Morino, Y., Kojima, H., & Tanase, S. (1979) *J. Biol. Chem. 254*, 279.
Ovchinnikov, Yu. A., Kiryushin, A. A., Egorov, C. A., Abdulaev, N. G., Kiselev, A. P., Modyanov, N. N., Grishin, E. V., Sukhikh, A. P., Vinogradova, E. I., Feigina, M. Yu., Aldanova, N. A., Lipkin, V. M., Braunstein, A. E., Polyanovsky, O. L., & Nosikov, V. V. (1971) *FEBS Lett. 17*, 133.
Rando, R. R. (1974) *Biochemistry 13*, 3859.
Sandler, M., Ed. (1980) *Enzyme Inhibitors as Drugs*, University Park Press, Baltimore, MD.
Scardi, V., Scotto, P., Iaccarino, M., & Scarano, E. (1963) *Biochem. J. 88*, 172.
Schnackerz, K. D., Ehrlich, J. H., Geisemann, W., & Reed, T. A. (1979) *Biochemistry 18*, 3557.
Seiler, N., Jung, J. J., & Koch-Weser, J., Eds. (1978) *Enzyme-Activated Irreversible Inhibitors*, Elsevier/North-Holland, New York.
Silverman, R. B., & Levy, M. A. (1981) *Biochemistry 20*, 1197.
Soper, T. S., & Manning, J. M. (1981) *J. Biol. Chem. 256*, 4263.
Ueno, H. (1982) Ph.D. Dissertation, Iowa State University.
Yang, B. I., & Metzler, D. E. (1979) *Methods Enzymol. 62D*, 528.

Nitrogenase Reactivity: Cyanide as Substrate and Inhibitor†

Jia-ge Li,‡ Barbara K. Burgess,* and James L. Corbin

ABSTRACT: We have examined the reduction of cyanide by using the purified component proteins of nitrogenase (*Av*1 and *Av*2). The previously reported self-inhibition phenomenon was found to be an artifact. One of the two species present in cyanide solutions, CN^-, was shown to be a potent reversible inhibitor (K_i = 27 μM) of *total* electron flow, apparently uncoupling MgATP hydrolysis and electron transfer. There appears to be no differential effect of CN^- on the specific activities of *Av*1 and *Av*2 nor is there any apparent irreversible physical damage to *Av*2. CN^- inhibition is completely reversed by low levels of CO, implying a common binding site. Azide partially relieves the inhibitory effect, but other substrates and inhibitors (N_2, C_2H_2, N_2O, H_2) have no effect. The other species present in cyanide solutions, HCN, was shown to be the substrate (K_m = 4.5 mM at *Av*2/*Av*1 = 8), and extrapolation of the data indicates that at high enough HCN concentration H_2 evolution can be eliminated. The products are methane plus ammonia (six electrons), and methylamine (four electrons). There is an excess (relative to methane) of ammonia formed, which, according to electron balance studies, may arise from a two-electron intermediate. Both nitrous oxide and acetylene (but not N_2) influence the distribution of cyanide reduction products, implying simultaneous binding. HCN appears to bind to and be reduced at an enzyme state more oxidized than the one responsible for either H_2 evolution or N_2 reduction.

Nitrogen fixation and all other reductions catalyzed by the nitrogenase system require two easily separated component proteins, called the molybdenum–iron protein (MoFe protein) and the iron protein (Fe protein). The physical properties of these two proteins have been recently reviewed (Orme-Johnson et al., 1977; Mortenson & Thorneley, 1979), and great similarity among proteins from different bacterial sources is evident (Emerich & Burris, 1976a,b). In addition to these two proteins, a source of reducing equivalents, MgATP, and protons are required for all substrate reductions (Bulen & LeComte, 1966). The MoFe protein is believed to contain the site of substrate reduction (Shah et al., 1973; Hageman & Burris, 1979), while the Fe protein is generally accepted as the specific one-electron donor for the MoFe protein (Hageman & Burris, 1978a,b; Ljones & Burris, 1978a,b). In addition to N_2, nitrogenase catalyzes the reduction of protons, nitrous oxide, acetylene, azide, cyanide, alkyl cyanides, alkyl isocyanides, hydrazine, cyclopropene, and allene. Alternative substrates have often been studied as probes for the number and nature of sites of substrate interaction on nitrogenase and the types of intermediates that might be formed during N_2 reduction. This paper will describe recent studies on the reduction of cyanide by the purified component proteins of nitrogenase.

Cyanide reduction by nitrogenase was first demonstrated by Hardy & Knight (1967). Using crude nitrogenase preparations, they showed that cyanide was reduced by six electrons to methane plus ammonia with small amounts (10% of the NH_3) of another base which was suggested to be a four-electron product, methylamine. The formation of all products required both component proteins, MgATP, and a reductant, and all were completely inhibited by carbon monoxide (0.9 atm). These observations were subsequently confirmed by a number of other investigators (Hwang & Burris, 1972; Rivera-Ortiz & Burris, 1975; Kelly et al., 1967; Kelly, 1968). Hardy & Knight (1967) suggested that cyanide reduction proceeded via the two-electron intermediates, methylenimine and methylamine, and was a good model for N_2 reduction. They were unable, however, to demonstrate the reduction of methylamine to methane and ammonia. Kelly et al. (1967) observed very small amounts of ethylene and ethane (0.08% of CH_4) during cyanide reduction catalyzed by nitrogenase. They suggested that these C_2 products were formed by interaction of adjacent C_1 radicals on adjacent cyanide binding sites.

Several investigators (Hardy & Knight, 1967; Hwang & Burris, 1972; Rivera-Ortiz & Burris, 1975) reported that the rate of nitrogenase-catalyzed cyanide reduction appeared to

† From the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received February 10, 1982.* Contribution No. 771. This study was supported by Project No. 79-59-2394-0-1-383-1 from SEA/CGO of the U.S. Department of Agriculture.
‡ Present address: Institute of Botany, Peking, People's Republic of China.

0006-2960/82/0421-4393$01.25/0

increase with increasing sodium cyanide concentration up to 5 mM and then to decrease dramatically at higher cyanide concentrations. This phenomenon was termed substrate self-inhibition and caused difficulties in determining the apparent K_m for cyanide reduction. K_m values ranging from 0.19 to 4 mM have been reported (Kelly, 1968; Hardy & Knight, 1967; Rivera-Ortiz & Burris, 1975). Although the reported cyanide reduction experiments cover the pH range 7.0–8.4, no single investigation utilized more than one pH value. Not surprisingly then, there is no consensus on the form of the substrate (CN^- or HCN). Both have been suggested [CN^- by Rivera-Ortiz & Burris (1975) and Burns & Hardy (1975), HCN by Hardy & Knight (1967)], although the major species present in that pH range is HCN.

Conflicting reports of the effect of other added substrates on nitrogenase-catalyzed cyanide reduction have appeared. Hardy & Knight (1967) reported that azide partially inhibits cyanide reduction, while N_2O and N_2 inhibit slightly, and H_2 stimulates cyanide reduction. River-Ortiz & Burris (1975) observed that azide competitively inhibits cyanide reduction and N_2 inhibits it slightly but that N_2O, C_2H_2, and H_2 all enhance cyanide reduction. The latter workers also reported that cyanide was a competitive inhibitor of azide reduction, a noncompetitive inhibitor of N_2 and C_2H_2 reduction, and an inhibitor of H_2 evolution. These results have been used to propose various models for multiple substrate binding sites on nitrogenase (Burns & Hardy, 1975; Burris & Orme-Johnson, 1976).

In general, total electron flow through nitrogenase is independent of the substrate being reduced (Watt & Burns, 1977). However, there are several reports that indicate cyanide does affect total electron flow. Hardy & Knight (1967) showed that the total electrons forming H_2 in the absence of cyanide were greater than the total electrons producing $H_2 + CH_4 + NH_3 + CH_3NH_2$ in the presence of cyanide. Because total electron flow (dithionite utilization) was not directly measured, it was not clear that total electron flow was being inhibited, and they suggested a cyanide reduction product was going unobserved. Biggins & Kelly (1970) showed C_2H_2 reduction was inhibited by cyanide and that reduction products of cyanide did not account for all electrons lost from C_2H_2 reduction. Because H_2 evolution was not measured in this study, again it was not clear if total electron flow was actually being inhibited. The several reports of decreased rates of CH_4 formation at high NaCN concentrations (see above) also did not measure H_2 evolution so that total electron flow was unknown. Ljones' (1973) direct measurement of dithionite utilization by nitrogenase in the presence of cyanide showed a 70% decrease by 2 mM KCN. Although the time course of cyanide reduction had previously been shown to be linear (Hwang & Burris, 1972), Ljones (1973) attributed the decrease in total electron flow to irreversible inactivation of the Fe protein. He showed that 10 mM KCN at pH 8.0 rapidly (less than 1 h) bleached the Fe protein. Studies for the effect of cyanide on adenosine 5′-triphosphate (ATP)[1] hydrolysis have also produced conflicting results. Hardy & Knight (1967) reported that NaCN from 1 to 5 mM did not affect ATP hydrolysis while Hwang & Burris (1972) reported a 60% enhancement of ATP hydrolysis by 3 mM KCN with decreased enhancement at higher concentrations.

