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Inhibition of Nitrogenase-Catalyzed NH_3 Formation by H_2[†]

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ABSTRACT: We have investigated the inhibition by H_2 (D_2) of NH_3 formation by nitrogenase from *Klebsiella pneumoniae* and have confirmed that the inhibition is competitive vs. N_2. D_2 inhibits NH_3 formation by diverting nitrogenase from production of NH_3 to production of HD (one electron per HD). By careful exclusion of N_2 from the reaction mixture, we have been able to place an upper limit on N_2-independent HD formation by nitrogenase, under 1 atm of D_2, at 1% of the total electron flux. Formation of NH_3 and formation of HD were inhibited identically by CO. We observed that as the ratio of dinitrogenase to dinitrogenase reductase is increased, the ratio of HD formed to NH_3 formed rises, and D_2 becomes a stronger inhibitor of N_2 reduction. This may be caused in part by an accompanying increase that is observed in the K_m of nitrogenase for N_2. We propose a model for D_2 inhibition of NH_3 formation in which D_2 and N_2 compete for the same form of nitrogenase. According to our proposal, when N_2 reacts with nitrogenase, either N_2 reduction proceeds to completion if H_2 (D_2) is absent or, if D_2 already is bound to nitrogenase, N_2 reduction is aborted and two molecules of HD are produced at the net expense of one electron per HD. Key consequences of the model are that it predicts that H_2 (D_2) is a competitive inhibitor of NH_3 formation and that the apparent $K_m(N_2)$ for formation of HD and NH_3 may differ.

Nitrogenase participates in several reactions involving H_2. In the absence of other substrates, nitrogenase catalyzes ATP- and reductant-dependent H_2 evolution from protons (Bulen et al., 1965). H_2 evolution in vitro cannot be completely suppressed by N_2 (Hadfield & Bulen, 1969), though the substrates C_2H_2 and HCN (Rivera-Ortiz & Burris, 1975) can completely suppress H_2 evolution in the extrapolated limit of infinite substrate concentration.

H_2 not only is a product of nitrogenase but also is an inhibitor of N_2 reduction by nitrogenase (Wilson & Umbreit, 1937). Hoch et al. (1960) observed the production of HD when N_2 fixation by soybean nodules was inhibited by D_2 (rather than H_2) and proposed that HD is formed when D_2 reacts with an enzyme-bound intermediate in N_2 reduction. Bulen (1976) combined these observations by proposing that inhibition of NH_3 formation by H_2 (D_2) and N_2-dependent HD formation from D_2 and H_2O are different manifestations of the same molecular process.

There is convincing experimental support for Bulen's proposal. First, H_2 inhibits N_2 reduction specifically, and HD formation is supported specifically by N_2. Thus, H_2 (D_2) inhibits reduction of N_2, but not of H^+ (Burns & Bulen, 1965), nitrous oxide (Hoch et al., 1960), azide, acetylene, cyanide, methylisonitrile (Hwang et al., 1972), or hydrazine (Burgess et al., 1981), all of which are substrates of nitrogenase. Correspondingly, HD formation from D_2 and H_2O is greatly enhanced by N_2 (Hoch et al., 1960) but not by N_2O (Hoch et al., 1960), azide, acetylene, cyanide, methylisonitrile (Jackson et al., 1968), or hydrazine (Newton et al., 1977). [It has been proposed (Wherland et al., 1981) and challenged (Li & Burris, 1983) that there is a low rate of HD formation during reduction of protons.] Second, several studies have led to the conclusion that HD formation is an electron-utilizing reaction, requiring one electron per HD formed. Under N_2, H_2 does not inhibit ATP hydrolysis or total product formation but redirects nitrogenase from production of NH_3 to increased production of H_2 (Hadfield & Bulen, 1969). Burgess et al. (1981) directly established that H_2 does not alter the total electron flux under N_2 by following the rate of dithionite oxidation in the presence and absence of H_2. These same electron allocation effects hold in the presence of D_2 only if the production of HD is allotted one electron per HD (Bulen, 1976; Newton et al., 1977). In addition, HD formation occurs almost entirely at the expense of NH_3 formation, and very few electrons, if any, are diverted by D_2 from H_2 evolution into HD production (Newton et al., 1977). Similarly, it is thought that H_2 inhibits NH_3 formation by causing production of H_2 in an N_2-dependent reaction without significantly affecting the other form of H_2 evolution, which occurs maximally in the absence of N_2.

Burgess et al. (1981) further ruled out the involvement of an exchange mechanism for HD production by demonstrating that the rate of incorporation of tritium from T_2 into the

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aqueous phase was only 2% of the rate of HD production in parallel reactions under D_2. Jackson et al. (1968) had demonstrated that there is no isotope effect in the inhibition of NH_3 formation by D_2 or H_2; there is presumably no significant difference in the rates of HT or HD formation.

A few reports do not support the equation of inhibition of NH_3 formation by H_2 (D_2) and N_2-dependent HD formation under D_2. For example, there was an early report that N_2 slightly suppressed HD formation (Kelly, 1968), and Li & Burris (1983) reported that an assignment of one electron per HD was not always required in their electron-balance studies. But the greatest weight of evidence strongly supports the proposal that D_2 inhibits NH_3 formation by diverting nitrogenase from NH_3 formation to HD formation without changing the total electron flux through nitrogenase.

There are three additional consistently observed features of this reaction which must be considered in the development of a detailed model of the inhibition of NH_3 by H_2 (D_2). First, H_2 is a competitive inhibitor (vs. N_2) of growth on N_2 (Wilson & Umbreit, 1937; Wyss & Wilson, 1941), of NH_3 formation by extracts of *Azotobacter vinelandii* (Strandberg & Wilson, 1967; Jackson et al., 1968), and of NH_3 formation by partially purified nitrogenase from *A. vinelandii* (Hwang et al., 1973; Rivera-Ortiz & Burris, 1975). Second, the apparent $K_m(N_2)$ is much lower for HD formation than for NH_3 formation (Turner & Bergersen, 1969; Bulen, 1976; Burris & Orme-Johnson, 1976; Newton et al., 1977; Burgess et al., 1981; Li & Burris, 1983). Third, HD formation is usually observed in reactions nominally free of N_2.

These observations have been compiled by Bulen (1976) and Newton et al. (1977) into a detailed model based on their work with nitrogenase from *A. vinelandii*. HD formation is hypothesized to occur when D_2 reacts with an enzyme-bound intermediate in N_2 fixation, resulting in the destruction of the intermediate, the production of two molecules of HD, and the net utilization of two electrons. Thus, reduction of N_2 (and no other substrate) is inhibited by H_2 (D_2), HD formation is dependent on N_2 (and no other substrate), and HD formation requires one electron per HD. This model was extended recently (Wherland et al., 1981; Burgess et al., 1981) to provide for a second, minor, N_2-*independent* route for the production of HD by nitrogenase. In this reaction, D_2 was postulated to divert to HD formation a fixed percentage of the electron flux normally allocated to H_2 (this was 9% under 1 atm of D_2). This postulated reaction effectively explained the HD formation usually seen in the putative absence of N_2 and could account for the difference in the apparent $K_m(N_2)$ observed for the formation of HD and NH_3.

