia[1,4,7]; (2) N_2O or N_2 inhibit ATP-dependent H_2 evolution but not reductant-dependent ATPase[1,3,6,8]; (3) CO inhibits the reduction of both N_2O and N_2 (refs. 1,9); and (4) N_2O is a competitive inhibitor of N_2 fixation[9].

This paper reports the following reductions:

$$N_3^- \rightarrow N_2 + NH_3$$
$$HCN \rightarrow CH_4 + NH_3$$
$$HCN \rightarrow CH_3NH_2$$

by cell-free extracts of *A. vinelandii* and *C. pasteurianum*. SCHÖLLHORN AND BURRIS[10] also have evidence for azide reduction by extracts of *C. pasteurianum* and they and DILWORTH[11] have extended the list to include the reduction of acetylene to ethylene by extracts of *C. pasteurianum*. Requirements, distribution of activity, and characteristics of these new reductions are shown to be identical with those of N_2 fixation and N_2O reduction. The stoichiometry, rates of electron consumption and K_m's are reported. These results are discussed with respect to mechanism and intermediates of N_2 fixation. A subsequent publication will report the utilization of reductions of analogs of acetylene and cyanide to locate the binding site and electron donor site of the N_2-fixing system[12].

METHODS

Growth of cells and preparation of extracts

A. vinelandii, ATCC 12518, was grown, cells were broken, and extracts were fractionated as previously described for N_2 fixation or N_2O reduction[1,3,6,13]. Fractions were designated as follows: (1) crude extract—supernatant after 35 000 × *g* for 30 min; (2) heated extract—supernatant after 35 000 × *g* for 1 h of crude extract heated for 10 min at 60° under 0.5 atm of H_2; (3) $P_{1/2}$—pellet of heated extract after 100 000 × *g* for 0.5 h; (4) P_3—pellet of supernatant from $P_{1/2}$ after 225 000 × *g* for 3 h; (5) S_3— supernatant after 225 000 × *g* for 3 h; (6) protamine sulfate precipitates and supernatant—precipitates between 0.000 to 0.100 and 0.100 to 0.125 mg protamine sulfate per mg protein of heated extract and supernatant after 0.125 mg; and (7) DEAE-cellulose fractions—protamine sulfate, 0.100–0.125, fractionated on a DEAE-cellulose column in an anaerobic chamber[13]. Fractions 1–5 were stored anaerobically at 4°, and 6–7 were stored anaerobically at room temperature[13].

C. pasteurianum, ATCC 6013, was grown, cells were broken, and extracts were fractionated as previously described for N_2 fixation or N_2O reduction[1,14,15,*]. Fractions were designated as follows: (1) crude extract—supernatant after 35 000 × *g* for 30 min of autolysate; (2) heated extract— supernatant after 35 000 × *g* for 30 min

* U.S. Patent 3 236 741 (1965).

of crude extract heated for 10 min at 60° under 0.5 atm H_2; and (3) phosphate gel preparation—supernatant from crude extract treated with protamine sulfate to remove nucleic acids and with calcium phosphate gel to remove inactive protein[15]. The crude and heated extracts were stored under 0.8 atm H_2 at room temperature, and the phosphate gel preparation was stored anaerobically at 4°.

Assays

Reductions of N_2, HCN, N_3^-, N_2O, C_2H_2, CH_3NH_2, NCO^-, CO, or NO were performed in 36-ml sidearm flasks. Dithionite was dissolved in O_2-free water containing a predetermined quantity of acid or base to produce a final pH of 7 for extracts of *A. vinelandii* and 6.5 for those of *C. pasteurianum*. The energy source and reductant were placed in the sidearm, the extract and the N_3^-, HCN (as NaCN), CH_3NH_2, or NCO^- were placed in the main compartment, and the incubation flask was immediately evacuated. After repeated evacuation and covering with the indicated gas, the contents of the sidearm were tipped in to initiate the reaction. The reaction mixture was incubated on a rotary shaker at 30°.

Samples of evolved gases, *e.g.*, CH_4, H_2, and N_2, were analyzed with a mass spectrometer, and the initial gas phase of He or CO was used as an internal standard. CH_4 was determined after fractionation on molecular sieve 5A or activated alumina columns in a Perkin–Elmer 880 or 800 gas chromatograph with a dual H_2 flame ionization detector.

NH_3 synthesis from N_2, HCN, or N_3^- was measured by titration after microdiffusion[16]; a control *minus* N_2, HCN, or N_3^- and covered with He or in some cases CO was used. CH_3NH_2 synthesis from HCN was measured by titration after microdiffusion; $^{14}CH_3NH_2$ from $H^{14}CN$ was measured by liquid-scintillation counting after microdiffusion. For purposes of identification, CH_3NH_2 was measured colorimetrically[17]. Saturated K_2CO_3 was used as the base for microdiffusion and 1 ml of 2% H_3BO_3 was used as the trapping solution. For colorimetric experiments 1 ml of 0.2 M HCl was used as the trapping solution. The diffusion time of CH_3NH_2 at room temperature was slower than that of NH_3, and 20 h at room temperature was found to give 98% recovery of CN_3NH_2 standards. The $^{14}CH_3NH_2$ in H_3BO_3 solution was titrated, acidified, and counted in a Packard Tri-Carb liquid-scintillation counter. The counting solution was dioxane containing naphthalene, dimethyl-POPOP (1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene), and PPO (2,5-diphenyloxazole)[18].

ATPase was determined as phosphate released from ATP or phosphoenolpyruvate[19] or as creatine formed from creatine phosphate[20]. Reductant-dependent ATPase was measured as the increase in phosphate or creatine released by dithionite.

Reagents

ATP, phosphoenolpyruvate, pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), creatine phosphate, and creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2) were obtained from Sigma Chemical Co.; protamine sulfate from Nutritional Biochemicals Co.; $Na_2S_2O_4$, reagent grade, from Fisher Scientific Co.; NaCN, "Baker Analyzed" reagent from J. T. Baker Chemical Co.; NaN_3, practical, (98% by our analysis), and CH_3NH_2 (40% in water) from Eastman Organic Chemicals; $K^{14}CN$, 0.95 mC/mmole, from New England Nuclear Corp.; KOCN, reagent, from Allied Chemical and Dye Corporation; and N_2O, NO, C_2H_2, and CO from The Matheson Co.

RESULTS

ATPase effect of HCN and azide

The effects of increasing concentrations of HCN and azide on the ATPase activity of heated extracts of *A. vinelandii* were examined in order to determine levels of these substrates that would not inhibit the reductant-dependent ATPase[3]. The reductant-dependent ATPase (Δ $Na_2S_2O_4$) was more sensitive than the non-reductant-dependent ATPase ($-Na_2S_2O_4$) to both HCN and azide and the reductant-

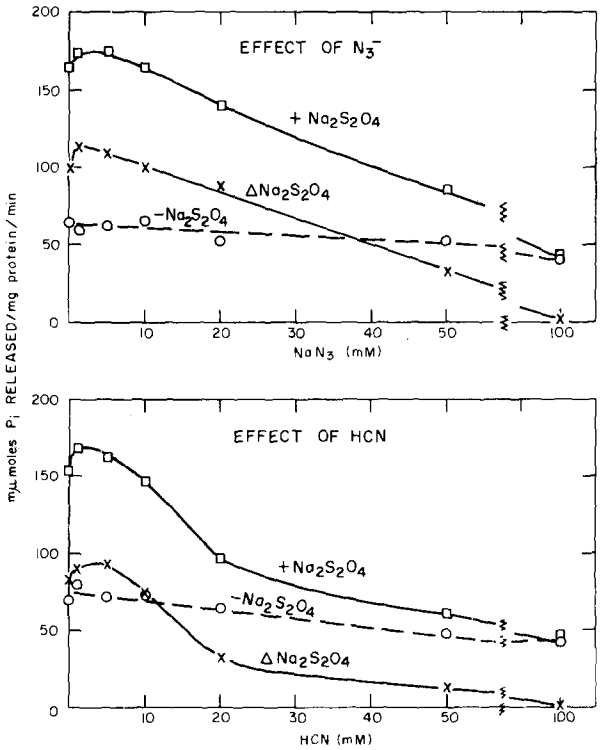


Fig. 1. Effect of HCN or azide on reductant-dependent ATPase of *A. vinelandii*. Incubation mixture contained in μmoles/ml: phosphoenolpyruvate, 30; ATP, 5; $Na_2S_2O_4$, 20; Tris·HCl, 50 (all at pH 7.0); $MgCl_2$, 5; and sodium azide or NaCN as indicated; and in mg protein/ml: pyruvate kinase, 0.1; and heated extract of N_2-grown *A. vinelandii*, 4. Vol., 1.0 ml; time, 30 min at 30°; gas phase, 0.9 atm He.

dependent ATPase was more sensitive to HCN than azide (Fig. 1). However, 0–5 mM HCN or 0–10 mM azide appeared to produce negligible effects on the reductant-dependent ATPase.