The binding of cyanide to the component proteins of nitrogenase has also been examined. Bui & Mortenson (1968) showed that cyanide bound to the MoFe protein, but not the Fe protein from *Clostridium pasteurianum*, and that binding was not dependent upon the presence of reductant and Mg-ATP. Biggins & Kelly (1970) showed binding to both component proteins from *Klebsiella pneumoniae* and to other proteins as well (e.g., bovine serum albumin). They suggested that binding was possibly nonspecific as CO did not decrease binding to either the Fe or the MoFe proteins.

Further studies probing cyanide reduction by using purified component proteins of nitrogenase are presented here.

Materials and Methods

Reagents and Chemicals. ATP, creatine phosphokinase, and Tes were obtained from Sigma Chemical Co., NaCN was Baker reagent grade, and NaN_3 was from MCB. K_2CO_3 was an MCB reagent (0.001% N). Gases were from the Matheson Co. Both creatine phosphate and dabsyl chloride were prepared in our laboratory. An improved yield (40%) of the latter over the published procedure of Lin & Lai (1980) resulted from slurrying the reactants in toluene and vigorous stirring overnight.

Nitrogenase Assay. Azotobacter vinelandii MoFe and Fe proteins, designated *Av*1 and *Av*2, respectively, were purified and analyzed as described elsewhere (Burgess et al., 1980). Specific activities of the proteins were ca. 1850 nmol of H_2 min^{-1} (mg of *Av*2)$^{-1}$ and 3000 nmol of H_2 min^{-1} (mg of *Av*1)$^{-1}$. Unless otherwise indicated, all assays were performed at 30 °C in 9.5-mL calibrated vials, fitted with butyl rubber serum caps, containing the appropriate gas mixture. The 1.0-mL reaction mixture contained 50 mM Tes–KOH (at the desired pH), 2.5 mM ATP, 5.0 mM $MgCl_2$, 30 mM creatine phosphate, 20 mM neutralized $Na_2S_2O_4$, and 0.125 mg of creatine phosphokinase. The vessel containing the reaction mixture was degassed and filled with the appropriate gas mixture by using a published procedure (Corbin, 1978), $Na_2S_2O_4$ was added, and the mixture was incubated at 30 °C for 5 min. NaCN was added where appropriate (see below), followed by *Av*2, and the reaction was started by adding *Av*1 to give the appropriate molar ratio of the two components. A total of 1 mg of protein per 1-mL reaction was used to avoid complications introduced by large protein concentrations (Wherland et al., 1981). Unless otherwise indicated, all experiments were performed at the *Av*2/*Av*1 molar ratio of 8. Molar ratios are based on molecular weights of 64000 for *Av*2 and 230000 for *Av*1. Molar ratio titration experiments were performed as described elsewhere (Wherland et al., 1981). For assays involving NaN_3, a 0.1 M stock solution was used and the NaN_3 added immediately following the addition of NaCN. All reactions were run for 5 min. All product formation was linear with time. Reactions were terminated with 0.1 mL of 1 N HCL containing KIO_3 (4.7 mg) unless otherwise indicated. The iodate was incorporated to oxidize the $S_2O_4^{2-}$ remaining which otherwise reduces the reagent used for the NH_3–CH_3NH_2 analysis (see below). Reactions were terminated with 0.10 mL of 37% HCHO when dithionite was to be determined (see below). Formaldehyde was shown to be effective by demonstrating that H_2 evolution ceased as soon as this reagent was introduced.

Preparation of NaCN Stock Solution. A 0.2 M NaCN solution was prepared anaerobically in 0.025 M Tes–KOH (at the desired pH). The pH of this solution was 10.5. After brief degassing, a 3.5-mL aliquot was transferred to a degassed serum vial. Degassed 3 N HCl (ca. 0.22 mL, the actual amount predetermined on a separate 3.5-mL aliquot) was added to attain the desired pH. Futher dilution with degassed

[1] Abbreviations: ATP, adenosine 5′-triphosphate; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; HPLC, high-performance liquid chromatography; dabsyl, 4-(dimethylamino)azobenzene-4′-sulfonyl.

Tes–KOH (at the appropriate pH) gave a final concentration of 0.1 M NaCN.

Product Analysis. With the exception of dithionite utilization studies (see below), *all products were measured on the same reaction vial.* All data points represent means of, typically, triplicate determinations (see paragraph at end of paper regarding supplementary material). Gas samples, 200 μL at bottle pressure, were taken with a pressure-lock syringe (Precision Sampling) and analyzed by using a Hewlett-Packard gas chromatograph with a Porapak N column (He) for CH_4, C_2H_4, and C_2H_6 detection, and a home-built gas chromatograph with a thermal conductivity detector and a molecular sieve 5A column (Ar) was used for H_2 evolution detection. Phosphate was determined by use of a published procedure (Fiske & Subbarow, 1925).

Amine Determinations. Methylamine and ammonia were separated and determined on a 0.5-mL aliquot of the reaction mixture via HPLC of their dabsyl derivatives. The method was essentially that reported by Lin & Lai (1980) with the following modifications: (1) a more concentrated (3 mg/mL) dabsyl chloride reagent solution was employed; (2) aqueous K_2CO_3 (20%, 0.10 mL) was substituted for solid Na_2CO_3; (3) after the reaction period, excess reagent was destroyed by addition of 15% NaCl (1.5 mL) containing 18 μmol of sodium valinate and then incubating 10 min (ca. 40 °C); (4) extraction was done with 2 mL of 1:1 *n*-BuOH–hexane after addition of 15% NaCl (2.5 mL). After the solution was washed with more 15% NaCl (4 mL), 10 μL of the upper (organic) phase was used directly for HPLC. Chromatography was carried out on a Waters Model 244 instrument (436-nm filter) with acetonitrile–95% ethanol–water (1:1:1, 1.4 mL/min) and a μBondapak C_{18} column (3.9 × 30 cm). Flow programming was used to flush the column after the desired peaks had eluted. Approximately 1 μmol of standard methylamine and ammonia solutions was subjected to the derivatization procedure for calibration. Reagent blanks were also run. The peak attributed to the CH_3NH_2 derivative was also shown to be coincident with that of the authentic derivative by using two other solvents (3:2 CH_3CN–H_2O and 4:1 methanol–H_2O) to confirm its identity.

The method was not suitable for really accurate ammonia determination at the levels encountered in our 5-min assay reactions, as the amount present was at best twice that of a control (no cyanide present). Only by using a 10-min reaction time at the optimal pH (7.3) was usable data for ammonia (e.g., that used in Tables II and IV) obtained, even though somewhat beyond the region of linear rate. Methylamine did not suffer from this problem as its background level in control reactions was only ca. $^1/_{20}$th that of the ammonia.

Dithionite Determinations. In these experiments, H_2, CH_4, and $S_2O_4^{2-}$ utilization were measured on the same reaction vial. The most convenient method for determination of $S_2O_4^{2-}$ in our case was as follows: Reactions were terminated by injection of 0.10 mL of 37% aqueous formaldehyde. After H_2 and/or CH_4 was measured, 0.05 mL of pH 4.5 2 M acetate buffer and 0.10 mL of 1% starch solution were injected, and the dithionite was titrated (starch end point) with 0.02 N iodine by using a Gilmont micrometer buret equipped with a hypodermic needle.

The formaldehyde immediately complexes both $S_2O_4^{2-}$ and SO_3^{2-} (the product of dithionite oxidation) as $HOCH_2SO_2^-$ and $HOCH_2SO_3^-$, respectively. The dithionite adduct (formaldehyde sulfoxylate) can be titrated directly with iodine (Wilson & Wilson, 1962) because the sulfite adduct is unreactive (free dithionite and sulfite both react with iodine).

Table I: Effect of pH on CN^-/HCN Distribution[a]

pH	HCN	CN^-
7.1	0.9904	0.0096
7.3	0.9848	0.0152
7.5	0.9760	0.0240
7.7	0.9625	0.0375

[a] For 1 mM NaCN added.

Formaldehyde also complexes any cyanide (which also would react with iodine) as $HOCH_2CN$ (von Richter, 1947). The acetate buffer is added to give a more stable end point for the titration. Less than 5 μL of the iodine would produce a readily visible end point. The accuracy of the titration was enhanced by using a dithionite level (5 mM) such that one-fifth to one-third was consumed in the reaction. Limiting the amount of dithionite in this way did not affect the rate of cyanide reduction or H_2 formation.