One important feature of the inhibition of N_2 reduction by H_2 (D_2) not explained by this model, with or without the provision for an N_2-independent mode of HD formation, is that H_2 (D_2) is *competitive* vs. N_2. If free H_2 (D_2) intercepted an enzyme-bound intermediate, the inhibition would be expected to be noncompetitive vs. N_2. A second difficulty with this model has arisen with the work of Li & Burris (1983). By carefully scrubbing N_2 from their tank gases, and by performing their experiments in an all-glass system, they were able to reduce HD formation to 0–2% of the total electron flux with purified nitrogenase from *A. vinelandii* (1.86–2.25%), *Clostridium pasteurianum* (0–0.85%), and *Klebsiella pneumoniae* (0–2.7%). They concluded that N_2-independent HD formation is minimal if it exists at all.

Thus, there are serious difficulties with two important aspects of the model for inhibition of NH_3 formation by H_2 (D_2) as formulated by the Kettering group: the model incorrectly predicts that H_2 (D_2) is a noncompetitive inhibitor of NH_3 formation, and the key feature of N_2-independent HD formation has been challenged. We have developed an alternative model for the inhibition of NH_3 formation by H_2 (D_2) which predicts that H_2 (D_2) is competitive vs. N_2, does not require an N_2-independent pathway for HD formation to explain the differing pN_2 dependence of the formation of HD and NH_3, and can account for several additional features of this interesting reaction.

Materials and Methods

Nitrogenase. Growth of *Klebsiella pneumoniae* and purification of nitrogenase components were performed as described by Li & Burris (1983). When assayed under the conditions described below, the specific activity of Kp1[1] was 1650 nmol of C_2H_2 produced min^{-1} (mg of protein)$^{-1}$, and the specific activity of Kp2 was 1200–1500 nmol of C_2H_4 produced min^{-1} (mg of protein)$^{-1}$.

We assumed the molecular weights of Kp1 (219 000) and Kp2 (66 800) given by Eady (1980). We observed no hydrogenase activity (measured as ATP-independent H_2 evolution from dithionite and methyl viologen) in any of our purified nitrogenase preparations and can place an upper limit on hydrogenase-catalyzed H_2 evolution in our nitrogenase preparations at 0.5 nmol min^{-1} (mg of protein)$^{-1}$. We can place the same upper limit on ATP-dependent H_2 evolution from each component in the absence of the other.

Nitrogenase Assays. All reactions were carried out at 30 °C, with a shaking rate of 150 cycles/min, in 1.0 mL containing 5 μmol of ATP (Sigma Chemical Co.), 10 μmol of $MgCl_2$, 40 μmol of creatine phosphate (United States Biochemical Co.), 0.1 mg of creatine phosphokinase (EC 2.7.3.2, Sigma Chemical Co.), 20 μmol of $Na_2S_2O_4$ (Sigma Chemical Co.), 50 μmol of Mops (Sigma Chemical Co.) adjusted to pH 7.0, and nitrogenase proteins as indicated. Product formation was linear with time to at least 5 μmol of total two-electron-reduced products.

C_2H_2-reduction assays were performed in 22-mL bottles fitted with vaccine stoppers. The gas phase was 10% C_2H_2 (generated by addition of CaC_2 to H_2O) in Ar. Assays were initiated by addition of an anaerobic solution of ATP and were terminated by addition of 0.3 mL of 25% trichloroacetic acid. Gas samples (0.5 mL) were removed with 1.0-mL plastic syringes, and ethylene was measured on a Varian 600D gas chromatographc unit equipped with a flame ionization detector and a column of Porapak R (150 cm long × 2 mm i.d.) operated at 50 °C with N_2 as the carrier gas. O_2 was scrubbed from cylinder Ar and N_2 by passage over hot (150 °C) BASF catalyst R3-11 purchased from Chemical Dynamics Corp.

In our experiments, three products were formed: H_2, HD, and NH_3. All three products were measured in each reaction vessel. Two types of reaction vessel were used; each was advantageous for particular purposes. When N_2 was present in the gas phase, 9.5-mL bottles fitted with serum stoppers were used. The volume of the gas phase was 8.2 mL. Ar was added through a glass manifold fitted with 22-gauge needles; N_2 and D_2 were removed from storage vessels with mercury as the displacing fluid and were added to the serum bottles with plastic syringes. The pN_2 and pD_2 in kilopascals were calculated as 100 times the ratio of added gas volume to 8.2

[1] Abbreviations: Av1 and Kp1, dinitrogenase from *Azotobacter vinelandii* and *Klebsiella pneumoniae*, respectively; Av2 and Kp2, dinitrogenase reductase from *A. vinelandii* and *K. pneumoniae*, respectively; kPa, kilopascal(s) (100 kPa is equivalent to 750 torr); Mops, 3-(*N*-morpholino)propanesulfonic acid.

mL multiplied by the ratio of atmospheric pressure to 750 torr and multiplied by the ratio of 303 K to room temperature. pAr was calculated by applying these pressure and temperature corrections to the pAr as measured by a Hg manometer on the manifold. After addition of gases, $Na_2S_2O_4$ was added, and the bottles were incubated at 30 °C for 10 min to equilibrate the temperature and scavenge O_2 from the gas phase. Reactions were initiated by addition of nitrogenase and terminated by addition of 0.3 mL of trichloroacetic acid. If D_2 was not present in the gas phase, H_2 was determined in a 0.3-mL gas sample with a thermal conductivity detector after gas chromatography through a molecular sieve 5A column at 80 °C with Ar as the carrier gas. The gas chromatograph was calibrated by using cylinder H_2 diluted into Ar. If D_2 was present, H_2, HD, and D_2 were determined by injection of 0.3 mL of the gas sample into an MAT 250 isotope ratio mass spectrometer, and the gases were analyzed as described below. NH_3 then was determined in each reaction vessel as described below.

For experiments in which N_2 was rigorously excluded, we employed an apparatus which contained only copper tubing and glass. The copper tubing and glass were joined with epoxy. Calibrated, single side-arm Warburg flasks were employed as reaction vessels and were fitted to the manifold by connectors which comprised a stopcock, a glass joint for attachment to the manifold, and a glass joint for attachment to the Warburg flask. The reaction components, excluding $Na_2S_2O_4$ and nitrogenase, were added to the Warburg flask which then was attached by the connector to the manifold. The flasks were evacuated and refilled with Ar (scrubbed of trace O_2 but not of trace N_2) 3 times. With Ar flowing through the connector, the Warburg flask was removed about 2 cm directly below the connector, $Na_2S_2O_4$ was added to the reaction mixture, and nitrogenase was added to the side arm. The Warburg flask was returned to the connector and evacuated immediately. D_2 was then passed through a molecular sieve column at dry ice temperature and into the manifold [see Domine & Haÿ (1968) for adsorption properties of the molecular sieve column]. This D_2 was free of mass 28 and mass 29 but contained 0.3% of mass 30. D_2 was let into the manifold slowly to ensure complete exposure of the gas to the molecular sieve pellets. D_2 was added to atmospheric pressure (measured by a Hg manometer on the manifold) and evacuated twice. After the manifold and Warburg flasks were filled the third time, all stopcocks were closed. The Warburg flasks were left attached to the connectors which were removed from the manifold. The Warburg flasks were incubated at 30 °C for 10 min before the reactions were initiated by mixing the enzyme from the side arm with the reaction mixture. After the mixture was shaken at 30 °C, the reactions were terminated by immersing the Warburg flasks in a dry ice/acetone bath. H_2, HD, and D_2 were measured in the gas phase by attaching the connector to the mass spectrometer and letting the gas sample flow from the Warburg flask into the mass spectrometer by briefly opening the stopcock on the connector. Li & Burris (1983) reported that commercial cylinder gases may contain up to 2% N_2. Treatment with a molecular sieve at low temperature is effective in removing this N_2. However, such treatment may be pointless unless reactions are performed in an all-glass system, as substantial leakage and diffusion of air may occur through and around rubber stoppers and through punctures in rubber stoppers under vacuum.