Azide reduction

Requirements and inhibitors

The requirements for and inhibitors of the reduction of azide to NH_3 are shown in Table I. A system identical to that for N_2 fixation or N_2O reduction—heated ex-

TABLE I

REQUIREMENTS AND INHIBITORS OF AZIDE REDUCTION

Complete system contained per 2 ml in μmoles: Tris·HCl, 50; creatine phosphate (Cr ~ P), 112; ATP, 10; $Na_2S_2O_4$, 40 (all at pH 7.0); $MgCl_2$, 10; and NaN_3, 20; and in mg protein: heated extract of N_2-grown *A. vinelandii*, 8; and creatine kinase, 0.4. Gas phase, 0.9 atm He, N_2, CO, or N_2O; incubation time, 45 min at 30°; incubation vol., 2 ml. Azide was omitted from complete system and gas phase was 0.9 atm of N_2 with 0.9 atm He as control for N_2-fixation assay.

Incubation system	*Gas phase*	*NH_3 (μmoles per incubation)*
Complete	He	10.3
−Extract	He	0.0
$-Na_2S_2O_4$	He	0.4
−ATP, −Cr ~ P, − creatine kinase	He	0.0
−Azide	He	0.0
Complete	CO	0.0
Complete	N_2O	2.5
N_2-fixation assay	N_2	7.7

tract of N_2-grown *A. vinelandii*, $Na_2S_2O_4$, and ATP-generator—is essential for NH_3 formation. H_2 (Table III) or KBH_4 can substitute for $Na_2S_2O_4$ with extracts of *C. pasteurianum* but not with those of *A. vinelandii*. Omission of azide eliminates NH_3 formation. The rate of NH_3 formation from azide is always greater than that of NH_3 from N_2, and the ratio (N_3^-/N_2) of 1.3 observed in this experiment (Table I) is typical for these conditions. Reduction of 10 mM azide to NH_3 is completely inhibited by 0.9 atm CO and 75% inhibited by 0.9 atm N_2O.

Dependence of azide reduction on extracts of N_2-grown cells

Reduction of azide to NH_3 is restricted to extracts of cells grown on N_2. Thus, a heated extract from N_2-grown cells of *A. vinelandii* reduces N_3^- or N_2 to NH_3, while a heated extract from urea-grown cells reduces neither N_3^- nor N_2 (Table II). A similar dependency was found with extracts of *C. pasteurianum*.

Distribution of azide-reducing activity

The distribution of azide-reducing activity in extracts of *A. vinelandii* or *C.*

TABLE II

DEPENDENCE OF AZIDE REDUCTION ON EXTRACTS OF N_2-GROWN CELLS

Complete system (see Table I) with heated extract of N_2-grown or urea-grown *A. vinelandii*, 8 mg protein/2 ml. Incubation time, 45 min at 30°; incubation vol., 2 ml; gas phase, 0.9 atm of He or N_2.

Extract	*Substrate*	*NH_3 (μmoles per incubation)*
N_2-grown cells	N_3^-	9.3
	N_2	8.3
Urea-grown cells	N_3^-	0.6
	N_2	0.6

TABLE III

DISTRIBUTION OF AZIDE-REDUCING ACTIVITY

A. vinelandii. See complete system in Table I, with mg protein per 2 ml: crude extract, 18; heated extract, 8.5; $P_{1/2}$, 6.2; P_3, 4.1; or S_3, 11.8; gas phase, 0.9 atm He for azide reduction and 0.9 atm N_2 or He for N_2 fixation; incubation time, 45 min; and incubation vol., 2 ml. *C. pasteurianum*. Incubation contained per 2 ml in μmoles: potassium cacodylate, 100; creatine phosphate, 112; ATP, 10 (all at pH 6.5); $MgCl_2$, 10; and NaN_3, 20 (only for azide reduction); and in mg protein: creatine kinase, 0.4; and crude extract, 18; heated extract, 12.8; phosphate gel preparation, 6.6; or a combination as indicated. Gas phase, 0.9 atm of He: H_2 (1:1) for azide reduction, and 0.9 atm of N_2:H_2 (1:1) or He:H_2 (1:1) for N_2 fixation; incubation time, 45 min; incubation vol., 2 ml.

Extract	*Substrate (mμmoles NH_3/mg protein per min)*		*Ratio N_2/N_3^-*
	N_2	NaN_3	
A. vinelandii			
Crude extract	6.5	7.2	0.90
Heated extract	10.8	13.5	0.80
$P_{1/2}$	9.2	8.3	1.1
P_3	27.0	28.0	0.96
S_3	1.8	3.0	0.6
C. pasteurianum			
Crude extract	4.6	5.8	0.80
Heated extract	0.4	0.5	0.80
Phosphate gel preparation	0.0	0.0	
Heated extract *plus* phosphate gel preparation (2:1)	11.6	11.6	1.0

pasteurianum parallels that of N_2-fixing activity (Table III). The azide-reducing and N_2-reducing activities of crude extract of *A. vinelandii* are concentrated in the heated fraction and the precipitate after centrifugation for 3 h at 225 000 × *g*. The ratios of the specific activities of N_2 reduction to those of N_3^- reduction are relatively constant (0.8–1.1) for all fractions except the supernatant after 3 h, S_3. The divergent ratio of this fraction is probably due to its low specific activities. Both the N_2-fixing and azide-reducing activities of the crude extract of *C. pasteurianum* are eliminated by heating at 60° for 10 min or by treatment with phosphate gel. Combination of the heated extract and the supernatant from phosphate gel treatment restores both activities. Again the ratios of the specific activities of N_2 reduction to those of N_3^- reduction are relatively constant (0.8–1.0) for all fractions.

Products and stoichiometry of azide reduction

Products of azide reduction are NH_3 and N_2. NH_3 was assayed by titration after microdiffusion from saturated K_2CO_3 to 2% H_3BO_3, and N_2 was identified and assayed by mass spectrometry. The ratio of these products varies with the incubation conditions (Table IV). With high concentrations of azide (10 mM) and a high ratio of gas-to-liquid volume (8:1), the final product contains approximately equal amounts of N_2 and NH_3. About 40% of the azide is reduced. Therefore, the products do not originate from impurities in the sodium azide. Under these conditions, only 1.2 μmoles (7%) of the N_2 formed is reduced to NH_3. Thus, azide appears to be reduced in preference to the N_2 formed from azide. The following stoichiometry is supported:

$$1\ N_3^- \rightarrow 1\ N_2 + 1\ NH_3$$

TABLE IV

PRODUCTS AND STOICHIOMETRY OF AZIDE REDUCTION

Complete system (see Table I) with heated extract of N_2-grown *A. vinelandii*. Gas phase, 0.9 atm He or H_2; incubation time, 45 min at 30°.

Volume (ml)		*Substrate*	*Gas phase*	*Product found (μmoles per incubation)*			*Substrate* reduced (μmoles)*	*Electrons** consumed (μmoles)*
Gas	*Liquid*			H_2	N_2	NH_3		
32	4	N_3^-, 10 mM	He	12.0	16.0	19.5	17.2	42
32	4		He	43.0				
		N_2	N_2			12.0	6.0	36
26	10	N_3^-, 5 mM	He	32.8	19.5	42.8	27.3	102
26	10		He	94.3	—	—		
		N_2	N_2			27.6	13.8	83

* N_3^- reduced = N_2 found + $^1/_3$ (NH_3 found − N_2 found).

** Electrons consumed for azide = (N_3^- reduced × 2) + [(N_3^- reduced − N_2 found) × 6) for N_2 = NH_3 found × 3.

With lower concentrations of azide and a lower ratio of gas-to-liquid volume (2.6:1), the final product contains more than 2 times as much NH_3 as N_2. Over 50% of the azide is reduced. Under these conditions 7.8 μmoles (29%) of the N_2 formed from azide is further reduced to NH_3. These results suggest that reduction of N_2 synthesized from azide occurs only under conditions favorable for the subsequent binding of N_2 on the site, *i.e.*, high partial pressure of N_2 and low azide concentration. Thus, the N_2 formed from azide does not appear to be in the correct position for further reduction, although it must be within 1–2 Å of the binding site.