Data Treatment. Calculation of the total amounts of H_2 and CH_4 was based on the calibrated vial volume minus 1.10 mL of liquid phase and then expressed as nanomoles per minute per milligram of total protein. No correction for solubility of the gases in the liquid phase was applied. Total electron flow was obtained either by the amount of dithionite consumed or by the amounts of products formed. Dithionite consumption was determined by subtracting the amount remaining in the reaction mixture from that found in a control reaction containing no protein. The relationship $2e/H_2$, $6e/CH_4 + NH_3$, $4e/CH_3NH_2$, and 2e/excess NH_3 is used in calculating electron flow from the products formed. In fact, because the CH_3NH_2/CH_4 ratio was effectively constant (0.35 ± 0.02 mol/mol; see Results) as well as the excess NH_3/CH_4 ratio (0.24 ± 0.07 mol/mol) at the $Av2/Av1$ ratio of 8 used in the variable pH cyanide reduction experiment, total electron flow was more conveniently obtained by $2 \times H_2 + 7.88 \times CH_4$ (and expressed in nanomoles of electrons per minute per milligram of protein). Thus, only H_2 and CH_4 needed to be determined for these.

Because HCN is a weak acid ($pK_a^{30°C} = 3.11$; Izatt et al., 1962), the relative amounts of HCN and CN^- in buffered solutions of NaCN are determined by the pH. The relative amounts of these two species at the pH values of interest are given in Table I. HCN is by far the dominant species. Although CN^- is minor, its concentration is dramatically changed by pH. The concentrations of both CN^- and HCN were calculated from the amount of NaCN used in each reaction by

$$[CN^-] = \frac{[NaCN]}{\text{antilog } (9.11 - pH) + 1}$$

which comes from the relationship of pK_a to pH, $pK_a^{30°C} = 9.11 = pH + \log ([HCN]/[CN^-])$ and $[NaCN] = [HCN] + [CN^-]$. In the text, CN^- and HCN will be used to refer to the actual species ("cyanide ion" and the neutral "hydrogen cyanide" molecule), while the term "cyanide" will be used in a general sense, e.g., cyanide reduction, without regard to the actual species involved.

The rate of nitrogenase turnover (as measured by H_2 evolution in the absence of cyanide) is also dependent on pH with values of 511 ± 22 nmol of H_2 min^{-1} mg^{-1} obtained at pH 7.1, 606 ± 31 nmol of H_2 min^{-1} mg^{-1} at pH 7.3, 567 ± 16 nmol of H_2 min^{-1} mg^{-1} at pH 7.5, and 469 ± 21 nmol of H_2 min^{-1} mg^{-1} at pH 7.7. Thus, the electron flow data in Figure 2 only have been "normalized" to the rate found at pH 7.3. That is, the actual rates observed at pH 7.1, 7.5, and 7.7 have been multiplied by the factors 1.18, 1.07, and 1.29, respectively, in

Table II: Product/Electron Balance

products[a] (nmol)				electron pairs as products (nmol)	nmol of $S_2O_4^{2-}$ consumed[c]
H_2	CH_4	NH_3	CH_3NH_2		
546	212	280	77	1404[b]	1380 ± 50

[a] The following conditions were used: 5 mM NaCN, pH 7.3, Ar atmosphere, 10-min reactions, average of five reactions. [b] Assuming two electrons for excess NH_3. If six electrons are given to excess NH_3, it would be 1540. [c] By titration (see Materials and Methods).

order to correct them to the activity found at pH 7.3.

Phenanthroline Method. To examine the effects of bathophenanthrolinesulfonate on *Av*2, we used the method of Blair & Diehl (1961), as modified for the nitrogenase system by Ljones & Burris (1978a,b).

Attempted Determination of Formaldehyde. The sensitive HPLC–dinitrophenylhydrazine method (Selim, 1977) was tried. It was successful in detecting *added* HCHO in the presence of dithionite, sulfide, and cyanide (at the normal levels of each), if first freed from its adduct state by careful treatment with KIO_3 and then NaS_2O_3. However, when added to an actual enzyme reaction, much HCHO did not survive, and its fate is unknown. The same should be true for any HCHO actually formed during HCN reduction.

Results

Reduction Product of Cyanide: Electron Balance. Using highly purified component proteins, we have confirmed the finding of Hardy & Knight (1967) that most of the cyanide is reduced (by six electrons) to give CH_4 and NH_3 and that CH_3NH_2 is the other base (a four-electron product). The ratio of CH_3NH_2 to CH_4 is constant at 0.35 ± 0.02, regardless of pH or substrate concentration at *Av*2/*Av*1 = 8.

Table II gives a typical balance of products and electrons and shows that the NH_3/CH_4 ratio is greater than the anticipated 1/1. A number of subsequent runs confirmed this excess NH_3 to be constant at 0.24 ± 0.07 per CH_4 over a wide range of added NaCN at *Av*2/*Av*1 = 8. Table II also shows that this excess NH_3 is best represented by a two-electron pathway. It seemed logical that this NH_3 could arise by hydrolysis of a two-electron-reduced intermediate and that HCHO was likely to be the missing carbonaceous product. Unfortunately, HCHO could not be successfully quantitated in our system by using HPLC (see Materials and Methods).

Hydrogen (in variable amounts) is formed concurrently as a two-electron reduction product. The formation of all cyanide reduction products is dependent on the presence of MgATP, dithionite, and both component proteins.

The data in Table II clearly show the equivalence of products formed to electrons consumed, and thus all major products are accounted for. Ethane and ethylene, products of cyanide reduction by nitrogenase (Kelly et al., 1967), were detected in very small amounts (0.036% and 0.02% of the methane, respectively), but only after doubling the amounts of proteins used in our assay. These products are insignificant with respect to electron–product balance and also cannot account for the excess ammonia.

Myth of Substrate Self-Inhibition. Several groups have reported (Hardy & Knight, 1967; Rivera-Ortiz & Burris, 1975) that at NaCN concentrations above 5 mM, the rate of formation of CH_4 decreases. This phenomenon was termed substrate self-inhibition. As shown in Figure 1, if this strongly basic stock solution is not preadjusted to a pH close to that

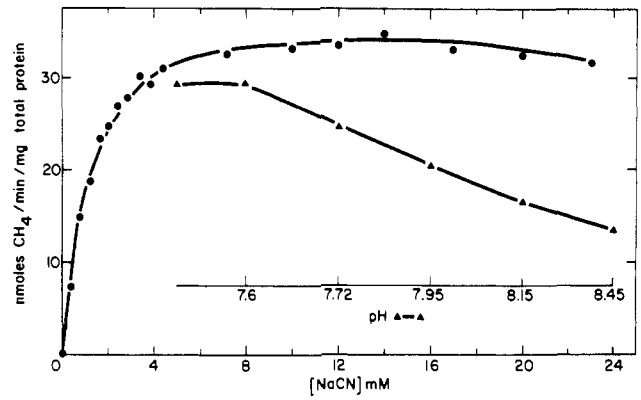


FIGURE 1: Plot of nanomoles of CH_4 formed per minute per milligram of total protein. Assay conditions are the same in both experiments with Tes–KOH, pH 7.3 (see Materials and Methods). (●) The pH of the (0.1 M) NaCN stock solution was adjusted to pH 7.3 (see Materials and Methods); (▲) the pH of the 0.1 M stock solution was not adjusted (pH 10.5). The pH values indicated on the plot are for the assay mixture after the addition of the unadjusted NaCN stock solution.

of the assay mixture, there is insufficient buffer capacity to maintain the desired pH at high NaCN concentrations. Thus, the reaction mixture is more basic at high NaCN concentrations, and not only is the nitrogenase turnover rate slower, resulting in less CH_4, but also the ratio of [HCN] to [CN^-] changes (see Materials and Methods) and has a similar effect on CH_4 formation (see next section). Figure 1 shows that preadjusting the pH of the cyanide stock solution eliminates this problem. Thus, the reported substrate self-inhibition is really an artifact arising from lack of pH control.

CN^- Inhibition of Total Electron Flow. Nitrogenase turnover has been shown to be essentially independent of the substrate being reduced [e.g., see Watt & Burns (1977)]. However, there is always a dramatic (ca. 60%) decrease in total electron flow when 5 mM NaCN is added to a nitrogenase assay under 1 atm Ar. In order to differentiate the possible roles of HCN and CN^- on cyanide reduction and total electron flow, we have performed a series of NaCN concentration dependence experiments at four different pH values: 7.1, 7.3, 7.5, and 7.7.