After measurement of H_2, HD, and D_2 in the gas phase, as detailed below, 1 mL of 4.5 M K_2CO_3 was added to the reaction mixtures to initiate the microdiffusion of NH_3 to glass rods dipped in 1 N H_2SO_4 (Burris, 1972). After overnight microdiffusion, NH_3 was assayed by the method of Chaykin (1969). Protein was measured by the microbiuret method of Goa (1953).

Analysis of H_2 and HD by the MAT 250 Isotope Ratio Mass Spectrometer. Masses 2, 3, and 4 were measured sequentially on the same detector by manually adjusting the accelerating voltage. In the absence of added gas sample, there was a negligible D_2 background, a small but measurable HD background, and a substantial H_2 background. The size of these background values was dependent somewhat on the gas pressure in the sample chamber. For example, addition of 0.3 mL of air or Ar into the sample chamber raised the apparent H_2 background by about 10%.

The procedure for measuring gas samples taken from serum bottles differed slightly from the procedure used for measuring gas samples from the Warburg flasks. For the serum bottles, 0.3 mL of the gas phase was removed with a plastic syringe and injected into the sample chamber of the mass spectrometer. After the signal stabilized, the voltage outputs for the D_2, HD, and H_2 peaks were read sequentially from the digital display on the machine. The sample chamber was evacuated, 0.3 mL of air was injected, and the H_2, HD, and D_2 backgrounds were measured. These background levels were subtracted from the sample peaks. Multiple measurements of the isotope ratios differed by less than 1% and depended on the sample size (about 0.3 mL) only in that the background value of H_2 was slightly dependent on the sample size.

In determining isotope ratios in gas samples from the Warburg flasks, we recognized that the volume between the Warburg flask and the stopcock on the connector probably did not mix with the gas phase of the Warburg flask. Though this volume was only 3–5% of the volume of the Warburg flasks, the first aliquot of gas let into the mass spectrometer was not analyzed but was evacuated. A second sample of the gas phase was let into the sample chamber, and the D_2, HD, and H_2 peaks were measured, the sample chamber was evacuated, and the background values were measured. When subsequent gas samples were measured, the gas pressure in the sample chamber was adjusted to the *same value* as for the first sample by measuring the D_2 peak (the volume of the sample chamber was variable by means of a bellows). The H_2, HD, and D_2 peaks were measured, the sample was evacuated, and the backgrounds were measured, as for the first sample. Thus, for these samples, the apparent percent of H_2 was not accurate because the true background is not known, but the *change* in the H_2 percentage between reaction vessels, caused by H_2 production, could be measured quite accurately.

Calculation of Nanomoles of H_2 and HD Evolved in the Presence of D_2. D_2 (Matheson) contained 1–2% each of H_2 and HD. In 8.2 mL of gas containing 50 kPa of this D_2, there are 1500–3000 nmol of H_2 and of HD; any production of H_2 and HD must be measured above this contaminating level. The total nanomoles of hydrogen present in a gas sample was calculated from the volume of "D_2" added to the reaction vessel, corrected for the room temperature and atmospheric pressure on that day. The nanomoles of H_2, HD, and D_2 present then was calculated from the ratios of the three species as measured mass spectrometrically. Since the H in H_2 and HD evolved by nitrogenase is from H_2O, the total hydrogen species is not constant; this can be significant at low pD_2. However, total D ($D_2 + {}^1/_2HD$) *is* constant and was therefore used as a reference quantity. Thus, we calculated the nanomoles of H_2 produced by comparing the $H_2/({}^1/_2HD + D_2)$ ratio in the initial gas phase to the $H_2/({}^1/_2HD + D_2)$ ratio

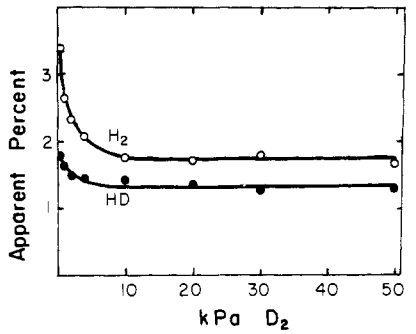

FIGURE 1: Apparent percentage of HD and H_2 contaminating tank D_2 vs. pD_2. Serum bottles (9.5 mL) were evacuated and filled with D_2 and Ar to a total pressure of 100 kPa. The partial pressure of Matheson-supplied D_2 was varied as shown. A 0.3-mL gas sample was removed from each bottle, and the H_2, HD, and D_2 peaks were measured as described under Materials and Methods. The apparent percentages of H_2 (○) and HD (●) were calculated after subtracting the H_2, HD, and D_2 backgrounds and were not corrected for the differences in sensitivity to each isotope.

after the reaction. The same procedure was followed for HD measurements. We assumed that all H_2 evolution was from H_2O while in fact a small fraction of the H_2 formed was due to contaminating HD and H_2 reacting in place of D_2. This H_2 evolution was presumably about 2–3% of the level of HD formation and was not important for interpretation of our results.

Our mass spectrometer does not give identical signal output for each hydrogen isotope. The machine was calibrated for H_2 by injecting a known amount of H_2 into a measured volume of D_2. Simple comparison of isotope ratios yielded a 17.5% overestimate of the added H_2; this correction was incorporated into our calculations. A similar analysis was not performed for HD, and we note that our HD measurements may be overestimated by as much as 10%.

Li & Burris (1983) showed that mass spectrometrically measured N_2/Ar ratios were underestimated at very low ratios. This was attributed to the suppression of the minor gas signal by the bulk gas. We have found the same phenomenon to hold when D_2 is the minor gas with either N_2 or Ar as the diluent gas. More importantly, the suppression of the three hydrogen isotopes is not uniform; this causes the apparent percentage of H_2 and HD to vary with the pressure of total hydrogen species present in the gas sample. Figure 1 shows that the apparent percentages H_2 and HD are constant as long as the total hydrogen species is over 10% of the total gas phase. We regarded 15 kPa in a 100-kPa gas phase an an operational lower limit for pD_2. Note: All raw data for experiments discussed in this paper are available from the authors upon request.