Although the rate of azide reduction relative to the rate of N_2 fixation varies with the different conditions, the rates of electron utilization are similar (Table IV).

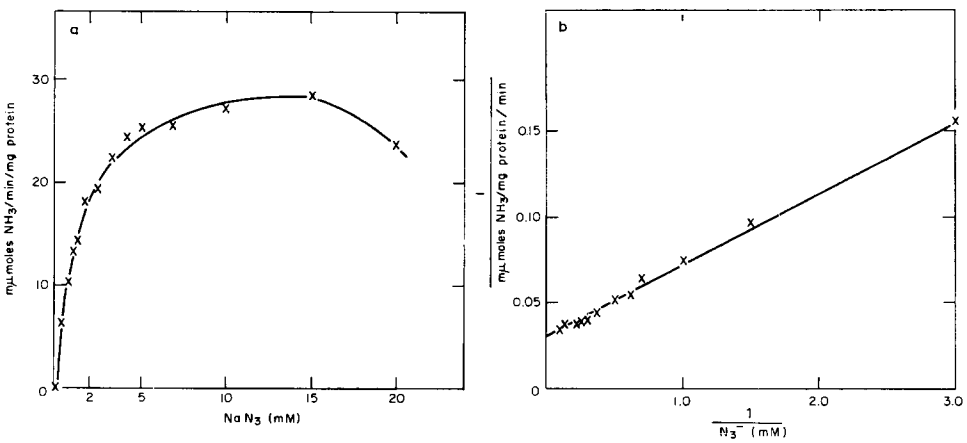


Fig. 2. a. Azide concentration and NH_3 synthesis. Incubation mixture, complete system (see Table I); time, 15 min at 30°; vol., 2.0 ml. b. Lineweaver–Burk plot with data of Fig. 2a for estimation of K_m of azide with heated extract of N_2-grown *A. vinelandii*.

The rate of azide reduction with high azide concentration and large gas volume is about 3 times that of N_2 fixation, while the rate with low azide concentration and low gas volume is only about 2 times that of N_2 fixation. Electron consumption for either azide reduction or N_2 fixation is similar under both sets of conditions, *e.g.*, 42 μmoles of electrons for azide reduction *vs.* 36 for N_2 fixation at high azide concentration and 102 *vs.* 83 at low azide concentration.

ATP-dependent H_2 evolution is decreased when azide is reduced to NH_3 and N_2 (Table IV). This reduction of H_2 evolution has been previously observed in the

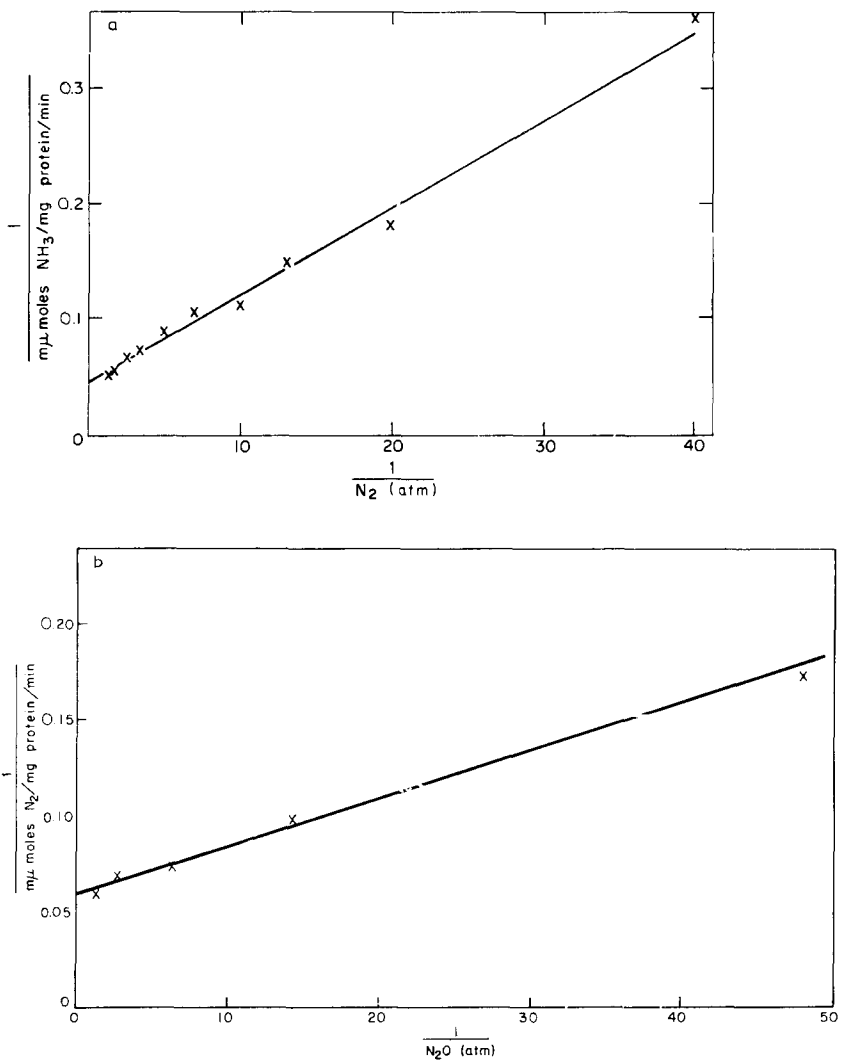


Fig. 3. Lineweaver–Burk plot for estimation of K_m of N_2 (a) and N_2O (b) with heated extract of N_2-grown *A. vinelandii*. Incubation mixture: complete system (see Table I), except that NaN_3 was omitted. Gas phase, 0.025–1.0 atm N_2 *plus* He to 1 atm was used with He, 1 atm, as control for N_2 fixation and 0.021–1.0 atm N_2O *plus* He to 1 atm for N_2O reduction. Time, 30 min at 30°; vol., 2.0 ml.

reduction of N_2 or N_2O (refs .1, 6). The decrease in electrons utilized for H_2 evolution in the presence of azide approximates the electrons utilized for reduction of azide to NH_3 and N_2 under both sets of conditions.

K_m of azide

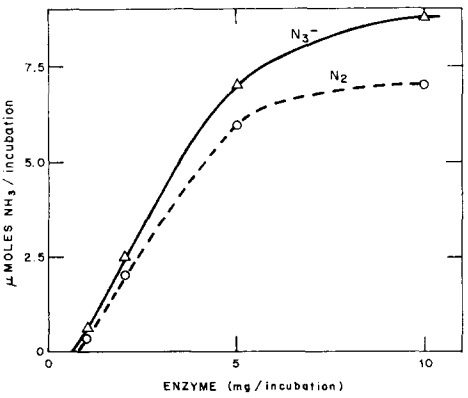
A typical experiment for the estimation of the K_m of azide in these reactions with heated extracts of N_2-grown *A. vinelandii* is shown in Fig. 2. All these assays were carried out for 15 min in a 2-ml liquid volume in order to minimize the conversion of product N_2 to NH_3, especially at low substrate concentrations of azide. Only NH_3 formation was assayed. A normal increase in rate occurs from 0–15 mM azide. Concentrations above 15 mM azide were inhibitory, presumably because of their inhibitory effect on the reductant-dependent ATPase (Fig. 1). The K_m for the reported experiment is 1.3 mM, and the range of values from three experiments is 1.27–1.45 mM.

K_m of N_2, N_2O and C_2H_2

The K_m's of N_2, N_2O, and C_2H_2 were determined with heated extracts of *A. vinelandii* for comparison with the K_m's of azide and HCN. The experiment with N_2 shown in Fig. 3a yielded a K_m of 0.16 atm (0.10 mM). The range of values from three experiments with N_2 is 0.14–0.19 atm with an average of 0.16 atm. This is similar to that reported for cell-free extracts of *C. pasteurianum*[7]. A typical experiment with N_2O is shown in Fig. 3b. The average K_m for N_2O was 0.05 atm (1.2 mM). This is identical to the K_i reported for N_2O inhibition of N_2 fixation with extracts of *C. pasteurianum*[9]. A tentative K_m for C_2H_2 is 0.03 atm (1.1 mM); data for C_2H_2 will be presented in a paper devoted to analogs of C_2H_2 and cyanide[12].