Figure 2a is a plot of the normalized rate of total electron flow (see data treatment) through nitrogenase as a function of the actual CN^- concentration at four different pH values. A definite correlation is found. At any given CN^- concentration, the same value of total electron flow is obtained, independent of the HCN concentration and the pH. Figure 2b is the same data as in Figure 2a but plotted vs. the calculated HCN concentration. At any given HCN concentration, four different values of total electron flow are obtained, and the higher the pH, the lower the total electron flow. Since [CN^-] increases with pH, there again appears to be a relationship between [CN^-] and total electron flow. Thus CN^- is the inhibitor of *total* electron flow through nitrogenase. A plot of 1/(total nmol of electrons min^{-1} mg^{-1}) vs. [CN^-] is linear and yields an apparent K_i of 27.2 ± 2.8 μM for this inhibition.

That the observed inhibition involved nitrogenase, and not the ATP generating system, was demonstrated by increasing the creatine phosphate, creatine phosphokinase, and MgATP concentrations 3-fold with no resultant increase in electron flow (5 mM NaCN, pH 7.3).

CN^- Uncouples MgATP Hydrolysis and Electron Transfer. As mentioned in the introduction, there are conflicting reports concerning the effect of added NaCN on the ATP/2e ratio

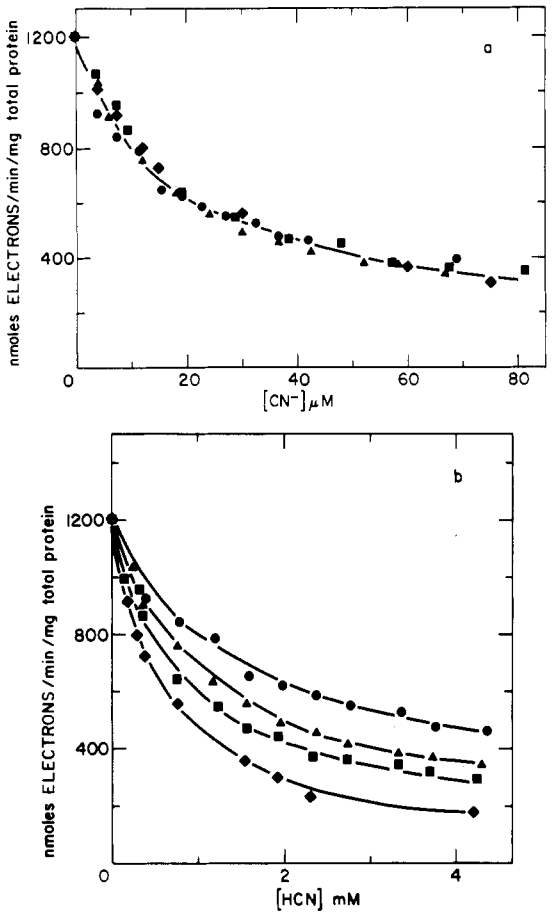


FIGURE 2: (a) Total nanomoles of electrons per minute per milligram of total protein vs. calculated CN^- concentration where electron flow has been normalized to correct for small changes in enzyme activity with pH (see Materials and Methods). (b) Normalized total nanomoles of electrons per minute per milligram of total protein vs. HCN concentration: (●) pH 7.1; (▲) pH 7.3; (■) pH 7.5; (♦) pH 7.7. Assay conditions and calculations are as described under Materials and Methods.

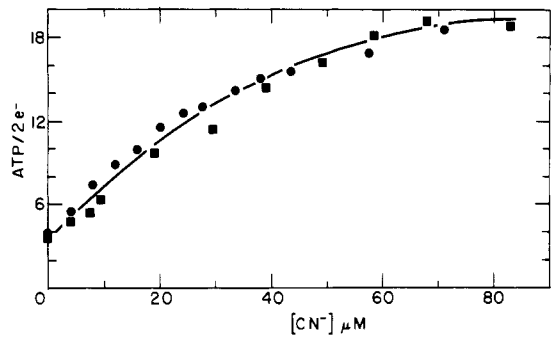


FIGURE 3: Plot of ATP hydrolyzed per 2e transferred (both in nanomoles per minute per milligram of total protein) vs. the calculated CN^- concentration. Assay conditions and calculations are as described under Materials and Methods: (●) pH 7.1; (■) pH 7.5.

(Hardy & Knight, 1967; Hwang & Burris, 1972). As is shown in Figure 3, there is an increase in the ATP/2e ratio with increasing HCN or CN^- concentration at pH 7.1 and 7.5. Figure 3 shows that, at any given CN^- concentration, the same ATP/2e ratio is obtained regardless of pH or HCN concentration. On the other hand, at any given HCN concentration, the higher the pH, the higher the CN^- concentration and the higher the ATP/2e ratio. The trend in Figure 3 is very similar to that shown in Figure 2a which suggests that the CN^- inhibition of total electron flow and the increase in the ATP/2e ratio with CN^- concentration are manifestations of the same

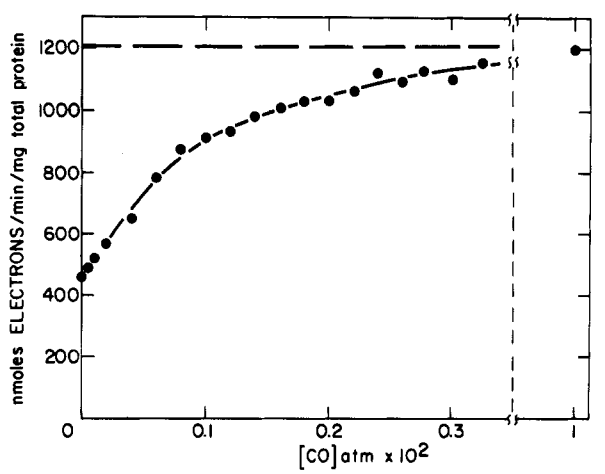


FIGURE 4: Total nanomoles of electrons per minute per milligram of total protein vs. [CO] in atmospheres at 5 mM NaCN, pH 7.3, $[CN^-]$ = 76 μM. Control with no CN^- present under 1 atm Ar or 1 atm CO total electron flow gave 1206 ± 37 nmol of electrons min^{-1} (mg of total protein)$^{-1}$.

Table III: Effect of Various Substrates and Inhibitors on Electron Flow in the Presence of Cyanide

gas phase[a]	(nmol of electrons/2) min^{-1} (mg of total protein)$^{-1}$ [b]
Ar	236 ± 5
C_2H_2	217 ± 21
N_2O	239 ± 4
N_2	226 ± 22
H_2	224 ± 3
Ar + 10 mM NaN_3	327 ± 29
CO	652 ± 29

[a] All at 1 atm with 5 mM NaCN at pH 7.3. [b] $S_2O_4^{2-}$ utilization (see Materials and Methods), sextuplicates.

phenomenon. It should be noted that both the total nanomoles of electrons per minute per milligram of total protein (Figure 2a) and also the total nanomoles of P_i per minute per milligram of total protein change with increasing CN^- concentration (see supplementary material). The P_i increases from about 2000 nmol min^{-1} mg^{-1} at zero CN^- to a maximum of 3000 nmol $min^{-1}mg^{-1}$ at all CN^- concentrations above about 25 μM.

Reversibility of CN^- Inhibition. Experiments were performed where (a) *Av*2 alone, (b) *Av*2 and the ATP generating system, (c) the ATP generating system alone, and (d) *Av*1 alone were preincubated under Ar with 5 mM NaCN at pH 7.5 for various time periods up to 15 min. Then either (1) the reaction was started by adding the appropriate component or (2) the atmosphere was switched to CO and then the reaction was started. In all cases 1, total electron flow was linear for the 5-min period studied at 330 nmol of total electrons min^{-1} (mg of total protein)$^{-1}$. In all cases 2, total electron flow was also linear at 1200 nmol of total electrons min^{-1} (mg of total protein)$^{-1}$, the same rate seen under Ar or CO without NaCN present. Even if nitrogenase is allowed to turn over under NaCN–Ar and then the atmosphere is switched to CO, total electron flow recovers. Therefore, CN^- inhibition does not represent irreversible damage to nitrogenase because it can be reversed by CO. CO relieves not only inhibition of total electron flow but also the effect on the ATP/2e ratio. An ATP/2e ratio of 3.87 is obtained at 5 mM NaCN, pH 7.3, with 1 atm of CO present, in good agreement with the zero CN^- value shown in Figure 3. CN^- inhibition of total electron flow can be even relieved by very low levels of CO as shown in Figure 4. Here, in the presence of 5 mM NaCN (pH 7.3), inhibition is almost completely counteracted by 0.002–0.003