Results

HD Formation Requires One Electron per HD Formed. Convincing and diverse experimental evidence has been presented that HD formation by nitrogenase from *A. vinelandii* is a one-electron-requiring process. The results of our electron-balance studies with nitrogenase from *K. pneumoniae* (not shown) are consistent with this proposal. In the absence of D_2, 50 kPa of N_2 decreases the total electron flux by 13–17% below that observed under Ar alone. In several experiments, inhibition by N_2 averaged 10–15%. This is in accord with the observations of Li & Burris (1983), who used nitrogenase from *K. pneumoniae*, *C. pasteurianum*, and *A. vinelandii*, and the observations of Burgess et al. (1981) with *A. vinelandii* nitrogenase but differs significantly from the report by Hageman & Burris (1980) that N_2 caused a 35% inhibition of the total electron flux through nitrogenase from *A. vinelandii*. CO inhibits reduction by *A. vinelandii* nitrogenase of substrates other than H^+ (Hwang et al., 1973) and reverses the inhibition of total electron flux by N_2 (Hageman & Burris, 1980); we observe this to be true with the *K. pneumoniae* enzyme as well.

These effects on electron flux by N_2 and CO are unchanged by the presence of D_2, if production of HD is assumed to utilize one electron per HD. Thus, N_2 causes a 15–20% drop in the electron flux, and CO reverses the inhibition by N_2. If HD formation does not require one electron per HD, however, then N_2 would cause a 36% drop in the total electron flux, much larger than the 13–17% drop seen in the absence of D_2. In light of these results and the extensive work done with nitrogenase with *A. vinelandii* described in the introduction, we have included one electron per molecule of HD in all electron-balance studies reported in this paper.

N_2 Dependence of HD Formation. Li & Burris (1983) showed that HD formation could be reduced consistently to less than 1–2% of the electron flux if all gases used were scrubbed free of N_2 by passage over a molecular sieve, and if rubber-stoppered bottles were avoided. Using these methods, we consistently obtained HD formation rates as low as 1–2% of the total electron flux under 100 kPa of D_2 and occasionally observed no HD formation (not shown). When using rubber-stoppered serum bottles, however, we observed 5–10% of the electron flux as HD, even using scrubbed gases (Figure 3d,e, for example). We consider this HD formation to be N_2 dependent and supported by N_2 which leaks into the serum bottles.

Burgess et al. (1981) reported that 1% CO inhibited NH_3 formation by *A. vinelandii* nitrogenase completely but inhibited HD formation by only 89%. This observation was cited in support of the existence of an N_2-independent mechanism for HD formation. In contrast, working with nitrogenase from *Clostridium pasteurianum*, Li & Burris (1983) found that CO inhibited HD formation more strongly than NH_3 formation. We investigated the CO dependence of H_2, NH_3, and HD formation with *K. pneumoniae* nitrogenase; our results are illustrated in Figure 2. CO had no effect on the total electron flux but diverted nitrogenase from formation of HD and NH_3 to H_2 evolution. CO inhibited the formation of HD and NH_3 identically and essentially completely with a K_i of about 0.1 kPa (Figure 2, insert), which is similar to the strength of inhibition of HD formation observed by Burgess et al. (1981). A complete CO inhibition profile should be completed for the nitrogenase in question before concluding that CO differentially inhibits NH_3 formation and HD formation by nitrogenase from organisms other than *K. pneumoniae*. Throughout this study, we observed essentially no difference, with respect to their NH_3 and HD contents, between reactions in which ATP was added after the reaction mixtures were acidified and complete reaction mixtures containing 5 kPa of CO. Measurable quantities of NH_3 and HD were usually produced under 1 kPa of CO.

On the basis of the very low rates of HD formation obtained when N_2 is rigorously excluded from, or when 5 kPa of CO is included in, reaction vessels, we conclude that N_2-independent HD formation has a maximum rate under 100 kPa of D_2 of less than 1% of the electron flux allotted to H_2 and is probably nonexistent.

Effects of pN_2 and pD_2 on the Distribution of Products. Figure 3a–e illustrates the pN_2 dependence of formation of H_2, HD, and NH_3 and total electron flux, under 0, 15, 30, 50, and 70 kPa of D_2, respectively. These data are replotted in Figures 4–8, as discussed below. Generally, HD formation

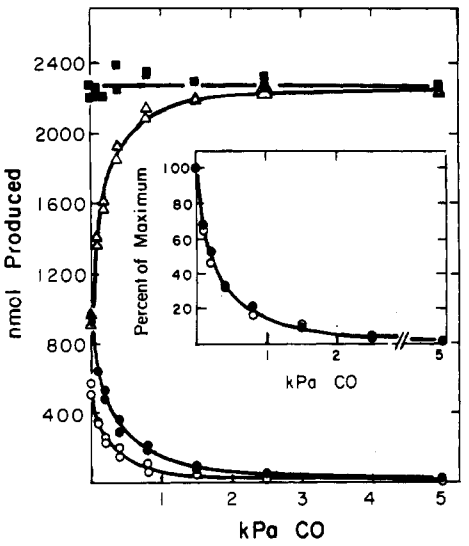

FIGURE 2: CO dependence of electron allocation to production of H_2, HD, and NH_3. Reactions were carried out in 9.5-mL seum bottles as described under Materials and Methods. The gas phase was made by injecting 4.1 mL of D_2 and CO as shown via syringe into bottles containing 50 kPa of N_2 supplied through a manifold. Reactions were initiated with 103 μg each of Kp1 and Kp2 and terminated after 15 min with trichloroacetic acid. NH_3 (○), HD (●), and H_2 (△) were assayed as described. The total electron flux (■) is expressed as the total nanomoles of electron pairs to products, and the production of HD (●) and NH_3 (○) is expressed in the insert as a percentage of the production of HD and NH_3 in the absence of CO.

increased with both pN_2 and pD_2, NH_3 formation was inhibited by D_2, and H_2 evolution was inhibited by N_2.

H_2 evolution was not affected by 15–70 kPa of D_2 at any pN_2 (Figure 4a). Similarly, electron allocation to HD and NH_3 was not affected by 15–70 kPa of D_2 at any pN_2 (Figure 4b). The data from Figures 3a and 4a,b are summarized in Figure 5 from which one can infer that D_2 between 0 and 70 kPa has no effect on the total electron flux or on the distribution of electrons by nitrogenase between H_2 and NH_3 plus HD. Thus, the partitioning of electrons between H_2 production and production of NH_3 plus HD is dependent only on the pN_2, while the distribution between NH_3 and HD at a given pN_2 is dependent on the pD_2. The results confirm the conclusions of Newton et al. (1977) and support the suggestions of Bulen (1976) that D_2 is a specific inhibitor of NH_3 formation and that N_2-dependent HD formation and inhibition of NH_3 formation by D_2 are different manifestations of the same molecular process.