Enzyme level

The reductions of azide and N_2 to NH_3 by different concentrations of heated extract of *A. vinelandii* are compared in Fig. 4. Although NH_3 formation is greater



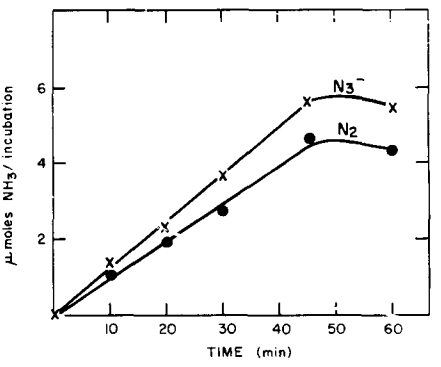


Fig. 4. Enzyme level (heated extract of *A. vinelandii*) and azide reduction or N_2 fixation. Incubation mixture, complete system (see Table I); time, 45 min; vol., 2.0 ml.

Fig. 5. Time courses of azide reduction and N_2 fixation by heated extract of *A. vinelandii*, 5 mg/ml. Incubation mixture, complete system (see Table I); vol., 2.0 ml.

from azide than from N_2, the curves appear to be identical and linear from 1.0–5.0 mg protein/2 ml. Neither curve extrapolates to zero enzyme concentration. This has previously been observed with N_2 fixation[6].

Time course of azide reduction

The time courses for the reduction of azide and N_2 to NH_3 by heated extracts of *A. vinelandii* are also similar (Fig. 5). Both are linear during the initial 45 min, and both stop at the same time because of depletion of the ATP-generator.

HCN reduction

Assays

Cyanide reduction by N_2-fixing extracts was assayed in four different ways: (1) base formed (NH_3 *plus* "CH_3NH_2") was titrated after microdiffusion from saturated K_2CO_3 into 2% H_3BO_3 in a Conway microdiffusion cell; (2) "$^{14}CH_3NH_2$" was measured by counting "$^{14}CH_3NH_2$" from $H^{14}CN$ after similar microdiffusion and trapping in H_3BO_3; (3) CH_4 was determined by H_2 flame ionization after gas chromatography; and (4) CH_4 and other gases were measured by mass spectrometry. Determination of base formed is less reliable than the other assays since titrations of diffused bases are corrected for background NH_3 of extracts and this correction is as high as 25–50% of the final titration. The CH_4 assay by gas chromatography is most sensitive and has a minimum detectability of $3 \cdot 10^{-7}$ g CH_4 per l of gas. CH_4 has been positively identified, while "CH_3NH_2" is suggested by indirect evidence. Methylamine will therefore be referred to as "CH_3NH_2". Although NaCN is the substance added, the substrate will be referred to as HCN since this is the principle form at the incubation pH.

Requirements and inhibitors

HCN is reduced to bases (NH_3 *plus* "CH_3NH_2") by extracts of N_2-grown *A. vinelandii*. The requirements for these reductions are again identical to those for N_2 fixation or N_2O reduction and include heated extract of N_2-grown *A. vinelandii*, $Na_2S_2O_4$, and an ATP-generator (Table V). NH_3 *plus* "CH_3NH_2" synthesis from HCN is 45% of NH_3 synthesis from N_2 by this extract; an average value of 41% was obtained with four different extracts. The reduction of HCN to NH_3 and "CH_3NH_2" is completely inhibited by 0.9 atm CO and partially inhibited by 0.9 atm N_2O. Addition of HCN (5 mM) to an extract covered with N_2 decreases the base formed and suggests competition between N_2 and HCN .

The requirements for and inhibitors of the reduction of $H^{14}CN$ to "$^{14}CH_3NH_2$" by *A. vinelandii* parallel those described above for the formation of NH_3 and "CH_3NH_2" (Table V). Heated extract of *A. vinelandii*, $Na_2S_2O_4$, and an ATP source are essential. CO, 0.9 atm, completely inhibits (97%); azide, 10 mM, partially inhibits (63%), and N_2O or N_2, 0.9 atm, inhibit only slightly (3–6%). Although H_2 is not an effective reductant, it appears to increase "$^{14}CH_3NH_2$" formation by the *A. vinelandii* enzymes. This stimulation by H_2 may be due to the displacement of some of the enzyme-bound "$^{14}CH_3NH_2$" so that it is not further reduced to CH_4 and NH_3.

The requirements for CH_4 formation from HCN by extracts of *A. vinelandii* parallel those described for "CH_3NH_2" *plus* NH_3 and for "$^{14}CH_3NH_2$". (Table V con-

tains results from gas chromatography and Table VIII contains results from mass spectrometry.) CO also completely inhibits CH_4 formation.

Crude extracts of N_2-grown *C. pasteurianum* also formed NH_3 *plus* "CH_3NH_2" and CH_4 from HCN, and "$^{14}CH_3NH_2$" from $H^{14}CN$. Requirements and inhibitions were similar to those observed with extracts of *A. vinelandii* except that $Na_2S_2O_4$ could be replaced by H_2 or KBH_4. The ability of H_2 and KBH_4 to act as reductants

TABLE V

REQUIREMENTS AND INHIBITORS OF HCN REDUCTION

A. vinelandii. Complete system (see Table I) except that NaCN, 10 μmoles/2 ml or $Na^{14}CN$, 63 000 counts/min per 10 μmoles replaced NaN_3. Different heated extracts of N_2-grown cells were used in each series of experiments. *C. pasteurianum*. Complete system contained per 2 ml in μmoles: potassium cacodylate, 100; creatine phosphate (Cr ~ P), 112; ATP, 10; $Na_2S_2O_4$, 20 (all at pH 6.5); $MgCl_2$, 10; and NaCN, 10 containing 63 000 counts per min $Na^{14}CN$; and in mg protein: creatine kinase, 0.40; and crude extract, 18. In the indicated cases $Na_2S_2O_4$ was replaced with H_2, 0.9 atm or KBH_4, 1 mg/2 ml. Gas phase, 0.9 atm indicated gas; incubation time, 45 min at 30°; incubation vol., 2 ml. The μmoles of base are differences between sample and an identical control with NaCN omitted. The limit of detectability of CH_4 by gas chromatography was 0.004 μmole per incubation in this experiment.

Incubation system	*Gas phase*	*NH_3 plus "CH_3NH_2" (μmoles base per incubation)*	*"$^{14}CH_3NH_2$" (counts/min per incubation)*		*CH_4 (μmoles per incubation)*
		A. vinelandii	*A. vinelandii*	*C. pasteurianum*	*A. vinelandii*
Complete	He	2.4	1210	1400	1.7
−Extract	He	−0.1	0	20	<0.004
$-Na_2S_2O_4$	He	0.0	10	55	<0.004
−ATP, −Cr ~ P, −Creatine kinase	He	0.5	20	0	<0.004
−Cyanide	He		0	0	<0.004
$-Na_2S_2O_4$	H_2	0.0	20	3700	
$-Na_2S_2O_4$, $+KBH_4$	He			1920	
$-Na_2S_2O_4$, −ATP −Cr ~ P, $+KBH_4$	He			35	
Complete	CO	0.3	40	45	<0.004
Complete + 10 mM N_3^-	He		450		
Complete	N_2O	2.1	1160		
Complete	N_2	−0.8	1130	1730	
Complete	H_2		1650	3160	
N_2-fixation assay	N_2	5.3			

for N_2 fixation with extracts of these bacteria has previously been established[4,7]. Typical data for "$^{14}CH_3NH_2$" formation from $H^{14}CN$ by crude extracts of N_2-grown *C. pasteurianum* are shown in Table V. H_2 is the most effective reductant, followed by KBH_4 and $Na_2S_2O_4$. ATP is essential with all reductants. Pyruvate could not be used as a reductant and energy source since it forms an addition product with cyanide. This addition product non-enzymically forms NH_3 in the microdiffusion system. CO inhibits the reductions completely.