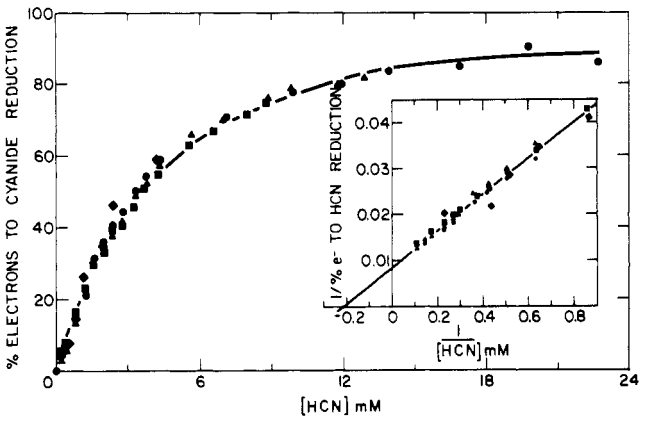


FIGURE 5: Plot of percentage of total electrons being used to produce CH_4, NH_3, and CH_3NH_2 vs. the calculated HCN concentration. The line is the computer fit to the Michaelis–Menten equation. Inset: Double-reciprocal plot using HCN concentrations in the range $(0.2–2)K_m$ (Segel, 1975). The line is the computer fit to the Michaelis–Menten equation. K_m = 4.5 ± 0.3 mM HCN; V_{max} = 113 ± 6%. Assay conditions and calculations are as described under Materials and Methods: (●) pH 7.1; (▲) pH 7.3; (■) pH 7.5; (♦) pH 7.7.

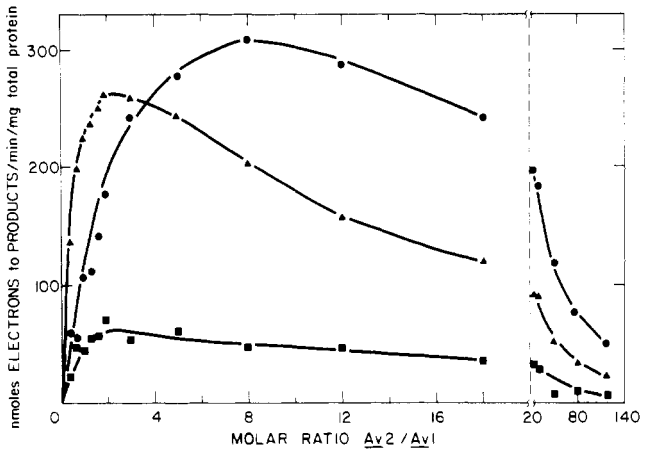


FIGURE 6: Plot of nanomoles of electrons to product per minute per milligram of total protein vs. the molar component ratio *Av*2/*Av*1: (●) 2 × H_2; (▲) 6 × CH_4; (■) 4 × CH_3NH_2. All assays and calculations are as described under Materials and Methods. Assay contained 5 mM NaCN at pH 7.3.

atm of CO to give an electron flow nearly that expected in the *absence* of CN^-. As shown in Table III, most other substrates and inhibitors of nitrogenase (C_2H_2, N_2, N_2O, and H_2) *do not* relieve CN^- inhibition of total electron flow. Interestingly, azide (10 mM) does partially relieve CN^- inhibition of total electron flow.

As mentioned in the introduction, a report has appeared (Ljones, 1973) suggesting that cyanide caused irreversible damage to *Av*2. We have monitored changes in the reactivity of the Fe_4S_4 center in *Av*2 toward bathophenanthroline sulfonate before and after addition of MgATP by using the method of Ljones & Burris (1978b). There was *no difference* in this reaction when *Av*2 was preincubated with 5 mM NaCN, pH 7.3, for varying time periods of up to 30 min when compared to *Av*2 in the absence of NaCN.

HCN as the Substrate. Since we have shown the CN^- species to be the inhibitor of total electron flow, is the neutral HCN species the substrate for cyanide reduction? Total electron flow should be *independent* of the substrate being reduced (Watt & Burns, 1977), and Figure 2 shows electron flow to be *independent* of [HCN], not [CN^-]. More compelling evidence that HCN is the substrate is found in Figure 5, where the data are plotted as the percentage of electrons to cyanide reduction vs. [HCN]. As the substrate (HCN) concentration is increased, the percentage of total electron flow going to cyanide reduction (methane, ammonia, and methylamine) should also increase, even though the overall total electron flow is not constant due to varying levels of inhibitor (CN^-). In other words, Figure 5 shows the distribution of electrons between cyanide reduction and hydrogen formation is dependent on [HCN] and is independent of pH and [CN^-] (each HCN value corresponds to four different [CN^-] levels).

If CN^- were the substrate, a plot of the same data, but using [CN^-] instead of [HCN], should have resulted in a single curve for the same reasons given above. In fact, one gets four curves, one for each pH. This result is again consistent with HCN being the substrate, because, at any given value for [CN^-], the amount of HCN present would depend on the pH, and this should be reflected in the percentage of electrons going to cyanide reduction. For example, at [CN^-] = 30 μM, [HCN] would be 3.12 mM at pH 7.1 but only 0.8 mM at pH 7.7, and the percentage of electrons going to cyanide reduction should be lower at the higher pH, as it is. CO inhibits HCN reduction with an apparent K_i of 0.00029 atm CO.

The data in Figure 5 represent a smooth saturation curve which fits Michaelis–Menten kinetics. A double-reciprocal plot (inset) is linear, with an apparent K_m of 4.5 ± 0.3 mM HCN. Previous attempts to obtain K_m data for cyanide reduction did not consider the aspect of CN^- inhibition of total electron flow. Thus, a simple plot of methane formation vs. NaCN concentration (e.g., Figure 1, pH adjusted) does not appear as a normal saturation curve. The V_{max} of 113 ± 6% demonstrates that H_2 evolution is eliminated at infinite HCN concentration.

Methylamine as a Substrate. The finding of CH_3NH_2 as a partially reduced product of cyanide reduction necessitated testing it as a substrate. When 5–60 mM CH_3NH_2 (added as the HCl salt) at pH 7.3 was used, *no* CH_4 was detected, and total electron flow was unaffected with all of the electrons appearing as H_2. Thus, CH_3NH_2 could not be demonstrated as a substrate of nitrogenase. Of course, it would exist in solution as the methylammonium cation under these conditions because of its high pK_a (10.6).

Component Ratio Titration. Figure 6 is a plot of the rate of product formation vs. the molar component ratio of *Av*2/*Av*1. In this experiment, cyanide reduction to either CH_4 (+NH_3) or CH_3NH_2 peaks at a ratio of about 2 while H_2 evolution peaks at a ratio of about 8. This trend is different from that seen in previous experiments for H_2 evolution, N_2 fixation, and HD formation where all products peak at the same ratio (Wherland et al., 1981).

Previous studies (Silverstein & Bulen, 1970; Davis et al., 1975) with nitrogenase have demonstrated that, in general, H_2 evolution is favored over other substrate reduction (especially N_2) at low Fe/MoFe protein molar ratios where the system is starved for electrons. For N_2 reduction (Wherland et al., 1981), the higher the *Av*2/*Av*1 ratio the greater the percentage of total electrons going to N_2 reduction. A different trend is seen for cyanide reduction where high ratios favor H_2 evolution over cyanide reduction (Figure 3 of supplementary material). At ratios below 0.7, the trend appears to be toward a smaller percentage to cyanide reduction, but there is not enough product formed at these ratios to be certain. The trend of high ratios favoring H_2 evolution over substrate reduction has previously been reported for hydrazine reduction (Burgess et al., 1981) and has also been seen for C_2H_2 reduction which receives its maximum percentage of electrons at *Av*2/*Av*1 ratios of 5 (J. Li, B. K. Burgess, and J. L. Corbin, unpublished

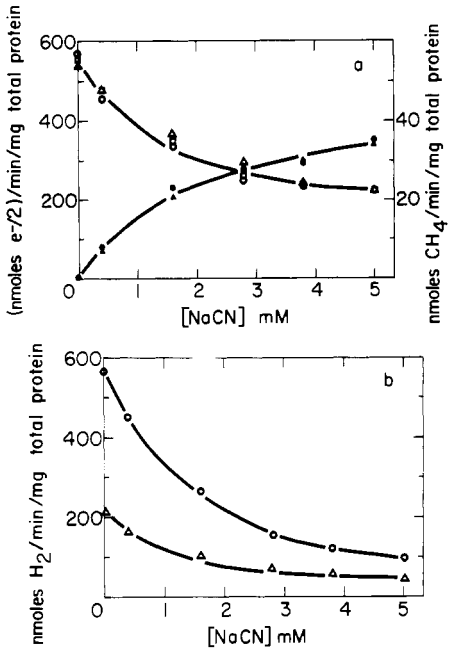


FIGURE 7: Effect of N_2 on HCN reduction. (a) (○) Nanomoles of $S_2O_4^{2-}$ used per minute per milligram of total protein without N_2; (△) nanomoles of $S_2O_4^{2-}$ used per minute per milligram of total protein with 1 atm N_2 present; (□) nanomoles of total electron pairs to all products per minute per milligram of total protein with no N_2 present; (●) nanomoles of CH_4 formed per minute per milligram of total protein without N_2; (▲) nanomoles of CH_4 formed per minute per milligram of total protein with 1 atm N_2 present; all vs. [NaCN], pH 7.3. (b) (○) Nanomoles of H_2 per minute per milligram of total protein without N_2 present; (△) nanomoles of H_2 per minute per milligram of total protein with 1 atm N_2 present vs. [NaCN], pH 7.3.