Proposed Model for Inhibition of NH_3 Formation by D_2. To facilitate further analysis of our data, we introduce the model for HD formation by nitrogenase shown in Figure 6. We propose that D_2 and N_2 compete for the same form of nitrogenase, E. Because N_2O is a competitive inhibitor of N_2 fixation (Repaske & Wilson, 1952), but H_2 (D_2) does not inhibit N_2O reduction (Hoch et al., 1960), it is logical to propose that H_2 (D_2) binds to a site on nitrogenase that is distinct from the N_2 and N_2O binding site. When N_2 reacts with nitrogenase, either N_2 reduction proceeds to completion if H_2 (D_2) is absent or N_2 reduction is aborted with the concomitant production of H_2 (HD) if H_2 (D_2) is already bound to E. In our model, free H_2 (D_2) cannot react with an enzyme-bound intermediate, possibly because of steric or chemical restrictions in the active site of nitrogenase which can only be overcome if H_2 (D_2) binds to nitrogenase before N_2. Because H_2 (D_2) does not inhibit reduction of any substrate other than N_2, E and ED_2 must be equally capable of reducing all substrates except N_2. All HD formation is N_2 dependent. Our

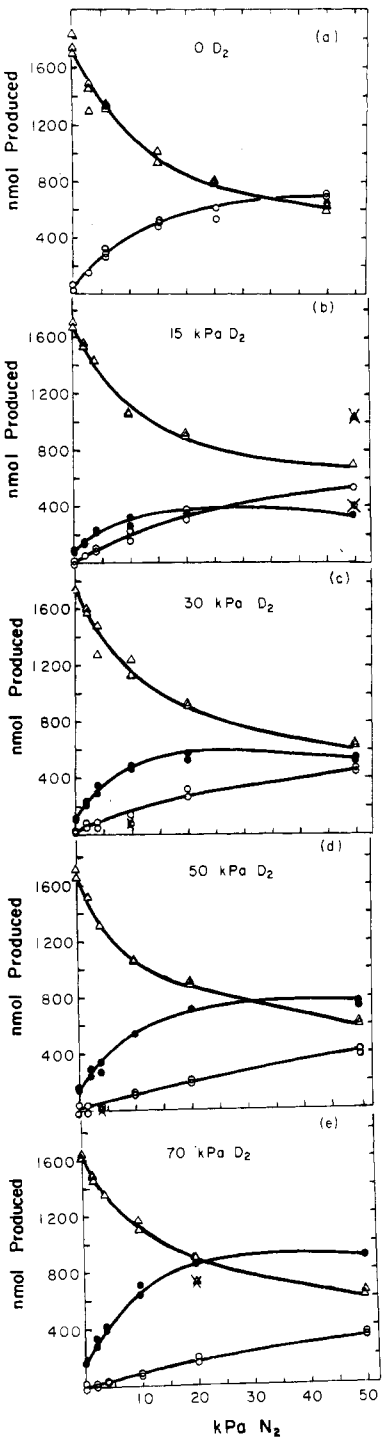

FIGURE 3: Dependence of H_2, HD, and NH_3 formation on pN_2 at different pD_2s. Reactions were performed in 9.5-mL serum bottles as described under Materials and Methods. Bottles were filled to 100 kPa of total pressure, except that one pair of bottles was filled to 120 kPa for Figure 3e. The pD_2s were 0 (a), 15 (b), 30 (c), 50 (d), or 70 kPa (e). Reactions were begun by the addition of 85 μg each of Kp1 and Kp2 and terminated after 12.5 min by addition of trichloroacetic acid. The experiment illustrated in Figure 3a was performed on a different day than those illustrated in Figure 3b–e and was normalized to Figure 3b–e by multiplying *all* points by a factor of 1.13. H_2 (△), HD (●), and NH_3 (○) were measured for each bottle as described. Data points crossed out were not used in the replots shown in Figures 4–8.

proposal retains the ideas that H_2 (D_2) reacts with an intermediate in N_2 fixation (Hoch et al., 1960) and that HD formation requires one electron per HD (Bulen, 1976); it merely suggests that H_2 (D_2) must somehow be activated by

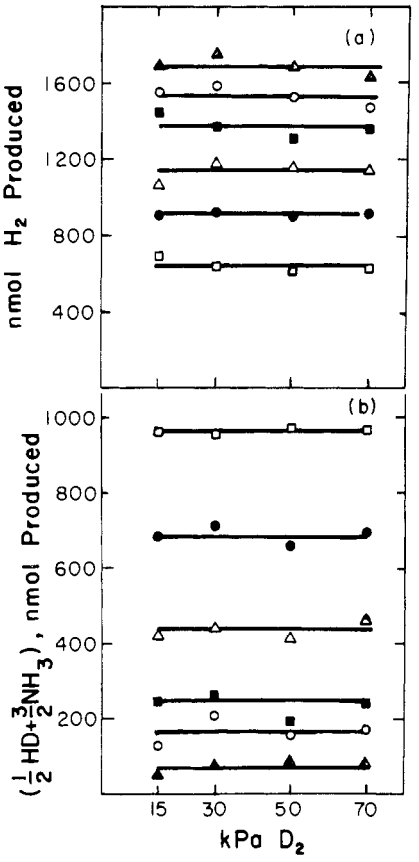

FIGURE 4: (a) Dependence of H_2 evolution on pD_2 and pN_2. Data were taken from Figure 3b–e. The average H_2 evolution is plotted vs. pD_2 at pN_2s of 0 (▲), 2 (○), 4 (■), 10 (△), 20 (●), and 50 kPa (□). (b) Dependence of total electrons in HD + NH_3 on pD_2 and pN_2. Data were taken from Figure 3b–e. The electrons partitioned to HD (one electron per HD) and NH_3 (three electrons per NH_3) were averaged and summed for each gas mixture, as shown. Symbols are as in Figure 4a.

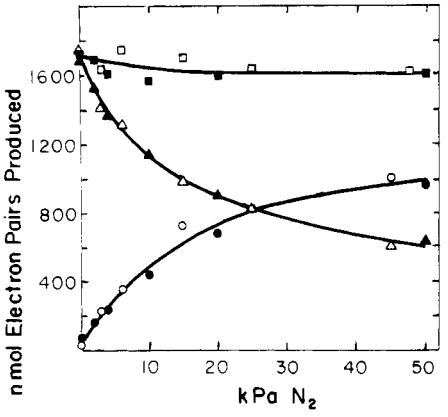

FIGURE 5: pN_2 dependence of total electron flux and of the allocation between H_2 evolution and production of HD + NH_3. Data were taken from Figure 3a–e. Plotted are electron pairs in NH_3 production (○), H_2 evolution (△), and total products (□) under 0 kPa of D_2 (data from Figure 3a). Also plotted are electron pairs in H_2 evolution (▲) averaged for all four pD_2s (Figure 4a), formation of NH_3 + HD (●) averaged for all four pD_2s, and the total electron pairs into products (■) averaged for all four pD_2s.

nitrogenase before formation of the intermediate with which it reacts.

This mechanism can be treated easily by the methods of Cleland (1963) to yield rate equations for the formation of NH_3 and HD (not shown). The rate equation for NH_3 formation predicts that D_2 is a competitive inhibitor of NH_3

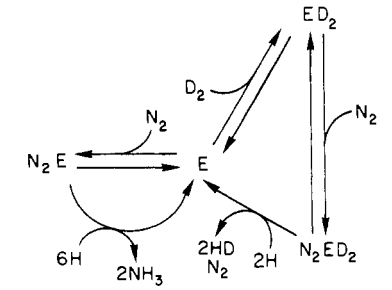

FIGURE 6: Model for inhibition of NH_3 formation by D_2.

