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# Nitrogenase Reactivity: Azide Reduction†

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ABSTRACT: We have examined the reduction of azide by the purified component proteins of nitrogenase (*Av*1 and *Av*2). One of the two species present in azide solutions, $HN_3$, was shown to be a potent substrate ($K_m$ = 12 $\mu$M) which is reduced by six electrons to $N_2H_4 + NH_3$. $HN_3$ reduction does not yield any less highly reduced products, which implies the presence of tightly bound intermediates. $HN_3$ appears to be an effective inhibitor of $H_2$ evolution and to bind to a redox state of the enzyme more oxidized than that responsible for $N_2$ fixation or $H_2$ evolution. The other species present in solution, $N_3^-$, was shown to be the substrate reduced by two electrons to yield $N_2 + NH_3$. $N_3^-$ is the only known anionic nitrogenase substrate, and its reduction is the only example of a nitrogenase reaction requiring inequivalent numbers of protons and electrons. Infinite [$N_3^-$] cannot eliminate $H_2$ evolution, and $N_3^-$ may bind to and be reduced by both high and low redox states of the enzyme. Some of the $N_2$ formed appears to be further reduced by six electrons to two $NH_3$ in a reaction that is inhibited by $D_2$. The $N_2$ formed from $N_3^-$ reduction, which is subsequently reduced to $2NH_3$, is not in equilibrium with $N_2$ in the gas phase. This suggests strongly that the $N_2$ must be formed at or near the $N_2$ reduction site.

Nitrogenase is composed of two separately purified proteins called the molybdenum-iron protein (MoFe protein) and the iron protein (Fe protein), whose physical properties have been reviewed recently (Orme-Johnson et al., 1977; Mortenson & Thorneley, 1979; Burgess, 1984). The MoFe protein contains the site of substrate reduction (Hageman & Burris, 1979; Shah et al., 1973) while the Fe protein is generally accepted as a specific electron donor for the MoFe protein (Hageman & Burris, 1978a; Ljones & Burris, 1978a,b). In addition to these two proteins, a source of reducing equivalents, MgATP, protons, and an anaerobic environment are required for all substrate reductions (Bulen & LeComte, 1966). Nitrogenase catalyzes not only the reduction of the physiological substrates dinitrogen and protons but also the reductions of the alternative substrates, nitrous oxide, acetylene, azide, cyanide, alkyl cyanides, alkyl isocyanides, hydrazine, cyclopropene, allene, and diazirine. 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 azide by the purified component proteins of *Azotobacter vinelandii* nitrogenase.

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Azide reduction by nitrogenase was first demonstrated by Schöllhorn & Burris (1967). Using crude preparations of nitrogenase from *Clostridium pasteurianum* (*Cp*) and *Azotobacter vinelandii* (*Av*), they showed that azide was apparently reduced by two electrons to give stoichiometric amounts of $N_2$ and $NH_3$. Like $N_2$ reduction, the formation of both products required MgATP and a reductant and was inhibited by CO. These results were confirmed and extended by Hardy & Knight (1967). Although the latter group supported the one $N_2$ to one $NH_3$ stoichiometry proposed earlier, they did observe a one $N_2$ to two $NH_3$ stoichiometry at low azide concentrations, and they suggested that the excess $NH_3$ arose from the further reduction of the $N_2$ formed. It was not until 1981, when Dilworth and Thorneley examined azide reduction using purified nitrogenase from *Klebsiella pneumoniae* (*Kp*) and *Av*, that it became clear that the stoichiometry was not one $N_2$ to one $NH_3$ and that there was a large relative excess of $NH_3$ formed. They further identified $N_2H_4$ as an additional product of azide reduction by nitrogenase. Electron balance was achieved in this study demonstrating that all major products were accounted for with the molar ratio of the products shown to be $\sim 1N_2H_4/2N_2/5\text{–}6NH_3$. This information led the authors to propose that nitrogenase catalyzes reactions 1–3.

$$N_3^- + 3H^+ + 2e^- \rightarrow N_2 + NH_3 \quad (1)$$

$$N_3^- + 7H^+ + 6e^- \rightarrow N_2H_4 + NH_3 \quad (2)$$

$$N_3^- + 9H^+ + 8e^- \rightarrow 3NH_3 \quad (3)$$

As azide is present as two species in solution, $N_3^-$ and $HN_3$, the question arises as to which of the two species is serving as substrate for each of the azide reduction reactions. Because $N_3^-$ is by far the major species in solution, it is generally believed to be the substrate [e.g., see Schöllhorn & Burris (1967), Hardy & Knight (1967), and Dilworth & Thorneley (1981)]. Dilworth & Thorneley (1981) performed azide concentration dependence experiments at pH 6.8 and 7.5. The data show that the apparent values for $K_m(N_3^-)$, as measured by either $N_2H_4$ or total $NH_3$ production, decreased with decreasing pH. The general direction, but not the magnitude, of the change suggested $HN_3$ as the substrate or cosubstrate (Dilworth & Thorneley, 1981; Li et al., 1982). Although apparent $K_m(N_3^-)$ values for azide reduction vary with the product being measured, the pH, the organism, and the Fe protein to MoFe protein ratio, reported values are on the order of 0.6–3.0 mM (Schöllhorn & Burris, 1967; Hardy & Knight, 1967; Parejko & Wilson, 1971; Hermann & Wilson, 1975; Dilworth & Thorneley, 1981) and quite similar to those of other alternative substrates. Dilworth & Thorneley (1981) pointed out that if $HN_3$ were the substrate, its apparent $K_m$ would be in the range 1–15 $\mu$M. As this value is much lower than the apparent $K_m$ for $N_2$ reduction, they argued that $HN_3$ was unlikely to be the substrate.

In general the rate of total electron flow through nitrogenase is believed to be essentially independent of the substrate being reduced [e.g., see Watt & Burns (1977)]. An early study of azide reduction showed that high concentrations of azide inhibited the rate of $NH_3$ production (Schöllhorn & Burris, 1967). Electron balance studies were not performed, however, and it was not clear if this substrate self-inhibition phenomenon was due to inhibition of electron flow. In 1967, Hardy and Knight reported that the rate of nitrogenase turnover (measured as products) was independent of azide concentration for crude preparations of nitrogenase from *Cp* (pH 6.5) and *Av* (pH 7.0). Interpretation of these early results is difficult because the azide reduction product, $N_2H_4$, was not measured (Dilworth & Thorneley, 1981). In detailed product balance studies with purified *Kp* nitrogenase (pH 6.8), it was demonstrated that total electron flow was independent of azide concentration at least up to 20 mM at pH 6.8 (Dilworth & Thorneley, 1981). Ljones' (1973) direct measurements, however, showed that azide was a weak inhibitor of dithionite utilization by *Cp* nitrogenase at pH 8.0. One report suggested that azide inhibited the rate of MgATP hydrolysis by nitrogenase (Hardy & Knight, 1967), although this result was not confirmed by others (Hwang et al., 1973). In the absence of added substrates, nitrogenase catalyzes an $H_2$-evolution reaction and all substrates are competing for a fixed number of electrons which would otherwise be used to evolve $H_2$. Dilworth & Thorneley (1981) demonstrated that, at pH 6.8, infinite azide concentration should not eliminate $H_2$ evolution completely and results in about 75% of the electrons reducing azide and the remainder going to $H_2$ evolution. They further suggested, however, that, at high pH (8.2) and infinite azide concentrations, azide should be able to suppress $H_2$ evolution completely.

A number of studies have focused on the effects of other substrates and inhibitors on azide reduction by nitrogenase. CO was shown to be a noncompetitive inhibitor of azide reduction to total $NH_3$ (Hwang & Burris, 1973; Rivera-Ortiz & Burris, 1975). $C_2H_2$ reduction is inhibited noncompetitively by azide, and azide reduction to total $NH_3$ is inhibited noncompetitively by $C_2H_2$ (Hwang & Burris, 1973). Studies of combinations of cyanide, azide, and $CH_3NC$ show that cyanide and $CH_3NC$ are competitive inhibitors of azide reduction to total $NH_3$ and that azide is a competitive inhibitor of cyanide reduction to $CH_4$ (Hwang & Burris, 1973; Rivera-Ortiz & Burris, 1975). These results were interpreted as evidence that cyanide, azide, and $CH_3NC$ were reduced at a common site which was distinct from the $N_2$-reduction site. More recent reports, however, have questioned this interpretation (Li et al., 1982; Rubinson et al., 1983) because the earlier studies did not consider the numerous problems of (1) pH control, (2) $CN^-$ and $CH_3NC$ inhibition of total electron flow, (3) azide relief of $CN^-$ and $CH_3NC$ inhibition, and (4) multiple products for the reduction of all three substrates. In addition, Dilworth & Thorneley (1981) demonstrated that $N_2$ was a competitive inhibitor of azide reduction to $N_2H_4$, which suggests that dinitrogen and azide reduction occur at the same site.

Further studies of azide reduction by the purified component proteins of *Av* nitrogenase are reported here.