results). Interestingly the CH_3NH_2/CH_4 ratio also changes with the *Av*2/*Av*1 ratio. In the *Av*2/*Av*1 ratio range 1–50, the CH_3NH_3/CH_4 ratio increases from ≃0.3 to 0.45. At ratios above 50, the relative amount of CH_3NH_2 decreases again to about 0.4 at *Av*2/*Av*1 = 70–130. Low ratios also give a larger excess of NH_3 with NH_3/CH_4 = 1.40 at *Av*2/*Av*1 in 1 vs. NH_3/CH_4 = 1.24 at *Av*2/*Av*1 = 8.

Total electron flow shows the same smooth variation with component ratio, peaking at a ratio of about 5 (Figure 3 of supplementary material), as has been seen in all previous experiments (Wherland et al., 1981). If the same data are normalized to *Av*2 and *Av*1 protein (Figure 3 of supplementary material), the general shape of these curves is very similar to that from previously reported titrations (Wherland et al., 1981). With CN^- (76 μM) present, total electron flow is decreased, and the specific activities of both component proteins are concomitantly decreased. *Av*2 specific activity is only 1000 nmol min^{-1} (mg of *Av*2)$^{-1}$ [vs. 1850 nmol min^{-1} (mg of *Av*2)$^{-1}$ with no CN^- at *Av*2/*Av*1 = 0.7], and *Av*1 is only 1300 nmol min^{-1} (mg of *Av*1)$^{-1}$ [vs. 3000 nmol min^{-1} (mg of *Av*1)$^{-1}$ with no CN^- at *Av*2/*Av*1 = 30].

Effect of Other Substrates and Inhibitors on HCN Reduction. Figure 7a shows that the addition of 1 atm N_2 to a cyanide reduction system has no effect on either total electron flow or HCN reduction to CH_4 (+NH_3). Figure 7b shows that the addition of N_2 to a cyanide reduction system does dramatically decrease the amount of H_2 being evolved. Electron balance requires the electrons lost from H_2 evolution appear as NH_3 from N_2 reduction, and we have demonstrated this at low HCN concentration (ca. 0.2 mM).

H_2 is known to be an inhibitor of N_2 fixation. The addition of H_2 to a cyanide reduction system has no effect on total electron flow (Table III) or on the amounts of CH_4, NH_3, or CH_3NH_2 formed.

Table IV: Effect of C_2H_2 and N_2O on the Distribution of HCN Reduction Products

no. of replicates[a]	gas phase	CH_4 (nmol)	CH_3NH_2/CH_4	NH_3/CH_4
8	Ar	210	0.35	1.25
5	C_2H_2	278	0.35	1.17
10	N_2O	335	0.29	1.00

[a] All 10-min reactions, 5 mM NaCN, pH 7.3.

C_2H_2 has previously been reported to stimulate cyanide reduction (Rivera-Ortiz & Burris, 1975). The addition of C_2H_2 to a HCN reduction system does not affect total electron flow (Table III) but does seem to increase the number of electrons going to HCN reduction and affects the distribution of those electrons. As shown in Table IV, when C_2H_2 is added to an HCN reduction system, the six-electron reduction to CH_4 + NH_3 is slightly favored over the four-electron reduction to CH_3NH_2 or the formation of the excess NH_3 (presumably via two-electron reduction) when compared to cyanide reduction under argon. C_2H_2 is reduced to C_2H_4 under these conditions at the expense of H_2 evolution.

N_2O has also been reported to stimulate cyanide reduction (Rivera-Ortiz & Burris, 1975). The addition of N_2O to a cyanide reduction system does not affect total electron flow (Table III) but does dramatically affect both the total number of electrons that are being used to reduce HCN and the distribution of these electrons (Table IV). In the presence of N_2O, the six-electron reduction to CH_4 + NH_3 is highly favored at the expense of the four-electron reduction to CH_3NH_2, and *no* excess NH_3 is found. N_2O reduction to N_2 was not directly measured, but electron balance strongly suggests that N_2O is reduced under these conditions.

Azide is the only substrate tested which inhibits HCN reduction to CH_4 (+NH_3). When 10 mM azide is added to an HCN reduction system (5 mM NaCN, pH 7.3), there is a 50% decrease in the amount of CH_4 formed. This effect is even more dramatic because azide simultaneously increases total electron flow (Table III). Unfortunately, azide reacts with the dabsyl reagent (to form dabsyl azide) and interferred with our HPLC detection of NH_3 and CH_3NH_2. Thus, we were unable to determine its effect on the distribution of the products of HCN reduction. However, the effect of azide on CH_4 formation is so large that it seems probable that azide is directly inhibiting HCN reduction. It is necessary to invoke azide reduction under these conditions to account for missing electrons.

Discussion

The importance of pH in studies of cyanide reduction by nitrogenase must be emphasized. Earlier results of "substrate self-inhibition" are probably attributable to lack of pH control. Equally as important in kinetic studies is to measure *all* products formed including H_2 and to be certain that all electrons have been accounted for by measuring dithionite utilization. The dithionite utilization assay reported here should greatly simplify future studies of this type. As indicated in the introduction, several investigators had some evidence that electron balance was not achieved in their cyanide reduction experiments (Hardy & Knight, 1967; Biggins & Kelly, 1970). The detailed electron balance studies reported here have demonstrated that CN^- is an inhibitor of total electron flow (see below) and, of course, the concentration of the species CN^- is highly pH dependent. Thus, the conclusions drawn from previous kinetic studies of cyanide reduction or its in-

teraction with other substrates cannot necessarily be assumed valid, because there is no indication that pH was controlled, and the inhibition of total electron flow by CN^- was not recognized.

The data presented here demonstrate that CN^- is a potent inhibitor (K_i of 27 μM) of total electron flow through nitrogenase, and at infinite [CN^-], all electron flow through the enzyme should cease. The presence of CN^- at less than infinite concentration (1–180 μM) in a nitrogenase assay system has no effect on (i) the distribution of electrons between HCN reduction and H_2 evolution and (ii) the apparent K_m for HCN reduction and (iii) does not prevent the reduction of HCN, N_2, C_2H_2, N_2O, azide, or H^+. These observations indicate that when CN^- is bound electron flow stops and that when it is not bound electrons flow and are distributed normally to whatever substrate(s) is (are) available. In the time period studied, total electron flow in the presence of CN^- is linear, and so the interaction of CN^- with nitrogenase appears rapid and nondestructive. CN^- thus seems to be reversibly bound to nitrogenase and in rapid equilibrium between free and bound forms.

Where does CN^- bind? The finding that N_2, C_2H_2, N_2O, HCN, and H_2 do not relieve CN^- inhibition of total electron flow, coupled with the failure of CN^- to inhibit specifically the reduction of H^+, HCN, N_2, C_2H_2, N_2O, or azide (see above), makes it unlikely that CN^- is binding to the site(s) where these substrates are being reduced. The discovery that CO can relieve CN^- inhibition of total electron flow suggests that the two are interacting at the same site. CO is known to be a noncompetitive inhibitor of the reduction of N_2 (Hwang et al., 1973), C_2H_2 (Davis et al., 1979), and azide (Dilworth & Thorneley, 1981) and an inhibitor of all other nitrogenase substrates [e.g., see Mortenson & Thorneley (1979)] except H^+. Both CO and CN^- are known to bind to metal atoms end on through the carbon, and some physical evidence for CO binding to the MoFe protein has been reported (Burris & Orme-Johnson, 1976; Davis et al., 1979). If CN^- and CO are binding to the same site, their effects are very different. When CN^- is bound, it stops electron flow through the enzyme. When CO is bound, electron flow continues, but all electrons appear as H_2 evolution. It is interesting that azide, which like cyanide is present as two species in solution, is the only substrate which partially relieves CN^- inhibition of total electron flow. Whether HN_3 or N_3^- is responsible for the effect is a question toward which future studies will be directed. The component ratio titration data show that CN^- is not simply removing one or the other of the two component proteins from participation in the reaction. CN^- does not change either the shape of the total electron flow vs. $Av2/Av1$ protein ratio plot or the $Av2$ or $Av1$ protein specific activity titration curves. It simply lowers the activity of both component proteins. Thus, we were unable to determine by this method which of the two components was binding CN^-. Attempts to demonstrate direct interaction with $Av2$ by using the bathophenanthroline sulfonate method (Ljones & Burris, 1978a,b) were also unenlightening.