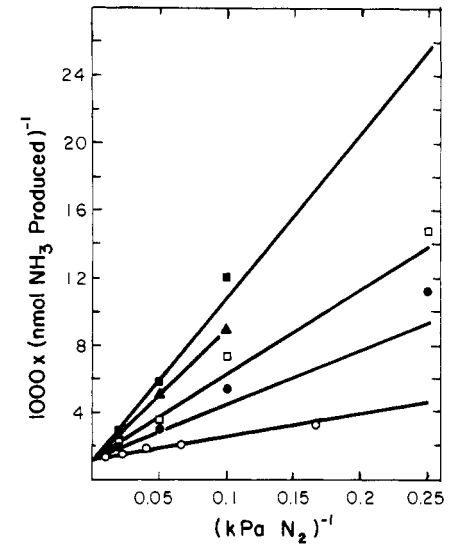

FIGURE 7: Competitive inhibition of NH_3 formation by D_2. Data were taken from Figure 3a–e and fitted to competitive inhibition by the COMPO program of W. W. Cleland. The lines are the computer-calculated fit to the data points as shown. D_2 pressures were 0 (○), 15 (●), 30 (□), 50 (▲), and 70 kPa (■). The $(pN_2)^{-1}$ values shown are calculated from the uncorrected pN_2s (see text). The point at 0.25 (kPa of $N_2)^{-1}$ and 70 kPa of D_2 (not shown) had a value of 0.037 (nmol of NH_3 produced)$^{-1}$ and was included in the data set. COMPO weights the high rates of product formation most strongly.

formation. A plot of (nanomoles of NH_3 produced)$^{-1}$ vs. $(pN_2)^{-1}$ at the five pD_2s is shown in Figure 7. D_2 is clearly a competitive inhibitor of NH_3 formation with purified nitrogenase from *K. pneumoniae*. A computer fit to the data (excluding data obtained at 0 and 2 kPa of N_2) provided estimates for the $K_m(N_2)$ of 11.7 ± 1.1 kPa and for the $K_{is}(D_2)$ of 11.5 ± 1.1 kPa. These data compare quite favorably with the $K_m(N_2)$ of 0.11 atm and the $K_{is}(D_2)$ of 0.112 atm reported by Hwang et al. (1973) for nitrogenase from *A. vinelandii*.

The rate equation for HD formation has exactly the same form as an ordered sequential reaction (D_2 adding first) with competitive substrate inhibition by the second substrate (N_2). When fitting our HD formation data to this model, we used the rates of HD production at both low and high pN_2s to calculate the inhibition constant for N_2. Since we obtained substantial HD formation in our "N_2-free" reactions (Figure 3b–e), some correction for pN_2 at low pN_2 was necessary. This correction was estimated by visually extrapolating the rate of HD formation to the abscissa in Figure 5. The pN_2 at the ordinate thereby was estimated to average 1.2 kPa. This was added to the intended pN_2 values to obtain the adjusted pN_2 values. The result of the computer fit of a sequential, ordered mechanism with competitive substrate inhibition to these adjusted HD formation data is shown in Figure 8.

Nine kinetic constants can be defined for this mechanism. The three constants defined for NH_3 formation are V_{max},

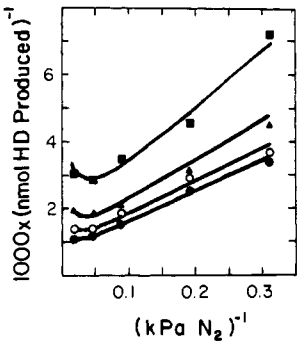

FIGURE 8: (HD formation)$^{-1}$ vs. $(pN_2)^{-1}$ at various pD_2s. The data were taken from Figure 3b–e and were fit to the SQCSINO program of W. W. Cleland. This program fit the data to an ordered, sequential mechanism (D_2 adding first) with competitive substrate inhibition by N_2. The lines are the computer fit to the data points, as shown. D_2 pressures were 15 (■), 30 (▲), 50 (○), and 70 kPa (●). N_2 pressures were corrected as described in the text.

Table I: Kinetic Constants Estimated by the Computer Fits to HD and NH_3 Formation As Shown in Figures 6 and 7[a]

NH_3 formation	HD formation
V_{max} = 849 ± 31 nmol/reaction	V_{max} = 2610 ± 350 nmol/reaction
$K_m(N_2)$ = 12.3 ± 1.5 kPa	$K_m(N_2)$ = 18.9 ± 4.3 kPa
	$K_i(N_2)$ = 13 ± 9 kPa
	$K_{is}(N_2)$ = 28 ± 26 kPa
$K_{is}(D_2)$ = 10.9 ± 1.3 kPa	$K_m(D_2)$ = 29 ± 16 kPa
	$K_i(D_2)$ = 20 ± 7 kPa

[a] All data were derived from Figure 3a–e. The computer programs were those of W. W. Cleland. NH_3 formation data were fit to competitive inhibition by D_2 (COMPO program), and the following kinetic constants were defined: $K_m(N_2)$, V_{max}, $K_{is}(D_2)$. Corrected pN_2's were used. HD formation data were fit to an ordered, sequential mechanism (D_2 adding first) with competitive substrate inhibition by N_2 (SQCSINO program). The following kinetic constants were defined: V_{max}, $K_m(N_2)$, $K_m(D_2)$, $K_{is}(N_2)$ (inhibition constant of N_2), and the dissociation constants of D_2 [$K_i(D_2)$] and N_2 [$K_i(N_2)$]. The K_m of each substrate was calculated by extrapolation to a very high concentration of the other substrate, while the dissociation constants were calculated by extrapolating to a very low concentration of the other substrate. Error values shown are 1 standard deviation. Corrected pN_2s were used.

$K_m(N_2)$, and $K_{is}(D_2)$. The six constants defined for HD formation are V_{max}, $K_m(D_2)$, $K_m(N_2)$, the dissociation constants $K_i(D_2)$ and $K_i(N_2)$, and a competitive inhibition constant for N_2, $K_{is}(N_2)$. The values of these constants derived from the computer fits to the NH_3 formation data and the HD formation data are compiled in Table I. We used the adjusted pN_2 values for these calculations on the NH_3 formation data, but this changed the results only slightly from those reported above by using the unadjusted pN_2 values.