## Materials and Methods

*Reagents and Chemicals.* ATP,[1] creatine phosphate, creatine phosphokinase, and Tes[1] were obtained from Sigma Chemical Co. $NaN_3$ labeled in one terminal nitrogen atom (99 atom % excess $^{15}N$) was from Stohler Isotope Chemicals. $NaN_3$ labeled in the central and one terminal nitrogen atom (91.9 atom % excess $^{15}N$) was from MSD Isotopes. Ultrahigh purity argon and $N_2$/Ar mixtures were purchased from AGA Burdox. Zero-grade $N_2$, $D_2$, and $C_2H_2$/Ar mixtures were obtained from Matheson.

*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. 1900 nmol of $H_2$ $min^{-1}$ (mg of *Av*2)$^{-1}$ and 2900 nmol of $H_2$ $min^{-1}$ (mg of *Av*1)$^{-1}$. All assays were performed at 30 °C in 9.5-mL calibrated vials,

[1] Abbreviations: ATP, adenosine 5′-triphosphate; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

fitted with butyl rubber serum caps, containing the appropriate gas mixtures. The 2.0-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, 15 mM neutralized $Na_2S_2O_4$, and 5.0 units of creatine phosphokinase. It was necessary to treat a solution of Tes with Dowex-50 ($H^+$ form) before pH adjustment and dilution to avoid problems with background $NH_3$.

The vessel containing the reaction mixture was either degassed and filled with the appropriate gas mixture on a previously described apparatus (Corbin, 1978) or degassed with ultrahigh purity Ar and an appropriate amount of inhibitor gas added. $Na_2S_2O_4$ was added, and the mixture was incubated with shaking at 30 °C for 5 min. *Av*1 was added, and the reaction was started by adding *Av*2 to give the appropriate molar ratio of the two components. A total of 2 mg of protein per 2-mL reaction was used to avoid complications introduced by large protein concentrations (Wherland et al., 1981). Unless otherwise indicated, all experiments were performed at an *Av*2/*Av*1 molar ratio of 8. Molar ratios are based on molecular weights of 64 000 for *Av*2 and 230 000 for *Av*1. Molar ratio experiments were performed as described elsewhere (Wherland et al., 1981). All reactions were run with shaking for 6 min, and all product formation was linear with time. Reactions were terminated with 0.2 mL of 37% HCHO when dithionite was to be determined. For all other experiments, reactions were terminated with 0.4 M EDTA, pH 7.4 (0.2 mL). Both termination methods were equally effective.

*Preparation of $NaN_3$ Stock Solution.* Solid $NaN_3$ was weighed out and diluted with demineralized water to produce a stock solution. $NaN_3$ was added to reaction vials before degassing by one of two methods. All concentration dependence experiments were performed by addition of 200 μL of various stock solutions which were 10 times the desired concentration. Other experiments involved addition of varying amounts of the same stock solution. The addition of azide stock solutions did not affect the pH of the reaction mixture. Experiments involving $C_2H_2$, CO, and $N_2$, added as inhibitors, used doubly labeled azide. All other experiments used azide labeled in one terminal position.

*Product Analysis.* With the exception of dithionite-utilization studies, *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). For $H_2$ analysis (if $D_2$ was not present), gas samples, 200 μL at bottle pressure, were taken with a pressure-lock syringe (Precision Sampling). $H_2$ was detected by using a home-built gas chromatograph with a thermal conductivity detector (5-Å molecular sieve column, Ar). $N_2$ formation was determined mass spectrometrically to avoid contamination with atmospheric $N_2$. Where inhibitors were not present, $^{29}N_2$ formation from the terminally labeled azide was monitored; where inhibitors were added and/or other products led to interference at mass 29, it was necessary to monitor $^{30}N_2$ formation from centrally and terminally $^{15}N$ labeled azide. For $^{29}N_2$ and $^{30}N_2$ analysis, gas samples, 1 mL at bottle pressure, were taken with a pressure-lock syringe (Precision Sampling) and were expanded to a fixed volume in a Finnigan mass spectrometer, and the spectrum was recorded at a preset time after expansion. For $^{29}N_2$ experiments, Ar was used as an internal standard. The relative instrumental sensitivies were obtained by using a 40% $N_2$–60% Ar mixture. For $^{30}N_2$ experiments, a 0.1-mL sample of $^{30}N_2$ (98%+) was injected as a standard. When $D_2$ was present, $H_2$ was measured by mass spectrometry on the same vial mixtures of known concentration as standards (Wherland et al., 1981).

In order to obtain low, reproducible backgrounds for $NH_3$ analysis, it was necessary to clean all reaction vials and serum stoppers by heating for at least 2 h in hot Micro (International Products Corp.) and then rinsing in demineralized water and absolute ethanol. Clean vials (oven dried) and stoppers were then stored in closed containers until used.

Attempts to scavenge $N_2H_2$ with 5 mM fumarate made no significant difference in electron allocation to azide at pH 6.8, which indicates that $N_2H_2$ is not released as an intermediate in azide reduction.

Since azide interferes with the Fiske & Subbarow (1925) method for phosphate and $N_2H_4$ interferes with the Ennor (1957) method for phosphocreatine consumption, ATP/$2e^-$ ratios are not reported.

Ammonia was determined by an HPLC–fluorescence method described elsewhere (Corbin, 1984). Dithionite was determined by using a published procedure (Li et al., 1982). In these experiments, $H_2$, $N_2$, and $S_2O_4^{2-}$ were measured on the same reaction vial.

*Data Treatment.* Calculations of the total amounts of $H_2$ and $N_2$ were based on the calibrated vial volume minus 2.2 mL of liquid phase and then expressed as nanomoles per minute per milligram of total protein. No correction for solubility of 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. The relationship $2e^-/H_2$, $2e^-/N_2$, $6e^-/N_2H_4$, or $2.67e^-$/excess $NH_3$ is used in calculating electron flow from products formed (Dilworth & Thorneley, 1981). Excess $NH_3$ is calculated as total $NH_3 - (N_2 + N_2H_4)$.

Because $N_3^-$ is a weak base ($pK_a^{30°C} = 4.6$; Boughton & Keller, 1966), the relative amounts of $N_3^-$ and $HN_3$ in buffered solutions of $NaN_3$ are determined by pH. $N_3^-$ is by far the dominant species at the pH values of interest. For 1 mM $NaN_3$ added, the $N_3^-$ concentration ranges from 0.9829 mM at pH 6.5 to 0.9986 mM at pH 7.6. Although $HN_3$ concentrations are low, they are dramatically changed by pH, ranging from 17.1 μM at pH 6.5 to 1.38 μM at pH 7.6 (for 1 mM $NaN_3$ added). The initial concentrations of both $N_3^-$ and $HN_3$ were calculated from the amounts of $NaN_3$ added in each reaction by

$$[HN_3] = \frac{[NaN_3]}{\text{antilog } (4.6 - \text{pH}) + 1}$$

which comes from the relationship of $pK_a$ to pH, $pK_a^{30°C} = 4.6 = \text{pH} + \log([N_3^-]/[HN_3])$ and $[NaN_3] = [N_3^-] + [HN_3]$. In the text, $N_3^-$ and $HN_3$ will be used to refer to the actual species ("azide ion" and the neutral "hydrazoic acid" molecule), while the term "azide" will be used in a general sense, e.g., azide reduction, without regard to the actual species involved. In all experiments, at low $NaN_3$ concentrations, a large proportion of the substrate is consumed in our 6-min reactions. Thus, for increased accuracy, we use the average $NaN_3$ concentration [(initial + final)/2] instead of the initial $NaN_3$ concentration for all calculations [e.g., see Rubinson et al. (1983)].

The rate of nitrogenase turnover is also dependent on pH. When data obtained at different pH values are compared, it is desirable to "normalize" to a constant electron flow. The simplest way to do this is to report the data in terms of the percentage of the total electron flow (at each point) that was used to produce a particular product.

## Results

*Electron Balance.* Using *Av* purified component proteins, we have confirmed the finding of Dilworth & Thorneley

Table I: Product/Electron Balance

| products[a] (nmol·mg$^{-1}$) | | | | electron pairs as products[b] (nmol·mg$^{-1}$) | nmol of $S_2O_4^{2-}$ consumed[c] |
|---|---|---|---|---|---|
| $H_2$ | $N_2H_4$ | $N_2$ | $NH_3$ | | |
| 1212 | 272 | 624 | 1350 | 3252 | 3288 ± 156 |

[a] The following conditions were used: 20 mM $NaN_3$, pH 7.3, Ar atmosphere, 6-min reactions, average of six reactions. [b] See Materials and Methods. [c] By titration (Li et al., 1982).