Dependence of HCN reduction on extracts of N_2-grown cells

Reduction of HCN to NH_3, "CH_3NH_2", and CH_4 is catalyzed by extracts of cells grown on N_2 but not by extracts of those grown on urea or NH_3 (Table VI). Thus, heated extracts of N_2-grown *A. vinelandii* reduce HCN and N_2, but heated extracts

TABLE VI

DEPENDENCE OF HCN REDUCTION ON EXTRACTS OF N_2-GROWN CELLS

A. vinelandii. See complete system (Table I) with NaCN, 10 μmoles/2 ml or $Na^{14}CN$, 63 000 counts/min per 10 μmoles in place of NaN_3. *C. pasteurianum*. See complete system (Table V), with dithionite as reductant and NaCN, 10 μmoles/2 ml or $Na^{14}CN$, 63 000 counts/min per 10 μmoles. Incubation vol., 2 ml; incubation time, 45 min at 30°; gas phase, 0.9 atm He for HCN reduction and 0.9 atm N_2 or He for N_2 fixation. CH_4 determined by gas chromatography.

Extract	*Substrate*	*$H^{14}CN$*	*HCN*		*N_2*
	Product	*"$^{14}CH_3NH_2$" (counts/min per incubation)*	*NH_3 plus "CH_3NH_2" (μmoles per incubation)*	*CH_4*	*NH_3 (μmoles per incubation)*
A. vinelandii-heated					
N_2-grown cells		1210	2.0	1.34	5.1
Urea-grown cells		0	0.7	0.012	0.0
C. pasteurianum-crude					
N_2-grown cells		3300	0.9	0.30	3.2
Urea-grown cells		0	0.0	0.014	0.0

of urea-grown *A. vinelandii* reduce neither. The sensitive "$^{14}CH_3NH_2$" and CH_4 assays indicate that the HCN-reducing activity of extracts of urea-grown cells is less than 1% of that of N_2-grown cells. Similarly, crude extracts of N_2-grown *C. pasteurianum* reduce both HCN and N_2, but crude extracts of NH_3-grown *C. pasteurianum* reduce neither.

Distribution of HCN-reducing activity

HCN-reducing activities of both *A. vinelandii* and *C. pasteurianum* accompany N_2-fixing activities during purification. The HCN-reducing activity measured as base formation (NH_3 *plus* "CH_3NH_2") from HCN or "$^{14}CH_3NH_2$" formation from $H^{14}CN$ and the N_2-fixing activity of crude extracts of *A. vinelandii* are both concentrated in the heated extract and the precipitate after 3 h at 225 000 × *g* (P_3) (Table VII, Expt. 1). The specific activities of "$^{14}CH_3NH_2$" formation from $H^{14}CN$ and NH_3 formation from N_2 by the P_3 fraction show similar-fold purifications (5.1- and 5.6-fold, respectively) from crude extract. The specific activity of HCN reduction measured as CH_4 formation is also increased in the heated extract. The CH_4-forming and N_2-reducing activities of the heated extract are both concentrated in the precipitate that forms between 0.100 and 0.125 mg protamine sulfate/mg protein. The specific activities of CH_4 formation from HCN and NH_3 formation from N_2 by this fraction also show parallel-fold purification. It has recently been demonstrated that the N_2-fixing particle of *A. vinelandii* can be separated into two fractions by anaerobic chromatography on DEAE-cellulose[13]. One fraction is eluted with 0.02 M Tris·HCl (pH 7.0) containing 0.23 M NaCl (DEAE–0.23 M NaCl) and the other fraction is

TABLE VII

DISTRIBUTION OF HCN-REDUCING ACTIVITY

A. vinelandii. See complete system (Table I) with NaCN, 10 μmoles/2 ml or $Na^{14}CN$, 63 000 counts/min per 10 μmoles in place of NaN_3. Extracts in mg protein/2 ml: crude extract, 18; heated extract, 8; $P_{1/2}$, 6; P_3, 4; S_3, 12; protamine sulfate 0.00–0.100, 6; protamine sulfate 0.100–0.125, 2; protamine sulfate 0.125 +, 3; DEAE–0.23 M NaCl, 0.8 mg; and DEAE–0.35 M NaCl, 0.8 mg. *C. pasteurianum.* See complete system (Table V) with $Na_2S_2O_4$ as reductant and extracts in mg protein/2 ml: crude extract, 18; heated extract, 14; and phosphate gel preparation, 8. Incubation time, 45 min at 30°; vol., 2 ml; gas phase, 0.9 atm of He for HCN reduction, and 0.9 atm of N_2 with 0.9 atm of He as control for N_2 reduction. CH_4 determined by gas chromatography.

Extract	*Expt. 1*			*Expt. 2*	
Substrate	$H^{14}CN$	*HCN*	N_2	*HCN*	N_2
Product measured	"$^{14}CH_3NH_2$ (*counts/min per mg protein per min*)	"CH_3NH_2" + NH_3 (*mμmoles/mg protein per min*)	NH_3	CH_4 (*mμmoles/mg protein per min*)	NH_3
A. vinelandii					
Crude extract	0.9	2.0	6.5	1.7	8.7
Heated extract	2.0	3.9	15.0	3.5	17.0
$P_{1/2}$	0.5	3.1	7.4		
P_3	4.6	7.3	37		
S_3	0.1	1.1	1.7		
Protamine sulfate 0.0–0.100				1.5	5.7
Protamine sulfate 0.1–0.125				8.2	46.0
Protamine sulfate 0.125 +				0.0	0.0
DEAE–0.23 M NaCl				0.22	
DEAE–0.35 M NaCl				0.00	
DEAE–0.23 M NaCl + DEAE–0.35 M NaCl (1:1)				0.9–4.5	
C. pasteurianum					
Crude extract	0.36	1.0	2.0	0.30	2.1
Heated extract	0.012	0.0	0.3	0.00	0.0
Phosphate gel preparation	0.000	0.1	0.0	0.02	0.0
Heated extract + phosphate gel preparation (2:1)	0.10	1.3	4.1	0.21	5.0

eluted with 0.02 M Tris·HCl (pH 7.0) containing 0.35 M NaCl (DEAE–0.35 M NaCl). We have confirmed that a combination of both fractions is synergistic for N_2 fixation. We have also found that a combination of both fractions is synergistic for HCN reduction (Table VII). With extracts of *C. pasteurianum*, both the HCN-reducing activity as measured by total base, "$^{14}CH_3NH_2$", or CH_4 formation and the N_2-fixing activity are removed by heating at 60° or treatment with phosphate gel; however, combination of the two fractions restores both activities (Table VII).

Products and stoichiometry of HCN reduction

The products of HCN reduction have been identified as CH_4 and a base that distills at room temperature. This base is assumed to be about 90% NH_3 and 10% CH_3NH_2. CH_4 was identified by gas chromatography (Tables V, VI and VII) and by mass spectrometry (Table VIII). CH_3NH_2 is a suggested product and has not been

TABLE VIII

PRODUCTS AND STOICHIOMETRY OF HCN REDUCTION BY *A. vinelandii*

Complete system (Table I) with heated extract of *A. vinelandii*, 100 mg/20 ml, and NaCN or $Na^{14}CN$, 100 μmoles/20 ml, in place of NaN_3. Incubation volume for HCN reduction was 20 ml and all components were added accordingly. CH_4 and H_2 were determined by mass spectrometry and 0.2 atm of indicated gas was used in these experiments. "$^{14}CH_3NH_2$" was calculated from conversion of $H^{14}CN$ containing 630 000 counts/min per 100 μmoles. N_2 fixation was determined in 2 ml volume with a gas phase of 0.9 atm N_2 or He and reported results are multiplied by 10 for comparison with HCN reductions. Incubation time, 45 min at 30°.

Incubation system	*Substrate*	*Gas phase*	*Product (μmoles/100 mg protein)*				*Substrate* reduced (μmoles)*	*Electrons** consumed (μmoles)*
			H_2	CH_4	NH_3 + "CH_3NH_2"	"$^{14}CH_3NH_2$"		
Complete	HCN or $H^{14}CN$	He	29	8.4	11.2	0.9	9.3–11.2	54
Complete	—	He	173	0.0	0.0			
Complete $-Na_2S_2O_4$ or −extract or −ATP, −Cr ~ P, −creatine kinase	HCN or $H^{14}CN$	CO	100	0.2	0.0	0.06		
	HCN	He	0.0–1.6	0.0	0.0			
Complete (N_2 fixation)	N_2	N_2			56.0		28	168

* Substrate reduced for HCN = CH_4 + "$^{14}CH_3NH_2$" or NH_3 + "CH_3NH_2"; for N_2 = $^1/_2 \times NH_3$.