Several interesting points arise from the calculations summarized in Table I. First, the V_{max} for NH_3 formation is the same as the V_{max} for HD formation (assuming HD formation is a one-electron process and NH_3 formation is a three-electron process). This means that under saturating N_2, nitrogenase can be completely diverted to HD formation by the presence of a very high pD_2 and that the rate of HD formation is limited by the same factor(s) that limit(s) the rate of NH_3 formation. Second, according to our model, the $K_m(N_2)$ for NH_3 formation (12.3 ± 1.5 kPa) is equivalent to, and should be numerically equal to, the $K_{is}(N_2)$ for HD formation (28 ± 26 kPa; Table I). Since we did not investigate low pD_2 levels in our experiments, the inhibition of HD formation by pN_2 was neither very pronounced (Figure 8) nor well-defined (Table I). Third, according to our model, $K_{is}(D_2)$ for NH_3 formation (11 ± 1.3 kPa) is equivalent to and should be numerically equal to the $K_i(D_2)$ for HD formation (20 ± 7 kPa). The $K_i(D_2)$ is calculated by an extrapolation to very dilute pN_2, and the fact that our data are not very reliable in this region probably accounts for this overestimate of $K_i(D_2)$. Fourth, the $K_m(N_2)$ for NH_3 formation (12.3 ± 1.5 kPa) is similar to the $K_m(N_2)$ for HD formation (18.9 ± 4.3 kPa). Our model does not force any particular relationship between the affinity of N_2 for E and ED_2, though Figure 4a,b shows that as increasing pD_2 shifts nitrogenase from E to ED_2, causing nitrogenase to shift from production of NH_3 to production of HD, the rate of electron flow to HD plus NH_3 does not change, even at nonsaturating pN_2. This indicates that E and ED_2 have the same affinity for N_2. Finally, for HD formation, the dissociation constants for N_2 and D_2 are quite similar to the respective Michaelis constants. This suggests that the dissociation rates of D_2 from ED_2 and of N_2 from N_2ED_2 are much faster than product formation.

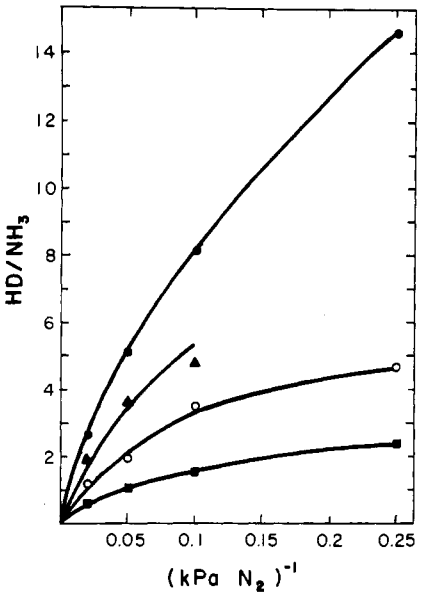

FIGURE 9: HD/NH_3 vs. $(pN_2)^{-1}$ at various pD_2s. HD/NH_3 ratios were calculated directly from the data presented in Figure 3b–e. N_2 pressures are uncorrected, and the lines were drawn visually. D_2 pressures were 15 (■), 30 (○), 50 (▲), and 70 kPa (●).

We also have derived a rate equation for the ratio of the rates of production of HD to NH_3 (not shown). Our model predicts that for a fixed pD_2, the HD/NH_3 ratio should approach 0 as $(pN_2)^{-1}$ approaches 0 and that the HD/NH_3 ratio should be independent of pN_2 at very low pN_2. The data, derived from Figure 3b–e, are shown in Figure 9 and conform to the predictions of our model. This is simply another manifestation of the competitive nature of the inhibition by D_2 and the consequent ability of high pN_2 to overcome the inhibition.

Effect of Component Ratio on Electron Partitioning. The effect of changing the Kp1/Kp2 ratio on the allocation of electrons to the formation of HD, NH_3, and H_2 is shown in Figure 10a. In this experiment, the Kp1 level was varied, but Kp2 was held constant. There was an increase in the total electron flux as the optimal Kp1/Kp2 ratio was reached and then a decrease in the total electron flux at higher Kp1 levels. This decrease in activity may have been due to inhibition of Kp2 by high Kp1 concentrations in spite of the high dithionite concentration [Hageman & Burris (1978) showed that dithionite can reverse the inhibition by excess dinitrogenase], or possibly to the increased NaCl content of the assays at high

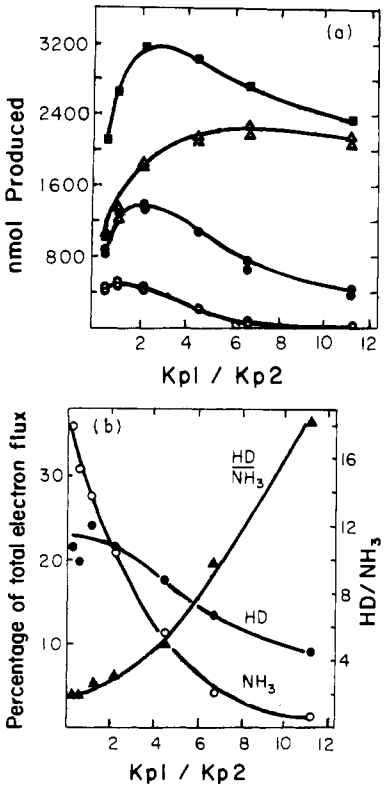

FIGURE 10: (a) Dependence on the Kpl/Kp2 ratio of the electron allocation to HD, H_2, and NH_3 production. Reactions were performed in 9.5-mL serum bottles as described under Materials and Methods except that the dithionite concentration was 40 mM. The gas phase was 50 kPa of D_2 and 50 kPa of N_2; 75 μg of Kp2 was included in all assays, and Kpl was varied as shown. Reactions were initiated by addition of ATP and were terminated after 20 min by addition of trichloroacetic acid. H_2 (△), HD (●), NH_3 (○), and total electron pairs into products (■) were assayed (or calculated) for each bottle as described earlier. (b) Dependence on the Kpl/Kp2 ratio of the percent of electron flux allocated to HD and NH_3, and of the HD/NH_3 ratio. Data were adapted from Figure 10a and expressed as the percentage of total electron flux allocated to HD (●) or to NH_3 (○) or as the ratios of nanomoles of HD produced to the nanomoles of NH_3 produced (▲) vs. the Kpl/Kp2 ratio. The points at the lowest Kpl/Kp2 ratios are derived from Figure 3d.

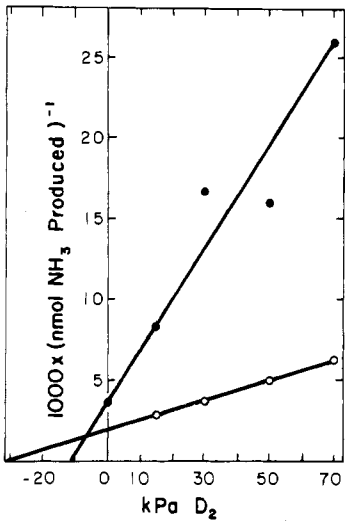

FIGURE 11: Inhibition of NH_3 formation by D_2 at two different Kpl/Kp2 ratios. Data for the Kpl/Kp2 ratio of 0.25 (○) were adapted from Figure 3b–e at a pN_2 of 20 kPa. Data for the Kpl/Kp2 ratio of 5.5 (●) were obtained from reactions performed in 9.5-mL bottles as described under Materials and Methods. The gas phase was 20 kPa of N_2, pD_2 was varied as shown, and the total pressure was 100 kPa; 33 μg of Kp2 and 595 μg of Kpl were incubated in each reaction bottle. Reactions were done in duplicate, initiated by the addition of ATP and terminated after 20 min by the addition of trichloroacetic acid. NH_3 was determined as described, and the average amounts of NH_3 formed at each pD_2 were used in the calculations. Lines were drawn visually.