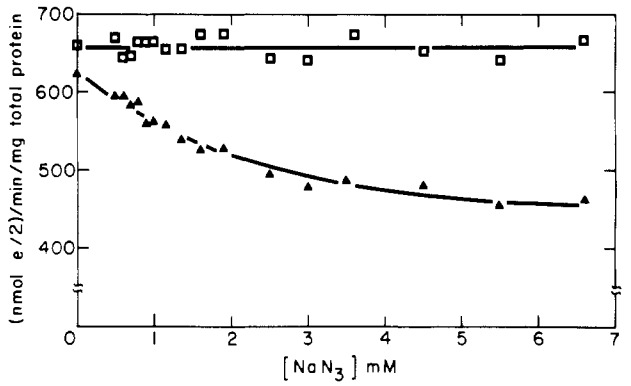



FIGURE 1: Plot of rate of total electron flow through nitrogenase vs. initial $NaN_3$ concentration: (□) pH 6.9; (▲) pH 7.6. Total electron pairs were calculated from products as described under Materials and Methods. In the same $NaN_3$ concentration range, electron flow was constant at 464 ± 15 (pH 6.5), 533 ± 17 (pH 6.7), 644 ± 30 (pH 7.1), and 616 ± 15 (pH 7.3) all in nmol of electron pairs min$^{-1}$ (mg of total protein)$^{-1}$.

(1981) with *Kp* that azide is reduced by six electrons to give $N_2H_4$ and $NH_3$ and by two electrons to give $N_2$ and $NH_3$ and that there is a large relative excess of $NH_3$ formed. Table I clearly shows the equivalence of products formed to electrons consumed, and thus all major products are accounted for. The product ratio for this experiment was $1N_2H_4/2.3N_2/5NH_3$ which also is quite similar to that previously reported for *Kp* at pH 6.8.

A control experiment, performed under the same conditions but with no azide present, gave 3270 ± 66 (nmol of $H_2$ formed in 6 min) (mg of total protein)$^{-1}$, which demonstrates that azide concentrations, at least up to 20 mM (*Av*, pH 7.3), do not affect the rate of total electron flow through nitrogenase as previously reported for *Kp* at pH 6.8 (Dilworth & Thorneley, 1981). As shown in Figure 1, however, azide does appear to weakly inhibit the rate of total electron flow through nitrogenase at high (>7.3) pH. This has also been observed for *Cp* nitrogenase (at pH 8.0), in dithionite-utilization studies, and attributed to irreversible inactivation of *Cp*2 (Ljones, 1973). In contrast to our previous studies of $CN^-$ (Li et al., 1982) and $CH_3NC$ (Rubinson et al., 1983) inhibition we were unable to relieve azide inhibition of total electron flow by addition of CO. This observation supports the idea that the inhibition is nonspecific. Thus, in order to avoid the problems of variable electron flow, all other experiments were performed in the pH range 6.5–7.3, where such inhibition was not observed.

*Reduction of Azide to $N_2H_4$.* In order to determine which species, $HN_3$ or $N_3^-$, is a substrate for nitrogenase, we have performed azide concentration dependence experiments at several pH values. Figure 2a is a plot of the percentage of the total electrons used to reduce azide to $N_2H_4$ as a function of the calculated $HN_3$ concentration at five different pH values. A definite correlation is found. At any given $HN_3$ concentration, the same value of percent electrons to $N_2H_4$ is obtained, independent of the $N_3^-$ concentration and the pH. Figure 2b is the same data as in Figure 2a but plotted vs. the

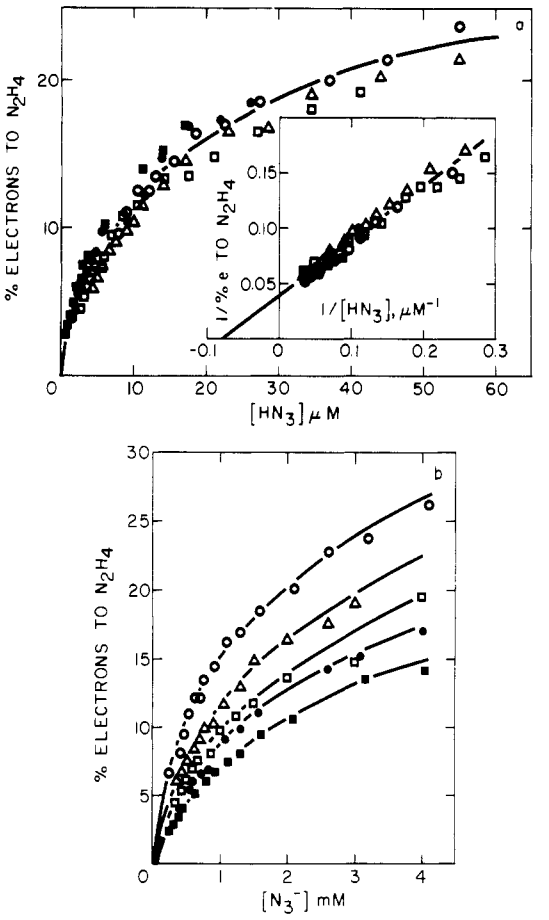



FIGURE 2: (a) Plot of percentage of total electrons being used to produce $N_2H_4$ vs. the calculated $HN_3$ concentration. Inset: Double-reciprocal plot using $HN_3$ concentrations in the range $(0.2–2)K_m$ (Segal, 1975). The line is a computer fit of data points at all pH values to the Michaelis–Menten equation giving $K_m$ = 12.3 ± 0.4 μM $HN_3$; $V_{max}$ = 25.4 ± 1.3%. (b) Percentage of total electrons being used to produce $N_2H_4$ vs. $N_3^-$ concentration. Assay conditions and calculations are as described under Materials and Methods: (○) pH 6.5; (△) pH 6.7; (□) pH 6.9; (●) pH 7.1; (■) pH 7.3.

$N_3^-$ concentration. At any given $N_3^-$ concentration, five different values of percentage electrons to $N_2H_4$ are obtained and the higher the pH the lower the percentage electrons to $N_2H_4$. Since $[HN_3]$ decreases with increasing pH, this is the expected result if $HN_3$ is the source of $N_2H_4$. We conclude that $HN_3$ is evidently the substrate and is reduced by nitrogenase in a six-electron reaction to $N_2H_4$ plus $NH_3$. A plot of 1/(percentage electrons to $N_2H_4$) vs. $[HN_3]$ is linear and yields an apparent $K_m$ of 12.2 ± 0.41 μM $HN_3$ and a $V_{max}$ of 25.4 ± 1.3% (inset of Figure 2a). We did not observe any less highly reduced products of $HN_3$ reduction, and attempts to trap a diazine-level intermediate were unsuccessful (see Materials and Methods).

*Reduction of Azide to $N_2$.* Since $HN_3$ is the substrate for the $N_2H_4$ pathway, is it also the substrate for the two-electron reduction to $N_2$? Apparently not, as Figure 3 shows a good correlation between the percentage of the total electrons used to reduce azide to $N_2$ vs. the calculated $N_3^-$ concentration at five pH values. At any given $N_3^-$ concentration, about the same value of percent electrons to $N_2$ is obtained, essentially independent of the $HN_3$ concentration or pH (but see excess $NH_3$ section below). If $HN_3$ were the substrate, a plot of the same data, but when $[HN_3]$ is used instead of $[N_3^-]$, should have resulted in a single curve for the same reasons given above. In fact, one gets five very different curves (Figure 1 of supplementary material). This result is again consistent

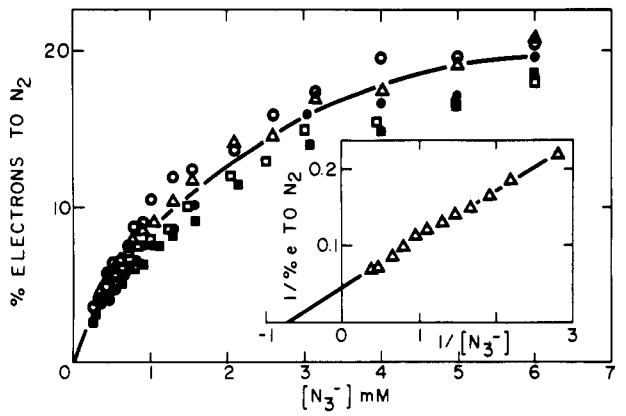



FIGURE 3: Plot of percentage of total electrons being used to produce $N_2$ vs. the $N_3^-$ concentration. Assay conditions and calculations are as described under Materials and Methods: (○) pH 6.5; (△) pH 6.7; (□) pH 6.9; (●) pH 7.1; (■) pH 7.3. Inset: Double-reciprocal plot of pH 6.7 data using $N_3^-$ concentration in the range (0.2–2)$K_m$ (Segal, 1975). The line is a computer fit to the Michaelis–Menten equation. $K_m$ = 1.35 ± 0.031 mM $N_3^-$; $V_{max}$ = 21.4 ± 0.93%. Slight pH trend discussed in excess $NH_3$ section.