** Electrons consumed for HCN = (CH_4 formed × 6) + ("$^{14}CH_3NH_2$" formed × 4); for N_2 = N_2 reduced × 6.

absolutely identified. Attempts to identify enzymatically produced "CH_3NH_2" or known CH_3NH_2 by gas chromatography or mass spectrometry have been unsuccessful because of the small amount of product formed even in large incubations (Table VIII). Evidence for "CH_3NH_2" is the following: (1) The total base that distills at room temperature from saturated K_2CO_3 into H_3BO_3 exceeds the CH_4 evolved by 10–20%; (2) $H^{14}CN$ is enzymatically transformed to a radioactive base that is distilled at room temperature and trapped by H_3BO_3; (3) the amount of this base is about 10–20% of total base and approximates the difference between total base distilled at room temperature and CH_4 (Table VIII); (4) this radioactive product distills more slowly at room temperature from saturated K_2CO_3 than NH_3 and this slower rate of distillation corresponds to that of known CH_3NH_2; and (5) colorimetric determination of "CH_3NH_2" (ref. 17) from an incubation indicates the presence of small amounts of CH_3NH_2. Thus, the major products of HCN reduction are equimolar amounts of CH_4 and a base proposed to be NH_3 (Table VIII). The minor product of HCN reduction is a base proposed to be CH_3NH_2. The following equations represent the reductions:

$$HCN \rightarrow CH_4 + NH_3$$
$$HCN \rightarrow CH_3NH_2$$

The minor product observed amounts to about 10% of the major products.

The conversion of 5 mM HCN to CH_4 *plus* NH_3, and "CH_3NH_2" is about 10% and 1%, respectively (Table VIII). Thus, the major products, CH_4 and NH_3, do not arise from impurities in NaCN. The conversion of $H^{14}CN$ to "$^{14}CH_3NH_2$" with

TABLE IX

CONVERSION OF $H^{14}CN$ TO "$^{14}CH_3NH_2$"

Complete system (Table I) except that $Na^{14}CN$ containing 56 000 counts/10 μmoles replaced azide. Incubation vol., 2 ml; time, 45 min at 30°; gas phase, 0.9 atm He.

$H^{14}CN$		*"$^{14}CH_3NH_2$"*	
mM	*counts/min per incubation*	*counts/min per incubation*	*% yield*
1.25	14 000	1110	7.8
2.50	28 000	1360	4.9
5.00	56 000	1390	2.5
10.0	112 000	730	0.7

1.25–10.0 mM $H^{14}CN$ is recorded in Table IX. With 1.25 mM $H^{14}CN$ the conversion is 8%. Thus, the minor product, "CH_3NH_2", is not synthesized from impurities in $H^{14}CN$.

The rate of HCN reduction measured as the sum of CH_4 or NH_3 *plus* "CH_3NH_2" is about 30–40% of that of N_2 reduction. Based on 4 electrons required for CH_3NH_2 formation from HCN, 6 for CH_4 and NH_3 from HCN, and 6 for 2 NH_3's from N_2, the rate of electron consumption for HCN reduction is about 30% of that for N_2 reduction (Table VIII).

ATP-dependent H_2 evolution is decreased by HCN as it is by N_2, N_2O, N_3^- and C_2H_2 (refs. 1, 6 and R. W. F. HARDY AND E. KNIGHT, JR., unpublished data). Surprisingly, however, the electrons utilized for reduction of HCN to CH_4, NH_3, and "CH_3NH_2" do not account for the decrease in electrons evolved as H_2. This may suggest that other unidentified products of HCN reduction utilize these electrons or that HCN inhibits the reaction. Addition of CO stops reduction of HCN but only partially restores H_2 evolution. This observation suggests that the presence of HCN, even though it is not being reduced, may inhibit H_2 evolution.

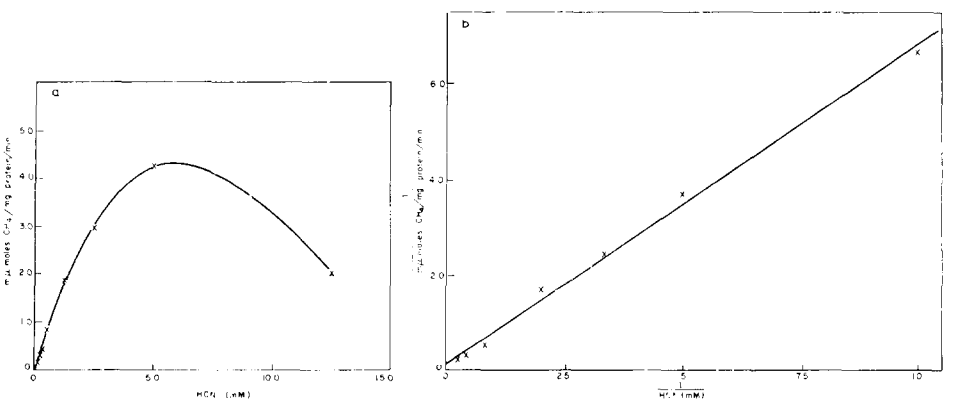


Fig. 6. a. HCN concentration and CH_4 synthesis by heated extract of N_2-grown *A. vinelandii*. Incubation mixture, complete system (Table V); time, 30 min at 30°; vol., 2.0 ml. b. Lineweaver–Burk plot with data of Fig. 6a for estimation of K_m of HCN with heated extract of N_2-grown *A. vinelandii*.

K_m for HCN reduction

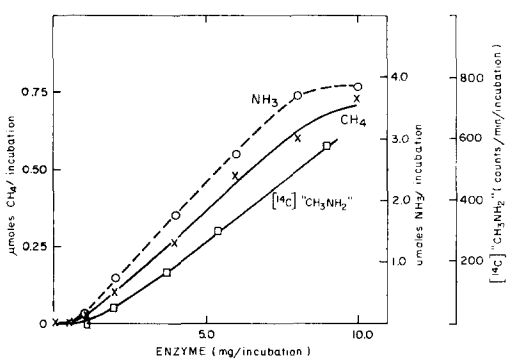
HCN reduction is very sensitive to the concentration of substrate (Fig. 6a). Formation of both "$^{14}CH_3NH_2$" from $H^{14}CN$ and CH_4 from HCN increases as substrate is increased from 0–5 mM. Above this concentration inhibition occurs. The average K_m of HCN was 4 mM with 3 different heated extracts of *A. vinelandii* (Fig. 6b). This value is susceptible to error because of substrate inhibition which occurs above 5 mM.

Enzyme level for HCN reduction

Syntheses of NH_3 from N_2, CH_4 from HCN, and "$^{14}CH_3NH_2$" from $H^{14}CN$ show a similar dependence on enzyme concentration (heated extract of *A. vinelandii*). All syntheses are linear from 1–8 mg protein/2 ml incubation mixture. As observed with N_3^- or N_2, the lines do not extrapolate to zero mg protein (Fig. 7).

Time course of HCN reduction

The reduction of HCN measured as CH_4 or NH_3 *plus* "CH_3NH_2" synthesized is linear during the initial 40–50 min of incubation (Fig. 8). This again parallels the previous observations on N_2 fixation and N_3^- reduction.



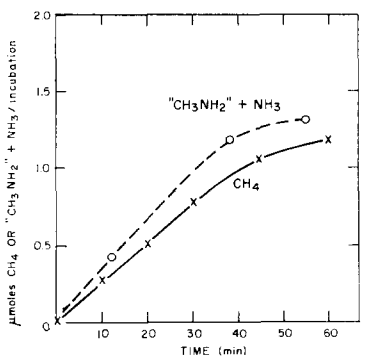


Fig. 7. Enzyme level (heated extract of *A. vinelandii*) and HCN reduction to CH_4, $H^{14}CN$ reduction to "$^{14}CH_3NH_2$", and N_2 fixation. Incubation mixture, complete system (Table V); time, 30 min at 30°; vol., 2.0 ml; $H^{14}CN$, 51 000 counts/10 μmoles.

Fig. 8. Time courses of HCN reduction to CH_4 and NH_3 *plus* "CH_3NH_2" by heated extract of *A. vinelandii*. Incubation mixture, complete system (Table V); vol., 2.0 ml.