CO relieves both the CN^- inhibition of electron flow and the uncoupling of MgATP hydrolysis from electron transfer. It is probable that the two effects of CN^- are manifestations of the same phenomenon. One interesting proposal for the uncoupling of MgATP hydrolysis and electron transfer to substrate is the "futile cycle" (Orme-Johnson et al., 1977). In such a scheme, CN^- binding to the MoFe protein would prevent the electrons transferred from the Fe protein (concomitant with MgATP hydrolysis) from reaching the point of substrate reduction. Thus, with nowhere to go, the electron falls back to the Fe protein. This scheme would explain why CN^- both eliminates total electron flow and uncouples MgATP hydrolysis and has no apparent differential effect on the specific activity of either of the two component proteins.

Mechanism of HCN Reduction. Unlike CN^-, HCN should bind to metal atoms through its nitrogen. It is not necessary to invoke CN^- reduction to explain any of the results reported here. If CN^- is reduced by this enzyme, it is not making a measurable contribution to the reduction products and thus must have a very large K_m.

We demonstrate that nitrogenase catalyzes the following reactions:

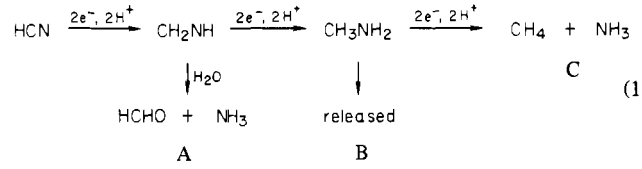
$$HCN + 4e^- + 4H^+ \rightarrow CH_3NH_2$$

$$HCN + 6e^- + 6H^+ \rightarrow CH_4 + NH_3$$

and suggest

$$HCN + 2e^- + 2H^+ \rightarrow CH_2NH \xrightarrow{H_2O} HCHO + NH_3$$

Our preferred scheme for HCN reduction is



(1)

At an $Av2/Av1$ ratio of 8, the stoichiometry of A:B:C is 0.24:0.35:1. The fact that this stoichiometry is independent of HCN concentration or pH suggests that A, B, and C occur in a common pathway and at the same site as indicated in eq 1. Changing the $Av2/Av1$ ratio should influence the rate at which the next two electrons become available, and this might be expected to change the distribution of A:B:C. The distribution of A:B:C does change with the $Av2/Av1$ ratio. Pathway A receives its maximum distribution at the lowest ratios (<8), B at medium ratios (12–50), and C at the highest ratios (70–130). This is consistent with the number of electrons needed for C > B > A.

Surprisingly, some substrates of nitrogenase also influence the distribution of A:B:C. Our data show that, like N_2 and H_2, N_2O and C_2H_2 do not change total electron flow in the presence of cyanide. N_2O and C_2H_2 do change the total number of electrons that are being used to reduce HCN and the distribution of these electrons to the various products of HCN reduction. N_2O eliminates pathway A (i.e., CH_4/NH_3 = 1), decreases B, and increases C. C_2H_2 has a similar but less dramatic effect where pathway A is decreased slightly, B stays the same, and C is increased slightly. The mechanism by which N_2O and C_2H_2 influence this redistribution is a question toward which future studies will be directed. Two general types of explanation are possible. First, N_2O and C_2H_2 influence electron flow in such a way that the six-electron reduction (pathway C) is preferred, or second, N_2O and C_2H_2 physically interfere with the chemical mechanism of HCN reduction. In either case, it is difficult to envision how N_2O or C_2H_2 could influence the product balance without being bound to the enzyme simultaneously with HCN. Further experimentation is needed to determine if this second site is the same as that discussed above for interaction with CN^- and CO.

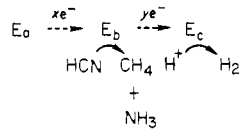
Is HCN reduction a good model for N_2 reduction by nitrogenase? Like HCN, N_2 is reduced by six electrons, and Burgess et al. (1981), Jackson et al. (1968), and Hoch et al. (1960) have suggested analogous intermediates of N_2H_2 and N_2H_4. N_2 and HCN reduction, like all other substrate re-

ductions except H^+, are inhibited by CO. This, however, is where the similarity between N_2 reduction and HCN reduction appears to end.

The apparent K_m for HCN reduction measured at an $Av2/Av1$ ratio of 8 is much greater than the apparent K_m for N_2 reduction at similar ratios, 4.5 mM vs. 0.08 mM (Rivera-Ortiz & Burris, 1975). Both the apparent $K_m(N_2)$ and the apparent K_m(HCN) are dependent upon the component ratio, but in opposite directions. For N_2, the apparent K_m decreases with increased $Av2/Av1$ ratios (Wherland et al., 1981). For HCN, the K_m increases with increased ratios. Thus, N_2 fixation is maximally favored over H_2 evolution at high ratios (greater than 5), while HCN is maximally favored over H_2 evolution at low ratios ($\simeq 1$). These results are also consistent with recent studies of a mutant of *Klebsiella pneumoniae* (*Kp* nif V^-) (McLean & Dixon, 1981). The isolated *Kp* 1 from this mutant can reduce HCN under usual assay conditions but can only reduce N_2 when the *Kp* 2 levels are greatly increased.

We show that H_2 evolution should be eliminated at high [HCN] and that, even at fairly low HCN concentrations (8 mM), less than one H_2 is evoluted for every HCN reduced. This result is not observed for N_2 reduction, where no one has yet demonstrated less than one H_2 evolved per N_2 fixed. The latter stoichiometry has been interpreted in terms of H_2 evolution being an intimate part of the mechanism of N_2 reduction. This involvement is clearly not the case for HCN reduction.

Our study also demonstrates that N_2 cannot compete with HCN reduction, even in situations where the HCN concentration is $\simeq$5-fold lower than the K_m(HCN) and the N_2 concentration is $\simeq$10-fold higher than the $K_m(N_2)$ and at an $Av2/Av1$ ratio of 8 that should favor N_2 reduction over HCN reduction. This result strongly suggests either that the two substrates are reduced at different sites or that they bind and are reduced at different redox states of the same site. The latter proposal is preferred on the basis of our component ratio results and experiments in the presence of both HCN and N_2. Based on these data, we suggest that HCN binds to and is reduced at an enzyme state more oxidized than the one that evolves H_2 (eq 2) (where E_a is the resting state of $Av1$ and



(2)

where x and y are unknown but ≥ 1). In this view, HCN could effectively eliminate H_2 evolution by scavenging the intermediate E_b. At high $Av2/Av1$ ratios, the formation of E_c would be favored so more HCN is needed to prevent electrons from getting to E_c. This also explains why H_2 evolution during HCN reduction peaks at a higher ratio than HCN reduction (figure 6). When N_2 is added to the HCN reduction system, it has no effect on HCN reduction but is reduced to $2NH_3$ at the expense of H_2 evolution. This would suggest that N_2 is bound and reduced at the state E_c. N_2 cannot influence how much of E_c is formed and thus cannot influence HCN reduction, but it can compete with H_2 evolution for electrons at this state. It should be noted that, under conditions of simultaneous HCN and N_2 reduction, we see greater than one H_2 evolved per N_2 fixed. Whether N_2 fixation and H_2 evolution at E_c are concomitant as has been suggested [e.g., see Rivera-Ortiz & Burris (1975) and Hadfield & Bulen (1969)] or nonconcomitant cannot be treated on the basis of the data presented here.

A recent study of azide reduction by *Klebsiella pneumoniae* nitrogenase (Dilworth & Thorneley, 1981) showed that azide is a competitive inhibitor of N_2 reduction. Based on their component ratio titration, it was suggested that azide is reduced at the same site, but at a more oxidized redox state than N_2. The finding that azide inhibits HCN reduction suggests that HCN and azide are probably reduced at the same site. Azide, like HCN, has also been reported (Dilworth & Thorneley, 1981) to eliminate H_2 evolution. The same report suggested that N_3^- was the substrate for azide reduction, although the change in K_m with pH was in the direction expected if HN_3 was the substrate or a cosubstrate. The finding that HCN is the substrate for cyanide reduction leaves N_3^- as the only potential anionic substrate. Further studies are needed to be certain which of the species, HN_3 or N_3^-, is the substrate for azide reduction.