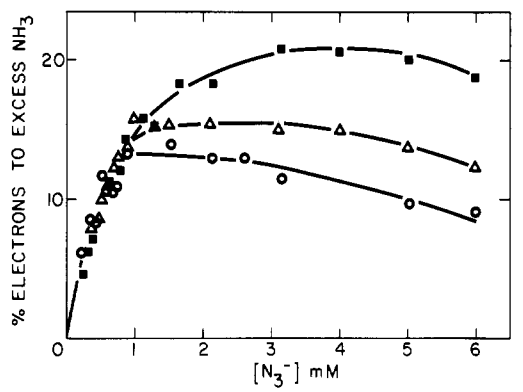



FIGURE 4: Plot of percentage of total electrons being used to produce excess $NH_3$ [$NH_3$ total − ($N_2H_4$ + $N_2$)] vs. the calculated $N_3^-$ concentration: (○) pH 6.5; (△) pH 6.7; (■) pH 7.3.

with $N_3^-$ being the substrate. At any given value for [$HN_3$], the amount of $N_3^-$ present would depend on the pH; for example, at [$HN_3$] = 15 μM, [$N_3^-$] would be 5.44 mM at pH 7.3 but only 0.86 mM at pH 6.5. The percentage electrons going to $N_2$ production should, therefore, be higher at the higher pH and it is (18% vs. 9%) (Figure 1 of supplementary material). These data demonstrate then that $N_3^-$ is the substrate which is reduced by two electrons to give $N_2$ plus $NH_3$.

*Reduction of Azide Leading to Excess $NH_3$.* Excess $NH_3$ is the amount of the total $NH_3$ formed that cannot be accounted for by the six-electron reduction of $HN_3$ to $N_2H_4$ plus $NH_3$ plus the two-electron reduction of $N_3^-$ to $N_2$ plus $NH_3$. Figure 4 is a plot of the percentage of total electrons being used to produce excess $NH_3$ vs. the calculated $N_3^-$ concentration at three pH values. Figure 4 shows a good correlation of excess $NH_3$ with [$N_3^-$] below about 1 mM. If the data in this range are plotted vs. [$HN_3$], three very different lines are observed for the three pH values (not shown). Thus, the rate of production of excess $NH_3$ is dependent either directly or indirectly on [$N_3^-$] and definitely not on [$HN_3$]. The data shown in Figure 4 do not follow Michaelis–Menten kinetics, with the curves at all pH values leveling off prematurely and even decreasing at high azide concentrations. Although this effect is less dramatic if total $NH_3$ is considered, especially at high pH, it is still noticeable, as previously observed by Schöllhorn & Burris (1967). The data in Figure 4 cannot therefore be used to obtain $K_m$ and $V_{max}$ values for excess $NH_3$ production. Interestingly, the inhibition of the formation of

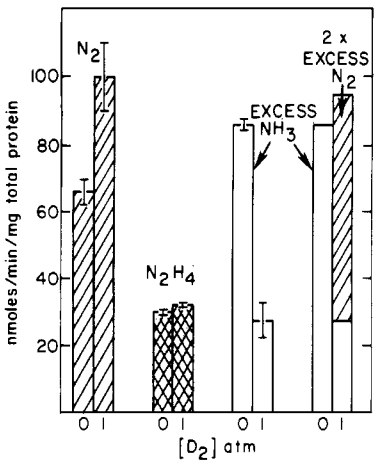



FIGURE 5: Effect of $D_2$ on products of azide reduction. Identical trend seen in separate experiments with 2 mM $NaN_3$ (Table I of supplementary material). The shaded area in the fourth set of bar graphs is 2 times the increase in $N_2$ seen when $D_2$ is added. This assumes that in the absence of $D_2$ that $N_2$ was reduced to two $NH_3$. $H_2$ evolution was 240 ± 13 under Ar (1 atm) and 214 ± 9 under $D_2$ (1 atm) both in nanomoles of electron pairs per minute per milligram of total protein. [$NaN_3$] = 4 mM.

excess $NH_3$ depends not only on increasing azide concentration but also on the pH. Much more inhibition is observed at low pH than at high pH, which strongly suggests that, whatever the mechanism for excess $NH_3$ formation, the reaction is dramatically inhibited by $HN_3$.

The simplest explanation for these observations (Hardy & Knight, 1967) is that the excess $NH_3$ arises from the reduction of the $N_2$ formed during azide reduction experiments. As the production of $N_2$ depends on the $N_3^-$ concentration, the excess $NH_3$ should similarly depend on [$N_3^-$]. It does (Figure 4). $N_2$ is a weak competitive inhibitor of $N_2H_4$ production (Dilworth & Thorneley, 1981), which we have shown arises from the reduction of $HN_3$. Conversely, one would expect $HN_3$ to be a competitive inhibitor of $N_2$ reduction, and in fact, $HN_3$ does appear to inhibit strongly the formation of excess $NH_3$. This observation would also explain the slight trend toward lower observed rates of $N_2$ production as the pH is raised (Figure 3). At high pH, there is less $HN_3$ present to inhibit the reduction of $N_2$ to excess $NH_3$ so that more $N_2$ should be reduced and the observed $N_2$ should be lower. These are the trends observed.

In order to determine definitively whether or not the excess $NH_3$ arises from the reduction of the $N_2$ formed, we have performed azide reduction experiments in the presence of $D_2$. $H_2(D_2)$ is known to specifically inhibit the reduction of $N_2$ to $2NH_3$ but does not inhibit the reduction of any other nitrogenase substrate [e.g., see Guth & Burris (1983)]. As shown in Figure 5 and supplemental Table I, the addition of $D_2$ to an azide reduction system does not affect $N_2H_4$ production but does cause a large decrease in excess $NH_3$ and a corresponding increase in $N_2$ production. The decrease in excess $NH_3$ formation is completely offset by the increase in $N_2$ which is exactly what is expected from the stoichiometry of the $H_2(D_2)$ inhibition reaction (eq 4; Burgess et al., 1981), where

$$N_2 + 2H^+ + 2e^- + H_2(D_2) \rightarrow N_2 + H_2(2HD) \quad (4)$$

every time $N_2$ is intercepted by $H_2(D_2)$, it is prevented from being reduced to $2NH_3$. Taken together, these data suggest strongly that excess $NH_3$ is not a direct product of azide reduction but rather a side product of the further reduction of the $N_2$ formed. Inhibition studies with CO (see inhibitor section below) are also consistent with this view.

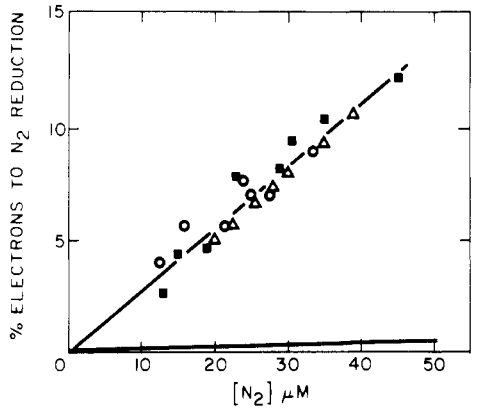



FIGURE 6: Plot of percentage electrons to $N_2$ reduction vs. micromolar $[N_2]$. Bottom line is theoretical curve for typical $N_2$ fixation experiment as discussed in the text. Top line is experimental showing percent electrons to $N_2$ reduction vs. $[N_2]$ during azide reduction experiments assuming that all excess $NH_3$ arises from reaction 5 under Results. Thus, one-third of excess $NH_3$ comes from two-electron reduction of $N_3^-$ to $N_2$, and the rest comes from six-electron reduction of that $N_2$ to $2NH_3$: (○) pH 6.5; (△) pH 6.7; (■) pH 7.3.

The question then arises as to whether or not the $N_2$ formed, that is, subsequently reduced to give excess $NH_3$, is in equilibrium with $N_2$ in the gas phase over the reaction. Reported values for $K_m(N_2)$ are on the order of 0.1 atm of $N_2$ (Hardy, 1979) which would be about 4 mM $N_2$ in the gas phase of the reaction vial. On the basis of this $K_m$ and a reported $V_{max}$ of about 75% electrons during $N_2$-reduction experiments (Rivera-Ortiz & Burris, 1975; Simpson & Burris, 1984), we have constructed a theoretical curve of percentage electrons to give $NH_3$ vs. $N_2$ at extremely low $[N_2]$ for the $N_2$-fixation reaction (bottom line in Figure 6). In Figure 6, we have also plotted our excess $NH_3$ data as a function of the average $N_2$ concentration (final $N_2/2$) present in the gas phase of our reaction vials during the course of the experiments. The points used are those below about 1 mM $N_3^-$ from Figure 4 or before the reaction is inhibited significantly by $HN_3$. Clearly, the amount of excess $NH_3$ formed cannot be explained if the $N_2$ produced is released from the enzyme and equilibrated with the gas phase prior to its being reduced to $NH_3$. Nonetheless, dissociation of the $N_2$ from the enzyme is indicated by $HN_3$ and CO inhibition studies (see inhibition section below).