Other attempted reductions

The reduction of CH_3NH_2 to CH_4 by *A. vinelandii* was examined. In an incubation mixture identical to that for HCN reduction, a trace of CH_4 above background was found with 10 mM CH_3NH_2 (Table X). No CH_4 above background was formed with 45 mM CH_3NH_2. The observed CH_4 synthesis from 10 mM CH_3NH_2 is less than 0.1% of that obtained from 5 mM HCN and represents a conversion of only 0.01% of the added CH_3NH_2. Thus, added CH_3NH_2 is a very poor substrate, and it is possible that the CH_4 synthesized originated from trace impurities rather than from CH_3NH_2. The failure to observe substantial CH_4 synthesis from CH_3NH_2 must

TABLE X

ATTEMPTED REDUCTION OF METHYLAMINE, NCO^- AND CO

Complete system (Table I), with heated extract of N_2-grown *A. vinelandii* and the indicated substrate in place of NaN_3. Incubation vol., 2 ml; incubation time, 45 min at 30°; and gas phase, 0.9 atm He or N_2 or 1.0 atm CO. CH_4 determined by gas chromatography.

Gas phase	*Substrate*	*CH_4 (μmoles per incubation)*	*NH_3 (μmoles per incubation)*
He	HCN, 5 mM	1.20	—
He	CH_3NH_2, 10 mM	0.0016	—
He	CH_3NH_2, 45 mM	0.0007	—
He	NCO^-, 5 mM	0.0015	—
CO	CO, 1 atm	0.0006	—
He	—	0.0007	—
N_2	N_2	—	6.2

represent weak binding and is not due to inhibition of reductant-dependent ATPase since 50 mM CH_3NH_2 does not affect this activity.

Reduction of cyanate to CH_4 was also examined with a complete incubation system identical to that for N_2 fixation. A trace of CH_4 similar to that produced from CH_3NH_2 was obtained. Similar reservations apply to this reduction as to the CH_3NH_2 reduction.

Reduction of CO was also examined. CO presumably binds to the substrate-binding site since it is a strong competitive inhibitor of N_2 fixation[9]. No CH_4 above background was obtained. Reduction of CO to CH_4 does not appear to occur; however, if it does, it must be less than 0.01% of the reduction of HCN to CH_4. Other less reduced products of CO are possible, *e.g.*, CH_2O, CH_3OH. Since CO has not been found to decrease ATP-dependent H_2 evolution even in large incubations (20 ml), it appears that anything but trace amounts of CO reduction products are excluded.

DISCUSSION

A number of reductions have now been demonstrated to be catalyzed by N_2-fixing extracts of *A. vinelandii* and *C. pasteurianum*[1-3,10-12,*]. These reductions include the following:

$N_2 \rightarrow 2\ NH_3$
$N_2O \rightarrow H_2O + N_2$
$N_3^- \rightarrow NH_3 + N_2$
$HCN \rightarrow CH_4 + NH_3$
$HCN \rightarrow CH_3NH_2$
$C_2H_2 \rightarrow C_2H_4$

The characteristics of each of these reductions are summarized under 16 different headings in Table XI. The striking parallelism of all these reductions by extracts of either the anaerobic or aerobic bacteria with respect to requirements, *e.g.*, ATP, reductant, and extract of N_2-grown cells, absolute dependence on N_2-grown *vs.* NH_3-grown or urea-grown cells, inhibition of energy-dependent H_2 evolution but not reductant-dependent ATPase, complete inhibition by CO, time course, and enzyme

* R. W. F. HARDY AND E. KNIGHT, JR., unpublished data.

TABLE XI

SUMMARY OF CHARACTERISTICS OF REDUCTIONS CATALYZED BY N_2-FIXING ENZYMES

Bacterial extract	*(A. vinelandii)* or (C. pasteurianum)**					
Substrate	N_2	N_2O	N_3^-	*HCN*		C_2H_2
Products	2 NH_3	$N_2 + H_2O$	$N_2 + NH_3$	$CH_4 + NH_3$	CH_3NH_2	C_2H_4
Proposed intermediates	*cis*-N_2H_2, N_2H_4	None	None	CH_2NH, CH_3NH_2	CH_2NH	None
Proposed bonding to site	n.d.	n.d.	n.d.	n.d.	n.d.	π, side on
Requirements	ATP $Na_2S_2O_4$ (H_2, BH_4^-) N_2-grown extracts	ATP $Na_2S_2O_4$ N_2-grown extracts	ATP $Na_2S_2O_4$ (H_2, BH_4^-) N_2-grown extracts	ATP $Na_2S_2O_4$ (H_2, BH_4^-) N_2-grown extracts	ATP $Na_2S_2O_4$ (H_2, BH_4^-) N_2-grown extracts	ATP [$Na_2S_2O_4$] (H_2) N_2-grown extracts
Distribution of activity	At least two components required	n.d.*	Parallels N_2 fixation	Parallels N_2 fixation	Parallels N_2 fixation	n.d.*
Effect on reductant-dependent ATPase	None	[None**]	[None**]	[None**]	[None**]	[None**]
Effect on ATP-dependent H_2 evolution	Inhibits	Inhibits	Inhibits	[Inhibits]	[Inhibits]	[Inhibits]
Inhibition of H_2 evolution $\cong$ electrons used for reductions	Yes	Yes (Competitive inhibition)	[Yes]	[No]	[No]	[Yes]
Effect on N_2 fixation		Inhibits	Inhibits	Inhibits	Inhibits	Inhibits
Effect of CO	Competitive inhibitor	Inhibits	Inhibits	Inhibits	Inhibits	Inhibits
Enzyme level	Linear but does not extrapolate to zero	n.d.	[Parallels N_2 fixation]	[Parallels N_2 fixation]	[Parallels N_2 fixation]	(Parallels N_2 fixation)
Time course	Linear until ATP exhausted	[Parallels N_2 fixation]	[Parallels N_2 fixation]	[Parallels N_2 fixation]	[Parallels N_2 fixation]	(Parallels N_2 fixation)
Estimated K_m (mM)	0.1	[1]	[1]	[4]	n.d.	(0.4) [1]
$v_{max.}$ relative to N_2 fixation	1	[3]	[3]	[0.4]	[0.04]	[3]
Electrons per molecule	6	2	2	6	4	2
Rate × electrons	6	[6]	[6]	[2–3]	[0.1 0.2]	[6]
References	3, 4, 5, 6, 7, 8, 9	1, 2, 3	1, 3, 10, 11	1, 3	1, 3	10, 11, 12, †

* [ ] indicates only determined or valid for *A. vinelandii*; ( ) indicates only determined or valid for *C. pasteurianum*; neither indicates determined for both *A. vinelandii* and *C. pasteurianum*; n.d. indicates not determined.

** None at concentrations used as substrate, *e.g.*, N_3^- 10 mM, HCN 5 mM, C_2H_2 0.1 atm, N_2O 0.5–1 atm.

† R. W. F. HARDY AND E. KNIGHT, JR., unpublished data.

level, provides convincing indirect proof that a common enzyme system is responsible for all these reductions. Such a proposal was initially introduced for the reduction of N_2O to N_2 (ref. 1). At that time it was concluded that at least part of the N_2-fixing system, the electron-activating reaction, *i.e.*, reductant-dependent ATPase, was involved in N_2O reduction. An additional essential site required for N_2O reduction was demonstrated by CO inhibition[1]. This site was suggested to be the N_2-binding site rather than a discrete N_2O-binding site. Similar conclusions are now drawn for the reduction of HCN, N_3^-, and C_2H_2 since the characteristics of these new reductions are identical to those of N_2O and N_2. The number of these additional reductions lends further support to the suggestion that the additional essential site or enzyme (demonstrated again by CO inhibition of each of these new reductions) is the suggested N_2-binding site rather than different binding sites for each substrate. (Inhibition of N_2 fixation by the new substrates occurs[9–11]; however, these inhibitions do not provide proof of a common binding site since there could be competition between N_2 and these other substrates for activated electrons.) One may then expect to find all of the above reactions catalyzed by all N_2-fixing systems. The symbiotic N_2-fixing system is currently being examined for catalysis of some of these reductions.