Our preferred mechanism for N_2 reduction is presented elsewhere (Burgess et al., 1981). An analogous scheme for HCN reduction is discussed above. Some thermodynamic considerations for the two schemes, based on free and not bound substrates and intermediates (Moore, 1955; Saudorfy, 1970), suggest some interesting possibilities. The overall reduction of HCN and especially its reduction to CH_2NH are more easily achieved thermodynamically (ΔH is −49 kcal/mol) than the overall reduction of N_2 (ΔH is −17 kcal/mol.). This might explain why higher $Av2/Av1$ ratios are needed for N_2 reduction than HCN reduction. The proposed reaction of N_2H_2 with D_2 to form N_2 and 2HD would be expected to be highly favored thermodynamically with a ΔH of −44 kcal/mol. An analogous reaction of CH_2NH with D_2 would, however, not be favored with a ΔH of +22 kcal/mol. This may explain why no stimulation of HD formation is seen with HCN as a substrate (Newton et al., 1977) and why H_2 does not inhibit HCN reduction. The reduction of N_2H_4 to $2NH_3$ should be more favorable (ΔH is −42 kcal/mol) than the analogous reduction of CH_3NH_2 to $CH_4 + NH_3$ (ΔH is −14 kcal/mol), which might explain why CH_3NH_2 is a major product of HCN reduction but not a substrate while N_2H_4 is not a measurable product of N_2 reduction but is a substrate.

Similar careful examination of other substrates may well yield results that will further clarify the complexities of cyanide reduction. It would be of value to know the fate of the carbonaceous portion of HCN that gives the excess NH_3.

Summary. In summary, our studies have shown the following: (1) the previously reported self-inhibition by cyanide is artifactual; (2) the CN^- species is a potent, reversible inhibitor (K_i = 27 μM) of *total* electron flow and appears to uncouple ATP hydrolysis and electron transfer to substrate; (3) the inhibition is completely reversed by CO (partially by azide), which implies a common binding site (other substrates tested have no effect); (4) cyanide has no differential effect on the specific activities of $Av1$ and $Av2$ and no apparent physical effect on $Av2$; (5) the HCN species is the substrate in cyanide reduction (K_m = 4.5 mM) and at infinite concentration can apparently eliminate H_2 evolution; (6) it is reduced (six electrons) to $CH_4 + NH_3$, (four electrons) to CH_3NH_2, and apparently to more NH_3 via hydrolysis of a two-electron intermediate; (7) both N_2O and C_2H_2 (but not N_2) influence the distribution of the cyanide reduction products, implying simultaneous binding; (8) HCN appears to bind to and be reduced at a state of nitrogenase which is more oxidized than the state responsible for H_2 evolution or N_2 reduction.

Acknowledgments

We are pleased to acknowledge the talents of Deloria Jacobs, Dorothy Lyons, and Samuel Lough in cell growth and protein

purification. We also thank Drs. John McDonald, Gary Watt, and William E. Newton for helpful discussions.

Supplementary Material Available

Supplementary Tables I–VIII showing all results in terms of number of replicates, means, and standard deviations and three figures (11 pages). Ordering information is given on any current masthead page.

References

Biggins, D. R., & Kelly, M. (1970) *Biochim. Biophys. Acta 205*, 288–299.

Blair, D., & Diehl, H. (1961) *Talanta 7*, 163–174.

Bui, P. T., & Mortenson, L. E. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 1021–1027.

Bulen, W. A., & LeComte, J. R. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 979–986.

Burgess, B. K., Jacobs, D. B., & Stiefel, E. I. (1980) *Biochim. Biophys. Acta 614*, 196–209.

Burgess, B. K., Wherland, S., Stiefel, E. I., & Newton, W. E. (1981) *Biochemistry 20*, 5141–5146.

Burns, R. C., & Hardy, R. W. F. (1975) in *Nitrogen Fixation in Bacteria and Higher Plants*, pp 72–132, Springer-Verlag, New York.

Burris, R. H., & Orme-Johnson, W. H. (1976) *Proc. Int. Symp. Nitrogen Fixation, 1st*, 208–233.

Corbin, J. L. (1978) *Anal. Biochem. 84*, 340–342.

Davis, L. C., Shah, V. K., & Brill, W. J. (1975) *Biochim. Biophys. Acta 403*, 67–78.

Davis, L. C., Henzl, M. T., Burris, R. H., & Orme-Johnson, W. H. (1979) *Biochemistry 18*, 4860–4869.

Dilworth, M. J., & Thorneley, R. N. F. (1981) *Biochem. J. 193*, 971–983.

Emerich, D. W., & Burris, R. H. (1976a) *J. Bacteriol. 134*, 936–943.

Emerich, D. W., & Burris, R. H. (1976b) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4369–4373.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 325–400.

Hadfield, K. L., & Bulen, W. A. (1969) *Biochemistry 8*, 5103–5108.

Hageman, R. V., & Burris, R. H. (1978a) *Biochemistry 17*, 4117–4124.

Hageman, R. V., & Burris, R. H. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2699–2702.

Hageman, R. V., & Burris, R. H. (1979) *J. Biol. Chem. 254*, 11189–11192.

Hardy, R. W. F., & Knight, E., Jr. (1967) *Biochim. Biophys. Acta 139*, 69–90.

Hoch, G. E., Schneider, K. C., & Burris, R. H. (1960) *Biochim. Biophys. Acta 37*, 273–279.

Hwang, J. C., & Burris, R. H. (1972) *Biochim. Biophys. Acta 283*, 339–350.

Hwang, J. C., Chen, C. H., & Burris, R. H. (1973) *Biochim. Biophys. Acta 292*, 256–270.

Izatt, R. M., Christensen, J. J., Pack, R. T., & Bench, R. (1962) *Inorg. Chem. 1*, 828–831.

Jackson, E. K., Parshall, G. W., & Hardy, R. N. F. (1968) *J. Biol. Chem. 243*, 4952–4958.

Kelly, M. (1968) *Biochem. J. 107*, 1–6.

Kelly, M., Postgate, J. R., & Richards, R. L. (1967) *Biochem. J. 102*, 1c–3c.

Lin, J.-K., & Lai, C.-C. (1980) *Anal. Chem. 52*, 630–635.

Ljones, T. (1973) *Biochim. Biophys. Acta 321*, 103–113.

Ljones, T., & Burris, R. H. (1978a) *Biochem. Biophys. Res. Commun. 80*, 22–25.

Ljones, T., & Burris, R. H. (1978b) *Biochemistry 17*, 1866–1872.

McLean, P. A., & Dixon, R. A. (1981) *Nature (London) 292*, 5824–5825.

Moore, W. J. (1955) *Physical Chemistry*, 2nd ed., p 342, Prentice-Hall, Englewood Cliffs, NJ.

Mortenson, L. E., & Thorneley, R. N. F. (1979) *Annu. Rev. Biochem. 48*, 387–418.

Newton, W. E., Bulen, W. A., Hadfield, K. L., Stiefel, E. I., & Watt, G. D. (1977) *Proc. Int. Symp. Recent Dev. Nitrogen Fixation, 2nd*, 119–130.

Orme-Johnson, W. H., Davis, L. C., Henzl, M. T., Averill, B. A., Orme-Johnson, N. R., Munck, E., & Zimmerman, R. (1977) *Proc. Int. Symp. Recent Dev. Nitrogen Fixation, 2nd*, 131–178.

Rivera-Ortiz, J. M., & Burris, R. H. (1975) *J. Bacteriol. 123*, 537–545.

Saudorfy, C. (1970) in *The Chemistry of the Carbon-Nitrogen Double Bond* (Patai, S., Ed.) p 6, Wiley-Interscience, New York.

Segel, I. H. (1975) *Enzyme Kinetics*, p 46, Wiley-Interscience, New York.

Selim, S. (1977) *J. Chromatogr. 136*, 271–277.

Shah, V. K., Davis, L. C., Gordon, J. K., Orme-Johnson, W. H., & Brill, W. J. (1973) *Biochim. Biophys. Acta 292*, 246–270.

Silverstein, R., & Bulen, W. A. (1970) *Biochemistry 9*, 3809–3815.

von Richter, V. (1947) *Richter's Organic Chemistry* (Anschütz, R., & Reindel, F., Eds.) 3rd Engl. ed., Vol. 1, p 245, Elsevier, London.

Watt, G. D., & Burns, A. (1977) *Biochemistry 16*, 265–270.

Wherland, S., Burgess, B. K., Stiefel, E. I., & Newton, W. E. (1981) *Biochemistry 20*, 5132–5140.

Wilson, C. L., & Wilson, D. W., Eds. (1962) *Comprehensive Analytical Chemistry*, p 289, Elsevier, London.