*A Second Look at the Reduction of $N_3^-$ to $N_2$.* As indicated above, a significant proportion of the $N_2$ produced from $N_3^-$ reduction appears to be further reduced to give excess $NH_3$. If that is the case, then Figure 3 is not an accurate representation of the kinetics of $N_3^-$ reduction to $N_2$ by nitrogenase. Figure 7 shows the kinetics of this reaction if one assumes that all of the excess $NH_3$ arises from reaction 5. As expected,

$$N_3^- \xrightarrow{+3H^+,\,2e^-} NH_3 + N_2 \xrightarrow{+6H^+,\,6e^-} 2NH_3 \qquad (5)$$

the data in Figure 7 follow Michaelis–Menten kinetics when plotted vs. $N_3^-$, and there is no trend with pH. The double-reciprocal plot is linear, giving an apparent $K_m = 0.86 \pm 0.06$ mM $N_3^-$ and a $V_{max} = 40 \pm 2.7\%$ for the two-electron reduction of $N_3^-$ to $N_2 + NH_3$.

*Hydrogen Evolution.* In 1981, Dilworth and Thorneley reported that the ability of azide to compete successfully with the $H_2$-evolution reaction was dependent on pH. By extrapolation to infinite azide concentration, they concluded that $H_2$ evolution could be eliminated at pH 8.0 but not at pH 6.8. That study, however, did not measure the products of azide reduction but rather the change in $H_2$ evolution as increasing azide is added. This approach is not valid if total electron flow is inhibited by increasing azide as appears to be the case at high pH (Figure 1; Ljones, 1973).

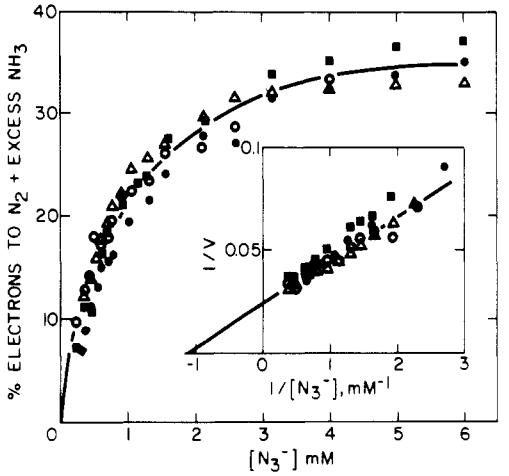



FIGURE 7: Plot of percentage of total electrons being used to reduce $[N_3^-]$ to $N_2$ and excess $NH_3$ vs. the calculated $[N_3^-]$. Inset: Double-reciprocal plot using $[N_3^-]$ in the range $(0.2–2)K_m$ (Segal, 1975). The line is a computer fit of data points at all pH values to the Michaelis–Menten equation giving $K_m = 0.86 \pm 0.06$ mM $N_3^-$ and $V_{max} = 40 \pm 2.7\%$. Assay conditions and calculations are as described under Materials and Methods: (○) pH 6.5; (△) pH 6.7; (□) pH 6.9; (●) pH 7.1; (■) pH 7.3.

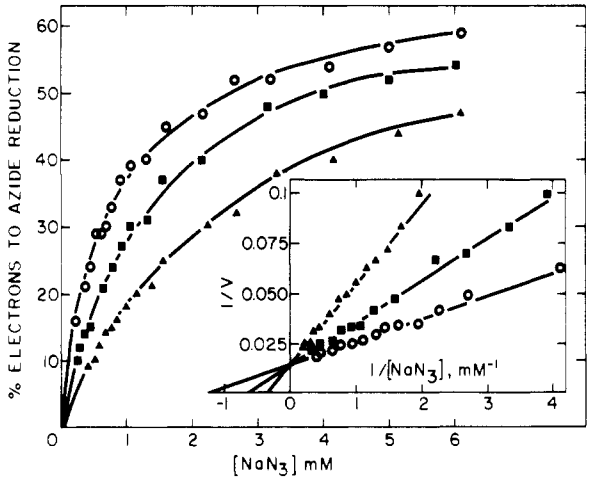



FIGURE 8: Plot of percentage electrons to azide reduction to all products (with the remainder of electrons going to $H_2$ evolution) vs. $[NaN_3]$ at (○) pH 6.5, (■) pH 7.3, and (▲) pH 7.6. Inset is double-reciprocal plot giving the following: $K_m = 0.68 \pm 0.04$ and $V_{max} = 63 \pm 2.2$ at (○) pH 6.5; $K_m = 1.46 \pm 0.05$ and $V_{max} = 68 \pm 5$ at (■) pH 7.3; $K_m = 2.64 \pm 0.06$ and $V_{max} = 65 \pm 3.8$ at (▲) pH 7.6.

Figure 8 is a plot of the percentage electrons to azide reduction (all products) vs. $[NaN_3]$. We have included the pH 7.6 data because high pH was of particular interest (Dilworth & Thorneley, 1981). Even though total electron flow was not constant at pH 7.6, we knew the electron flow at each point and thus could calculate the percentage electrons to azide reduction products. As shown in Figure 8, the $K_m$ values for total azide reduction increase as the pH is increased. This is expected because, at high pH, much more $[NaN_3]$ is required to maximize $HN_3$ reduction. As shown in the inset to Figure 8, however, the $V_{max}$ is independent of pH. This result is also expected because at infinite $[NaN_3]$, regardless of pH, the system will be saturated with both $[N_3^-]$ and $[HN_3]$. The $V_{max}$ of 65% electrons going to azide reduction (with the remainder going to $H_2$ evolution) from Figure 8 is the same as the sum of the $V_{max}[HN_3]$ (Figure 2a) and $V_{max}[N_3^-]$ (Figure 7) and is also the value we determined experimentally at 25 mM $NaN_3$ (pH 6.7, 7.6). Thus, $H_2$ evolution cannot be eliminated by azide with about 35% of the electrons still going to $H_2$

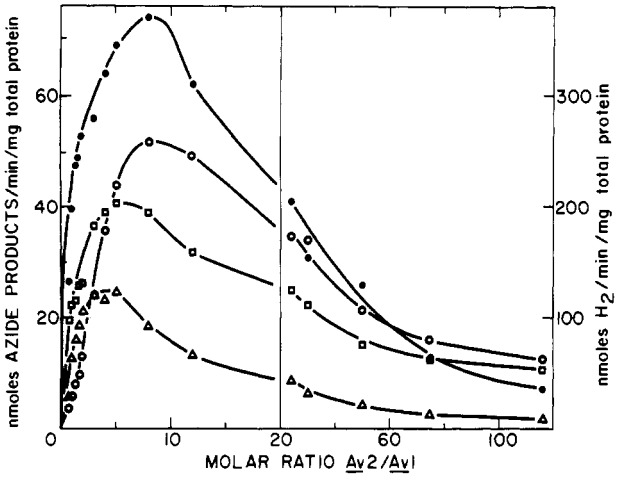



FIGURE 9: Plot of nanomoles of products per minute per milligram of total protein vs. the molar component ratio *Av*2/*Av*1: (○) $H_2$; (□) $N_2$; (△) $N_2H_4$; (●) excess $NH_3$. All assays and calculations are as described under Materials and Methods. Assays contained 2 mM $NaN_3$ at pH 7.1.

evolution at infinite [$NaN_3$] at *Av*2/*Av*1 = 8.

*Component Ratio Titration.* Figure 9 shows the rate of product formation vs. the molar component ratio of *Av*2 to *Av*1. In this experiment, $HN_3$ reduction to $N_2H_4$ + $NH_3$ peaks at the lowest ratio of about three while $N_3^-$ reduction to $N_2$ + $NH_3$ peaks at a ratio of about 5. $H_2$ evolution and excess $NH_3$ formation peak at the highest ratio of about 8. This observation is consistent with the observation of Dilworth & Thorneley (1981) where $N_2H_4$ formation was favored over total $NH_3$ production at low *Kp*2/*Kp*1 ratios. The trends observed in Figure 9 are different from those seen in previous experiments for $H_2$ evolution, $N_2$ fixation, and HD formation where all products peak at the same ratio (Whereland et al., 1981) but similar to those for HCN (Li et al., 1982) and $CH_3NC$ (Rubinson et al., 1983).

Previous studies (Silverstein & Bulen, 1970; Davis et al., 1975) with nitrogenase have led to the suggestion that, in general, $H_2$ evolution is favored over other substrate reductions (especially $N_2$) at low Fe/MoFe protein molar ratios where the system is electron limited. 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 HCN reduction (Li et al., 1982), $CH_3NC$ reduction (Rubinson et al., 1983), and azide reduction (Figure 2 of supplementary material) where high ratios favor $H_2$ evolution over HCN, $CH_3NC$, $HN_3$, and $N_3^-$ reductions.