The N_2-fixing system is thus proposed to consist of an electron-activating site

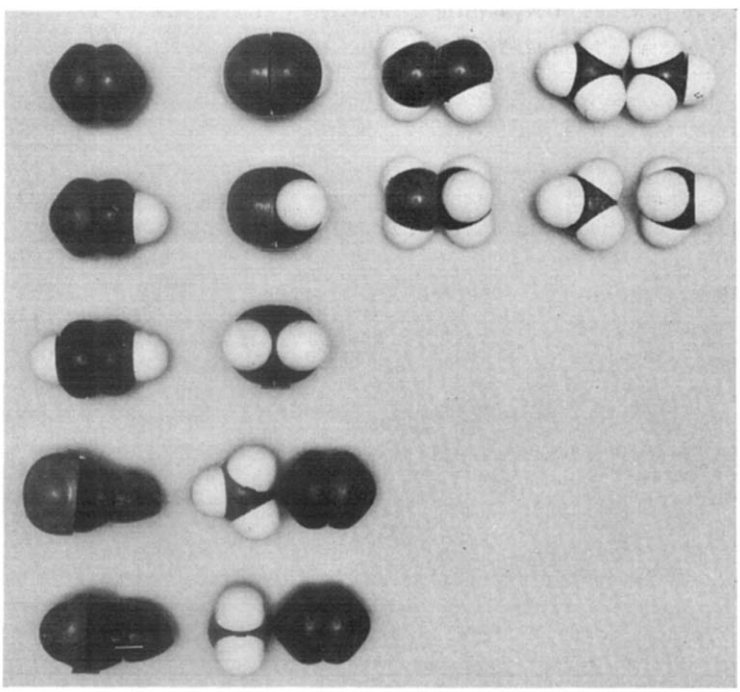


Fig. 9. Molecular models of substrates, postulated intermediates, and / or products of the N_2-fixing enzymes of *A. vinelandii* and *C. pasteurianum*. The organization of models follows:

N_2	*cis*-N_2H_2	N_2H_4	NH_3, NH_3
HCN	CH_2NH	CH_3NH_2	NH_3, CH_4
C_2H_2	C_2H_4		
N_3^-	NH_3, N_2		
N_2O	H_2O, N_2		

and a uniquely versatile substrate-binding site. This binding site is suggested to bind and/or activate N_2, N_2O, N_3^-, HCN, and C_2H_2 for reduction by electrons (possibly a metal hydride) donated by the electron-activating site. All substrates whose reductions are reported are small linear molecules; however, there appears to be considerable variability with respect to molecular length, *e.g.*, N_2O or N_3^- are about 2 times as long as N_2 (Fig. 9). Current reductions of analogues of HCN and acetylene indicate that even longer and in some cases non-linear molecules may be reduced[12]. The site appears to differentiate somewhat with respect to the binding of these different substrates. The observed K_m's with heated extract of *A. vinelandii* indicate that nitrogen is the preferred substrate followed by C_2H_2, N_2O, N_3^- and HCN. Although the site differentiates with respect to binding and/or activation, the rates of electron consumption are similar for N_2, C_2H_2, N_2O, and N_3^- (Table XI). This suggests that the electron-activating reaction and not the substrate-binding reaction is limiting in all these reductions. The small amount of "CH_3NH_2" observed as a product of HCN reduction coupled with the negligible reduction of CH_3NH_2 to CH_4 indicates that CH_3NH_2 binds poorly to the site. It is suggested that one of the hydrogens of the CH_3 group of CH_3NH_2 may distort the molecule from the site and prevent the required contact with the binding site (Fig. 9). In contrast, N_2H_4 is less distorted by its hydrogens (Fig. 9) and therefore binds strongly enough so that no N_2H_4 is observed as an enzyme-free product of N_2 fixation. Ethylene binds to the site less well than CH_3NH_2 since no C_2H_6 or CH_4 is observed from C_2H_2 (refs. 10, 11*). CO binds very tenaciously to the site ($K_i = 3.4 \cdot 10^{-7}$ M) (ref. 9) and inhibits all reductions. Yet there is no evidence for reduction of CO. This lack of reduction may mean incorrect orientation, lack of activation, or inability of the activated electrons to reduce CO. Nitric oxide also binds tenaciously to the site ($K_i = 4.3 \cdot 10^{-7}$ M) (ref. 9). We have observed reduction of NO to N_2, but this may not be completely enzymatic since the reductant used, $Na_2S_2O_4$, reduces NO to N_2O in the absence of enzyme.

Some preliminary observations on the possible orientations and bonding of the substrate and the binding and/or activating site are suggested by the various substrates. All substrates have a triple bond, *e.g.*, $N \equiv N$, $HC \equiv N$, $HC \equiv CH$ or a possible triple bond, *e.g.*, $N \equiv N^+-O^-$, $N \equiv N^+-N^{2-}$ (ref. 21) which would permit π bonding (side-on) and in all cases except C_2H_2 there is one or more pairs of non-bonding electrons, *e.g.*, $:N \equiv N:$, $HC \equiv N:$, $:N=N^+-\ddot{N}^{2-}: \longleftrightarrow :\ddot{N}^- \equiv N^+ = \ddot{N}^-:$, $:N \equiv N^+-\ddot{O}^-:$ (ref. 22) which would permit σ bonding (end-on). Thus, the major possible orientations of substrate ($N \equiv N$) and binding site(s) (M) are single or double side-on, *e.g.*,

$$\begin{matrix} & \mathrm{M} \\ & \uparrow \\ \mathrm{N} \equiv \mathrm{N} \text{ or } & \mathrm{N} \equiv \mathrm{N} \end{matrix}$$

and single or double end-on, *e.g.*, $N \equiv N^+-M$ or $M-{}^+N \equiv N^+-M$. Double

$$\begin{matrix} \downarrow & \downarrow \\ \mathrm{M} & \mathrm{M} \end{matrix}$$

end-on bonding of N_2 with a site that changes dimensions was suggessted by the Kettering group[6]. This is intriguing since the intermediates, N_2H_2 and N_2H_4, which we will propose between N_2 and NH_3 represent increasing molecular lengths. Such a variable site would also accommodate the new substrates N_3^- and N_2O that are about 2 times as long as N_2. However, this proposal which has never had any experimental

* R. W. F. HARDY AND E. KNIGHT, JR., unpublished data.

basis is now doubtful. N_2 formed from azide or N_2O does not appear to be further reduced, although a double binding site would require the product N_2 to be attached to one of the binding sites. Cyanide or C_2H_2 groups with at least one end blocked with carbon atoms are reduced even though double end-on binding is impossible[12]. Moreover acetylene appears to be bound side-on (π bonded) since its original hydrogens are not replaced during reduction in ^2H_2O with extracts of *C. pasteurianum*[11] and *A. vinelandii* (R. W. F. HARDY AND E. KNIGHT, JR., unpublished data). One is tempted to suggest that the orientation of the other substrates are analogous to that of C_2H_2. However, there is more precedent for single end-on bonding (σ) than for side-on bonding of nitriles[22,23]. Thus, it is not yet possible to select single or double side-on or single end-on bonding for any substrate except C_2H_2. However, it is difficult to imagine an organized binding and reducing system (nitrogenase) that shows similar rates of electron addition to different substrates when these substrates are oriented on the site in different ways.

The reduction products of N_2, HCN, N_2O, N_3^-, and C_2H_2 provide evidence for 2-, 4-, and 6-electron products. N_2 and NH_3 from azide, N_2 and H_2O from N_2O, and C_2H_4 from C_2H_2 are 2-electron products; "CH_3NH_2" from HCN is a 4-electron product; and CH_4 and NH_3 from HCN, and NH_3 from N_2 are 6-electron products. No evidence has been obtained for any oxidized products. Thus, we suggest that the N_2-fixing system consists of a stepwise reducing system. Two electrons are added at each step. On this basis the proposed intermediates for N_2 fixation are N_2H_2 and N_2H_4, and the proposed intermediates for HCN reduction are CH_2NH and CH_3NH_2. The failure to observe N_2H_4 and N_2H_2 suggests that they are enzyme bound. The presence of small amounts of "CH_3NH_2" indicates that about 10% of the CH_3NH_2 is lost from the site before the addition of the final 2 electrons. The presence of small amounts of methylene imine or its hydrolysis product, formaldehyde, has not been determined.

Utilization of the reduction of HCN to CH_4, of $H^{14}CN$ to $^{14}CH_3NH_2$, or of C_2H_2 to C_2H_4, and detection of CH_4 and C_2H_4 by hydrogen flame ionization after gas chromatography or detection of $^{14}CH_3NH_2$ may provide a sensitive new assay for detection of the N_2-fixing system. The gas chromatographic determination makes possible a range of about 10 000 times between minimum and maximum, in contrast to a 20-fold range with the NH_3 assay. C_2H_2 is the preferred assay substrate, since more product is formed because of its requirement for 2 electrons *versus* 6 electrons for HCN.

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