The data shown in Figure 9 and Figure 2 of supplementary material) indicate that the ability of azide to compete successfully with $H_2$ evolution should increase as the *Av*2/*Av*1 ratio is lowered. To test this indication, we performed an azide concentration dependence experiment (pH 6.9) at an *Av*2/*Av*1 molar ratio of 1.5 (data not shown). $HN_3$ is a markedly better substrate at this ratio. The apparent $K_m$ for $HN_3$ reduction to ($N_2H_4$ + $NH_3$) decreases from 12 μM at *Av*2/*Av*1 = 8 (Figure 2a) to 6.8 μM ± 0.06 at *Av*2/*Av*1 = 1.5. The $V_{max}$ increases dramatically from 25% at *Av*2/*Av*1 = 8 (Figure 2a) to 40.2 ± 4.8% at *Av*2/*Av*1 = 1.5.

The effect of component protein ratio on the production of $N_2$ is less dramatic. Although the apparent $K_m$ is lowered from about 1.3 mM at *Av*2/*Av*1 = 8 (Figure 3) to 0.78 mM ± 0.05 at *Av*2/*Av*1 = 1.5, the $V_{max}$ is unaffected at 21.4% (Figure 3) vs. 20.6 ± 1.4%. When the reduction of $N_3^-$ to both $N_2$ and excess $NH_3$ is considered (data not shown, pH 6.9), the $V_{max}$ decreases from about 40% to 35% at the lower ratio. This result is consistent with the trend shown in Figure 9 where excess $NH_3$ production peaks at a higher ratio than does $N_2$ formation. When all azide reduction products are considered together, the $V_{max}$ increases from 65% at *Av*2/*Av*1 = 8 (Figure 8) to 75.2 ± 2.4% (Figure 3 of supplementary material) at *Av*2/*Av*1 = 1.5, with the remainder of the electrons going to $H_2$ evolution. Thus, although the ability of azide to compete successfully with $H_2$ evolution is improved by lowering the *Av*2/*Av*1 ratio to 1.5, $H_2$ evolution still cannot be eliminated by infinite concentration of azide at this ratio.

Table II: Percent Inhibition

| inhibitor[a] | percent inhibition of $N_2H_4$ | $N_2$ | excess $NH_3$ |
|---|---|---|---|
| CO (0.06%) | 59 | 63 | 80 |
| $N_2$ (40%) | 39 | 20 | ND[b] |
| $C_2H_2$ (5%) | 24 | 38 | 90 |
| $C_2H_2$ (15%) | 39 | 46 | 94 |

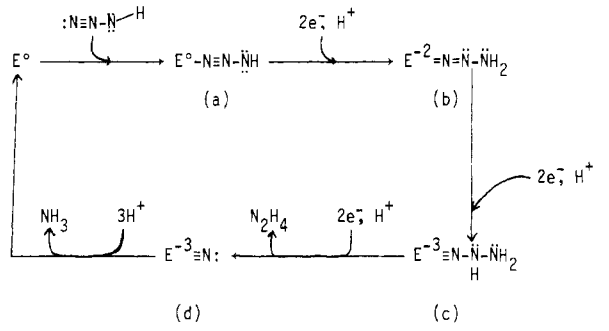
[a] Balance Ar. Conditions used were 2 mM $NaN_3$, pH 7.1, in triplicate determinations. [b] Not determined.

*Inhibition of Azide Reduction.* $HN_3$ and $N_3^-$ are reduced by nitrogenase to give different products, and it is of interest to know whether each of these substrates inhibits the reduction of the other. As shown in Figure 2a, the reduction of $HN_3$ to $N_2H_4$ + $NH_3$ is unaffected by the presence of $N_3^-$. For example, $N_2H_4$ formation is constant at [$HN_3$] = 25 μM, even though the [$N_3^-$] ranges from 0.14 (pH 6.5) to 9.07 mM (pH 7.3). Thus, $N_3^-$ does not inhibit $HN_3$ reduction at least in the concentration range tested. If $HN_3$ were to inhibit $N_3^-$ reduction to give $N_2$ + $NH_3$, then one would expect less $N_2$ to be formed as the pH is lowered (i.e., the [$HN_3$] is increased). As shown in Figure 3, however, we actually see somewhat more $N_2$ formed as the [$HN_3$] is increased (pH is lowered). Thus, $HN_3$ does not appear to inhibit [$N_3^-$] reduction to ($N_2$ + $NH_3$) in the concentration range tested. The data shown in Figure 4, however, show that $HN_3$ does appear to inhibit dramatically the formation of excess $NH_3$ (see excess $NH_3$ section above).

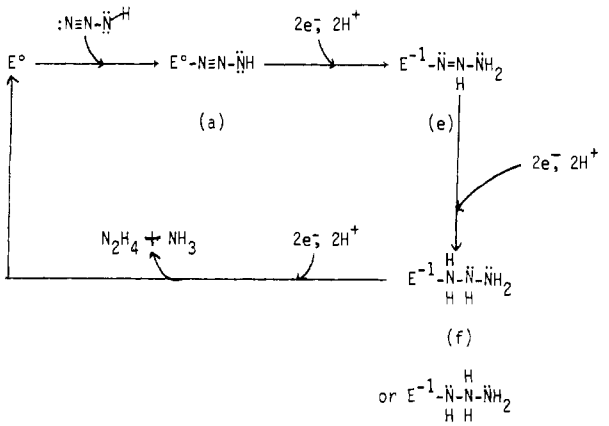
We have tested the effects of other substrates and inhibitors on azide reduction in order to determine if they inhibit the $HN_3$ and $N_3^-$ reduction pathways to different extents. In 1981, Dilworth and Thorneley reported that, for *Kp* nitrogenase, CO was a noncompetitive inhibitor of $N_2H_4$ production, which we have shown arises from the reduction of $HN_3$. We observe the same noncompetitive inhibition pattern for CO inhibition of $HN_3$ reduction (to $N_2H_4$ + $NH_3$) for *Av* nitrogenase (data not shown). As shown in Table II, CO also inhibits the production of both $N_2$ and excess $NH_3$, but its effect on excess $NH_3$ formation is much greater. If, as discussed in the excess $NH_3$ section, the excess $NH_3$ arises from the further reduction of the $N_2$ formed, then when CO inhibits the reduction of that $N_2$, it should cause both a decrease in the observed excess $NH_3$ and a corresponding increase in observed $N_2$. When we examined the inhibition pattern of CO on $N_2$ formation, we were surprised to see a competitive-type inhibition pattern (Figure 4 of supplementary material). This result is probably not meaningful because the observed $N_2$ is lowered by CO inhibition of $N_3^-$ reduction (to $N_2$ + $NH_3$) while simultaneously being raised by the more dramatic CO inhibition of the reduction of that $N_2$ to excess $NH_3$. When the reduction of $N_3^-$ to $N_2$ and excess $NH_3$ is considered, however, the inhibition pattern appears noncompetitive (Figure 5 of supplementary material) as it is for all other nitrogenase substrates.

Dilworth & Thorneley (1981) demonstrated that $N_2$ was a weak competitive inhibitor of $N_2H_4$ formation by *Kp* nitrogenase, a result we have confirmed for *Av* (data not shown). As shown in Table II, $N_2$ also inhibits the reduction of $N_3^-$

Scheme I



Scheme II



(to $N_2 + NH_3$). If only $N_2$ formation is considered, a competitive-type inhibition pattern is observed (data not shown) as it was for CO. Unfortunately, excess $NH_3$ could not be determined in this experiment with added $N_2$, and the competitive-type inhibition pattern may not be meaningful for the same reasons given above for CO. As shown in Table II, $C_2H_2$ inhibits $HN_3$ reduction (to $N_2H_4 + NH_3$), $N_3^-$ reduction (to $N_2 + NH_3$) and the formation of excess $NH_3$. Like CO, $C_2H_2$ has the most pronounced effect on the production of excess $NH_3$.

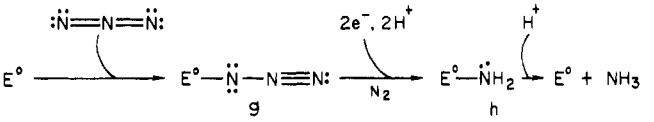
## Discussion

*Reduction of $HN_3$.* In 1981, Dilworth and Thorneley reported a previously unrecognized nitrogenase reaction, the six-electron reduction of azide to give $N_2H_4 + NH_3$. That report included a proposal for the chemical mechanism of azide reduction to $N_2H_4 + NH_3$ which relied on $N_3^-$ as the substrate. This cannot be correct because our data demonstrate that $HN_3$ is the substrate for that reaction. Schemes I and II shown here are mechanisms that rely on $HN_3$ as the substrate. The substrate



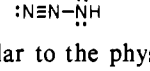
(unlike $N_3^-$; see below) is expected to bind to a metal atom in the enzyme to give species (a) which has the $N_2$ triple bond closest to the metal atom. Both Schemes I and II involve sequential two-electron reductions of that triple bond. Scheme I involves an inequivalent number of protons and electrons transferred to yield $N_2H_4$ and a nitride species, which would then be protonated to yield $NH_3$. This scheme is similar in some respects to the mechanism proposed by Dilworth & Thorneley (1981) for this reaction and that proposed by the Sussex group [e.g., see Chatt (1980) and Thorneley & Lowe (1982)] for $N_2$ reduction. Scheme II involves three sequential, two-electron, two-proton reductions and is similar to mechanisms we have proposed for the six-electron reductions of $N_2$ (Burgess et al., 1981), HCN (Li et al., 1982), and $CH_3NC$

Scheme III



(Rubinson et al., 1983). It also involves coupled proton–electron transfer which has been suggested to occur at all enzymic molybdenum sites, including nitrogenase (Stiefel, 1973).

To the enzyme, the substrate



probably looks quite similar to the physiological substrate :N≡N: which might explain the extremely low $K_m$ for $HN_3$ reduction to $N_2H_4 + NH_3$. Also like $N_2$ reduction, the six-electron reduction of $HN_3$ does not yield any lesser reduced products, which indicates that the intermediates formed remain tightly bound. This is not the case for $CH_3NC$ or HCN reduction both of which yield significant amounts of four-electron-reduced products (Li et al., 1982; Rubinson et al., 1983). Our attempts to trap a diazene-level intermediate in this reaction were unsuccessful. Pre-steady-state kinetic studies have shown that $N_2H_4$ appears upon acid quenching the reaction following about three electron transfers (Dilworth & Thorneley, 1981). Although both Schemes I and II do not allow for the appearance of free $N_2H_4$ prior to six electron transfers, intermediates b, c, e, and f all would be expected to yield $N_2H_4$ upon acid quenching, thus explaining the pre-steady-state kinetic data.

As previously demonstrated by Dilworth & Thorneley (1981) for *Kp* and now confirmed for *Av*, $N_2$ appears to be a competitive inhibitor of $HN_3$ reduction, and thus, the two reactions are likely to occur at the same site. On the other hand, $N_2$ is a weak inhibitor of $HN_3$ reduction with a $K_i$ about 5-fold greater than its $K_m$ (Dilworth & Thorneley, 1981). This could be explained if $HN_3$ and $N_2$ have different affinities for different redox states of the enzyme. The component protein ratio titration data shown here strongly suggest that, like HCN (Li et al., 1982) and $CH_3NC$ (Rubinson et al., 1983), $HN_3$ binds to a redox state of the enzyme more oxidized than that reactive toward $N_2$ reduction or $H_2$ evolution. This is also consistent with the observations that, like HCN and $CH_3NC$, but unlike $N_2$, $HN_3$ appears to be a fairly good inhibitor of $H_2$ evolution (see below).

*Reduction of $N_3^-$.* The two-electron reduction of azide to give $N_2 + NH_3$ was the earliest reaction to be recognized (Schöllhorn & Burris, 1967). Our data demonstrate $N_3^-$ to be the substrate for that reaction. Unlike $HN_3$, the substrate $N_3^-$ (:N̈=N=N̈:) is symmetrical and is expected to be polarized on binding to a metal site in the enzyme to yield species g (Scheme III), where the $N_2$ triple bond is now remote from the metal. Species g could be readily reduced by two electrons and two protons to yield $N_2$ and a bound amido species (h), which would rapidly be protonated to yield $NH_3$. The reduction of $N_3^-$ is the only example of nitrogenase reduction requiring unequal numbers of protons and electrons.

The data presented here strongly support the suggestion (Hardy & Knight, 1967) that some of the $N_2$ formed (Scheme III) is further reduced by six electrons to give $NH_3$ (excess $NH_3$). The evidence includes the observations that (a) both $N_2$ and excess $NH_3$ formation depend on $[N_3^-]$ and not $[HN_3]$, (b) the excess $NH_3$ reaction is strongly inhibited by $HN_3$ which, in turn, is competitively inhibited by $N_2$, (c) inhibition of the excess $NH_3$ reaction by $HN_3$ and CO (and possibly $N_2$ and $C_2H_2$) causes a corresponding increase in the observed $N_2$,

and most importantly, (d) like $N_2$ reduction, but unlike any other nitrogenase–substrate reduction, the formation of excess $NH_3$ is inhibited by $D_2$, which gives a corresponding increase in observed $N_2$.

Figure 6 shows that the $N_2$ formed from $N_3^-$ reduction, which appears to be further reduced to two $NH_3$, is not in equilibrium with $N_2$ in the gas phase as previously suggested (Hardy, 1979). Thus, the $N_2$ must be formed close enough to the $N_2$-reduction site to be rapidly trapped at that site. If $N_3^-$ and $N_2$ both bind to an identical site and if $N_3^-$ reduction occurs as shown in Scheme III, then the $N_2$ formed should be within about 2 Å of the site, when the site is vacated by $NH_3$ (Hardy, 1979). The data presented here, however, cannot be used to establish definitively whether or not $N_3^-$ and $HN_3$ (or $N_2$) bind to the same or different sites. Both reactions are inhibited by $N_2$ and $C_2H_2$ (although to different extents) which might suggest a common site. On the other hand, $N_3^-$ is the only known anionic substrate for nitrogenase, and $HN_3$ and $N_3^-$ do not appear to inhibit the reduction of each other which might suggest different sites exist for the two forms. Previous studies of the effects of azide on cyanide and $CH_3NC$ reduction systems also suggest different sites, an interpretation we currently prefer (Li et al., 1982; Rubinson et al., 1983). If two sites are involved, however, they must be close enough to each other to allow the $N_2$ formed from $N_3^-$ reduction to bind to the $N_2$-reduction site.

An equally important point is whether or not the $N_2$-reduction site, at the time of $N_2$ release following $N_3^-$ reduction, is in a redox state that would allow $N_2$ to bind and be reduced. The component protein ratio titration data (Figure 9) show that $N_3^-$ reduction to $N_2$ peaks at a ratio intermediate between $HN_3$ reduction and $H_2$ evolution (or excess $NH_3$). This observation suggests that $N_3^-$ is reactive toward a state of the enzyme more reduced than that reactive toward $HN_3$ reduction. The simplest explanation, which is consistent with all the avilable data, is that $N_3^-$ can bind to several different redox states of the enzyme at least one of which is the state reactive toward $N_2$ reduction. For example, if $N_3^-$ were to bind to the resting state of the enzyme ($E^0$ in Scheme III), then the enzyme would return to that state following the two-electron reduction to yield $N_2 + NH_3$. If the $N_2$ could not bind to that state, it would be released to the atmosphere to give the $N_2$ observed during azide reduction experiments. If, however, $N_3^-$ were to bind to a more reduced state of the enzyme [e.g., $E^{-3}$ (Thorneley & Lowe, 1982)], it would also return to that state upon release of $N_2 + NH_3$. In this case, the enzyme would be at the appropriate redox state to bind and subsequently reduce the $N_2$ formed. The binding of $N_3^-$ to that state might occur with concomitant $H_2$ evolution (see below) as has been suggested for $N_2$ binding (Thorneley & Lowe, 1982). The important point here is that the $N_2$-reduction site must already be in the appropriate redox state when $N_2$ is evolved from $N_3^-$ reduction as it is unlikely that the $N_2$ would remain trapped at the site, while the enzyme goes through additional, slow, catalytic cycles involving Fe protein/MoFe protein dissociation (Thorneley & Lowe, 1983).

*Inhibition of $H_2$ Evolution.* In 1969, Hadfield and Bulen, using *Av* nitrogenase, found that $H_2$ evolution could not be eliminated during $N_2$ reduction. This observation was later confirmed by a number of studies, which attempted to eliminate $H_2$ evolution by extrapolation of $N_2$ concentration dependence data (Rivera-Ortiz & Burris, 1975) or by direct measurements of the $H_2$ evolved to $N_2$ reduced ratio at extremely high (6–50 atm) $N_2$ concentrations (Bulen, 1976; Simpson & Burris, 1984). Others have presented data showing that the ratio of $H_2$ evolved to $N_2$ reduced varies with Fe protein/MoFe protein ratio [e.g., see Wherland et al. (1981) and Guth & Burris (1983)], the pH (Hageman & Burris, 1980), and the ADP/ATP ratio (Mortenson & Upchurch, 1981). None of these reports, however, demonstrated that $H_2$ evolution can ever be completely eliminated during $N_2$ reduction. Thus, it is generally accepted that, during $N_2$ reduction catalyzed by nitrogenase, some electrons will always be lost to $H_2$ evolution. For a number of alternative substrates, for example, $C_2H_2$ (Rivera-Ortiz & Burris, 1975), HCN (Li et al., 1982), and $CH_3NC$ (Rubinson et al., 1983), $H_2$ evolution can be eliminated completely. Thus, $N_2$ is sometimes considered to be unique among nitrogenase substrates in its inability to eliminate the $H_2$-evolution reaction.

Dilworth & Thorneley (1981) demonstrated that, at physiological pH values, azide could not completely eliminate $H_2$ evolution, with about 75% of the electrons being used to reduce azide and the remainder going to $H_2$ evolution. They further suggested, however, at high pH (8.2) and infinite concentration of azide, that unlike $N_2$, azide should be able to suppress $H_2$ evolution completely. That study did not measure the products of azide reduction but rather $\Delta H_2$ and assumed that no inhibition of nitrogenase turnover occurs with increasing azide concentration at pH 8.2. The data in Figure 1 and previous studies (Ljones, 1973) question the validity of that assumption. The data shown in Figure 8 clearly demonstrates that the ability of infinite concentration of azide to compete with $H_2$ evolution is independent of pH. Our extrapolation of azide concentration dependence data and actual measurements at extremely high (25 mM) azide concentrations demonstrate that $H_2$ evolution cannot be eliminated by azide with about 35% of the electrons still going to $H_2$ evolution at $Av2/Av1 = 8$ (Figure 8) and 25% at $Av2/Av1 = 1.5$ (Figure 3 of supplementary material). Whatever the reason for this phenomenon, the observation is in dramatic contrast to the situation with the alternative substrates $C_2H_2$, HCN, and $CH_3NC$ while quite similar to observations for the $N_2$-fixation reaction.

For the $N_2$-fixation reaction, Hadfield & Bulen (1969) interpreted their observation as evidence that $H_2$ evolution was, in some way, an intimate part of the chemical mechanism of $N_2$ reduction. Expanding on this concept, Newton et al. (1976) suggested that the minimum stoichiometry of the reaction might be one $H_2$ evolved per $N_2$ reduced. Rivera-Ortiz & Burris (1975) give values of 0.56–0.9 $H_2$ evolved per $N_2$ reduced by extrapolation to infinite $N_2$, while Simpson & Burris (1984) most recently reported values of 0.99–0.73 at 50 atm of $N_2$. Others have reported values of ~1:1 (Bulen, 1976; Mortenson & Upchurch, 1981). Although published values could be used to argue for or against a minimum 1:1 stoichiometry, some proposed mechanisms for $N_2$ reduction include an obligatory $H_2$ evolution step [e.g., see Thorneley & Lowe (1982) and Guth & Burris (1984)]. It is of interest, therefore, to determine the ratio of $H_2$ evolved to azide reduced for each of the azide reduction pathways.

Table III gives a compilation of the $V_{max}$ values, shown under Results, for the individual reductions of $HN_3$, $N_3^-$, $N_2$, and $H^+$ during azide-reduction experiments and the theoretical numbers of molecules of substrate reduced that those $V_{max}$ values represent. This information was used to construct Table IV, which gives the ratio of $H_2$ evolved per azide reduced for the various azide-reduction reactions. It should be noted that, if the chemical mechanism of any pathway of azide reduction requires $H_2$ (as has been suggested for $N_2$ reduction; Newton et al., 1976; Thorneley & Lowe, 1982), then a stoichiometry

Table III: $V_{max}$ Values and Theoretical Numbers of Molecules of Substrate Reduced That Those $V_{max}$ Values Represent

| reaction no. | reaction[c] | $V_{max}$ (% e), $Av2/Av1 = 8$ | molecules[a] reduced, $Av2/Av1 = 8$ | $V_{max}$ (% e), $Av2/Av1 = 1.5$ | molecule[a] reduced, $Av2/Av1 = 1.5$ |
|---|---|---|---|---|---|
| 1 | $HN_3 \xrightarrow{6} N_2H_4 + NH_3$ | 25 | 8.3 | 40 | 13.3 |
| 2 | $N_3^- \xrightarrow{2} NH_3 + N_2 \xrightarrow{6} 2NH_3$ | 40 | | 35 | |
| 3 | $N_3^- \xrightarrow{2} N_2 + NH_3$ | 20 | 26.76[b] | 20 | 25[b] |
| 4 | $N_3^- \xrightarrow{8} 3NH_3$ | 20 | | 15 | |
| 5 | $N_2 \xrightarrow{6} 2NH_3$ | | 4.56[b] | | 3.3[b] |
| 6 | $2H^+ \xrightarrow{2} H_2$ | 35 | 35 | 25 | 25 |

[a] Assumes total electron pairs = 100. [b] Assumes all excess $NH_3$ (4) arises from reaction 2; thus, one-third of excess $NH_3$ must be added to reaction 3 with the remaining two-thirds going to reaction 5. [c] Numbers on arrows indicate number of electrons.

Table IV: Ratio of $H_2$ Evolved per Azide Reduced for the Various Azide-Reduction Reactions

| azide reactions[a] considered | $H_2$/azide reduced, $Av2/Av1 = 8$ | $H_2$/azide reduced, $Av2/Av1 = 1.5$ |
|---|---|---|
| reactions 1 + 3 + 5 | 0.89 | 0.60 |
| reactions 1 + 3 | 1.00 | 0.65 |
| reactions 3 + 5 | 1.12 | 0.88 |
| reaction 3 | 1.31 | 1.00 |

[a] From Table III.

significantly less than 1:1 should never be obtained regardless of reaction conditions (e.g., $Av2/Av1$ ratio). Thus, $H_2$ evolution cannot be a part of the chemical mechanism for all azide-reduction reactions, where a ratio of 0.6 is obtained at $Av2/Av1 = 1.5$ (Table IV). Likewise, $H_2$ evolution cannot be part of the chemical mechanism of both $HN_3$ reduction to $N_2H_4$ and $N_3^-$ reduction to $N_2$, where a ratio of 0.65 is obtained. As the $Av2/Av1$ ratio is lowered, the $V_{max}$ for $N_2H_4$ formation increases from 25% to 40% and the $V_{max}$ for the remaining $H_2$ evolution decreases from 35% to 25%. These observations indicate that of all azide reactions the six-electron reduction of $HN_3$ to $N_2H_4 + NH_3$ is least likely to require $H_2$ evolution as an intimate part of its chemical mechanism. The data in Table IV could be used to argue that the two-electron reduction of $N_3^-$ to $N_2 + NH_3$ does require $H_2$, where a 1:1 stoichiometry is observed at $Av2/Av1 = 1.5$. There are not enough molecules of that $N_2$ further reduced to give excess $NH_3$ to suggest whether or not $H_2$ evolution might be required. It should be noted that, if we only had the $Av2/Av1 = 8$ data available, the interpretation would have been different (Table IV) and that further variation in reaction conditions for either $N_3^-$ or $N_2$ might yield ratios significantly less than 1:1. It can be stated definitively, however, that unlike the alternative substrates $C_2H_2$, HCN, and $CH_3NC$ but like the physiological substrate $N_2$, $N_3^-$ is not a very effective inhibitor of $H_2$ evolution.

Similar careful examination of other substrates may well yield results that will further clarify the complexities of azide reduction. In addition, it is hoped that biophysical techniques may soon provide some direct evidence concerning the sites and states of interaction of $HN_3$, $N_3^-$, and other substrates and inhibitors on nitrogenase or the types of intermediates formed during $HN_3$ reduction.

## Conclusions

In summary, our studies have shown the following: (1) $HN_3$ is an extremely "good" substrate for nitrogenase ($K_m = 12\ \mu M$ at $Av2/Av1 = 8$) which is reduced by six electrons to give $N_2H_4 + NH_3$; (2) $HN_3$ reduction does not yield any lesser reduced products; (3) $HN_3$ appears to bind to and be reduced by a redox state of the enzyme more oxidized than that reactive toward $N_2$ reduction or $H_2$ evolution; (4) $HN_3$ is an effective inhibitor of $H_2$ evolution; (5) $N_2$ is a weak competitive inhibitor and CO is a noncompetitive inhibitor of $HN_3$ reduction by *Av* nitrogenase, and $C_2H_2$ inhibits $HN_3$ reduction, while $N_3^-$ and $D_2$ do not inhibit $HN_3$ reduction; (6) $N_3^-$ is the substrate (the only known anionic substrate) reduced by two electrons to yield $N_2 + NH_3$ (the only known nitrogenase reaction which requires an inequivalent number of protons and electrons); (7) $N_3^-$ is *not* an effective inhibitor of $H_2$ evolution and may bind to and be reduced by a number of different redox states of the enzyme; (8) $N_3^-$ reduction is inhibited by CO, $N_2$, and $C_2H_2$ but not by $HN_3$ or $D_2$ both of which cause an increase in the amount of $N_2$ observed; (9) the $N_2$ formed from $N_3^-$ reduction appears to be further reduced to give excess $NH_3$; (10) the $N_2$ formed, which is further reduced, is *not* in equilibrium with $N_2$ in the gas phase and thus must be produced in close proximity to the $N_2$-reduction site; (11) the formation of excess $NH_3$ is strongly inhibited by CO, $C_2H_2$, $HN_3$ and $D_2$; (12) previous reports of high concentrations of azide inhibiting total $NH_3$ formation (substrate self-inhibition) are due to $HN_3$ inhibition of excess $NH_3$ formation.

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## Supplementary Material Available

Ten tables showing all results in terms of number of replicates, means, and standard deviations and five figures (16 pages). Ordering information is given on any current masthead page.

**Registry No.** $HN_3$, 7782-79-8; azide, 14343-69-2; nitrogenase, 9013-04-1.

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