Kpl levels (the NaCl content of the reaction mixtures was increased by 40 mM at the highest Kpl level shown). Figure 10a also shows that as the Kpl/Kp2 ratio increases, nitrogenase is diverted from production of NH_3 and HD to production of H_2. When the *percentage* of total electrons partitioned to each product is plotted vs. the Kpl/Kp2 ratio (Figure 10b), it becomes obvious that the rate of NH_3 formation is much more sensitive to the Kpl/Kp2 ratio than is the rate of HD formation. Thus, the HD/NH_3 ratio becomes very large at high Kpl/Kp2 ratios (Figure 10b). This was first observed by Wherland et al. (1981) with nitrogenase from *A. vinelandii*.

Because the results in Figure 10a,b were based on fixed-time assays, we considered the possibility that the measured ratio of HD/NH_3 formed at high Kpl/Kp2 may be due to the onset of a long lag in NH_3 formation but not in HD formation. Hageman & Burris (1980) observed a lag in NH_3 formation and a corresponding burst in H_2 evolution of about 1 min with a molar Avl/Av2 ratio of 5. We employed a molar Kpl/Kp2 ratio of about 5.5 which gave a measurable rate of NH_3 formation (Figure 10a) and an HD/NH_3 ratio of 6 (Figure 10b). Since the lag in NH_3 formation is presumably short compared to 15 min when the HD/NH_3 ratio is 2 (low Kpl/Kp2, Figure 10b), a tripling of the HD/NH_3 ratio in a 15-min fixed-time assay would require that there be a 10-min lag in NH_3 formation, but no lag in HD formation. In fact, we observed a lag in NH_3 and HD formation of at most 1 min and a burst of about 1 min in the rate of H_2 evolution (not shown). Therefore, the lag in NH_3 formation is not long enough to account for the increase of the HD/NH_3 ratio from 2 to 6.

We investigated further the effect of the component ratio in the electron allocation to the formation of H_2, HD, and NH_3 by examining the effect of pD_2 on the distribution of products at a molar Kpl/Kp2 ratio of about 5.5. We observed that D_2 inhibited NH_3 formation and enhanced HD formation but had no effect on the total electron flux to HD plus NH_3 (not shown). This was also true when the Kpl/Kp2 ratio was 0.25 (Figure 4a,b). In one experiment with a component ratio of 5.5 (not shown), we observed that H_2 evolution was inhibited by D_2, but Wherland et al. (1981) showed clearly that the total electron flux was not altered by the presence of D_2 at any Av2/Avl ratio. Thus, it seems that the observation that the distribution of products between H_2 and HD plus NH_3 is determined only by the pN_2 but the distribution between HD and NH_3 at a given pN_2 is determined by the pD_2 (Newton et al., 1977) holds for all component ratios. Therefore, the observation that increasing the Kpl/Kp2 ratio causes an increase in the HD/NH_3 ratio (Figure 10b) must mean that D_2 is a stronger inhibitor of NH_3 formation at high Kpl/Kp2 than at low Kpl/Kp2. Figure 11 illustrates the effect of pD_2 on NH_3 formation at two different Kpl/Kp2 ratios but at the same pN_2. D_2 is indeed a stronger inhibitor of NH_3 formation at the higher Kpl/Kp2, and this is why the HD/NH_3 ratio at a given pD_2/pN_2 increases with increasing Kpl/Kp2 (Figure 10b).

How might the component ratio influence the ability of D_2 to inhibit NH_3 formation? Since D_2 is a competitive inhibitor of NH_3 formation, a decrease in the affinity of nitrogenase for N_2 relative to its affinity for D_2 as Kpl/Kp2 increased would cause D_2 to become a stronger inhibitor. We found,

in fact, that the $K_m(N_2)$ for NH_3 formation is 26.8 ± 3 kPa with a Kp1/Kp2 ratio of 5.5 (not shown) whereas the $K_m(N_2)$ with a Kp1/Kp2 ratio of 0.25 is only 12.3 kPa (Table I).

When the data shown in Figure 3a are plotted as H_2 evolved per N_2 fixed vs. $(pN_2)^{-1}$ (not shown), we observe an extrapolated minimum value of 1.0 H_2 evolved per N_2 fixed, as was first reported by Hwang et al. (1973). Interestingly, when a similar plot is made from the data used above to estimate the $K_m(N_2)$ with a component ratio of 5.5, the minimum extrapolated value was 2.4 H_2's evolved per N_2 fixed (not shown). The slopes of the two plots also differed by a factor of 2.4, as expected from the observed difference in $K_m(N_2)$ for NH_3 formation (27 vs. 12 kPa). Thus, varying the component ratio changes the $K_m(N_2)$ *and* the percentage of the total electron flux which can be allocated to NH_3 formation under saturating N_2.

Discussion

Our results support the suggestion of Bulen (1976) that the inhibition of NH_3 formation by H_2 (D_2) and N_2-dependent HD formation under D_2 occur by the same reaction. Our electron-balance studies support a one-electron per HD requirement for HD formation, and we have confirmed the findings of Newton et al. (1977) that D_2 diverts nitrogenase from NH_3 production to HD production without significantly affecting the distribution of products between H_2 and HD plus NH_3, or the total electron flux through nitrogenase (Figures 4 and 5).

Several proposals for the mechanism of HD formation by nitrogenase have been elaborated recently. Chatt (1980) proposed a mechanism based on a trihydride species in the active site of nitrogenase. This mechanism, however, conflicts with many reported observations. According to Chatt's mechanism, HD formation should be at the expense of H_2 evolution, not of NH_3 formation. This is clearly not the case (Newton et al., 1977; Wherland et al., 1981; Figures 4 and 5 of this paper). Second, as was pointed out by Burgess et al. (1981), a *maximum* of one HD formed per N_2 fixed is predicted by Chatt's mechanism. In fact, the HD/NH_3 ratio varies with experimental conditions, and values as high as 6 (Newton et al., 1977), 8 (Wherland et al., 1981), and 18 (Figure 10b, this paper) have been reported.

A very different proposal for the mechanism of inhibition of NH_3 formation by H_2 has been elaborated by the Kettering group (Bulen, 1976; Newton et al., 1977; Burgess et al., 1981). In this model, H_2 (D_2) interacts with an enzyme-bound intermediate in N_2 fixation and causes the destruction of the intermediate with the net utilization of two electrons to form two molecules of H_2 (HD). A second, N_2-independent mechanism for HD formation was postulated to account for several observations regarding HD formation (Wherland et al., 1981; Burgess et al., 1981). However, the work of Li & Burris (1983), supported by our findings, strongly challenges the existence of an N_2-independent mechanism for HD production. Without this N_2-independent mode of HD formation, the ability of the Kettering group's model to explain several observations becomes drastically limited, as will be described below.

In this paper, we have proposed a model for the inhibition of NH_3 formation by H_2 (D_2) which differs from the Kettering group's proposed mechanism in several respects (Figure 6). Our model predicts that H_2 (D_2) is a competitive inhibitor of NH_3 formation whereas the Kettering group's model, with or without the provision for an N_2-independent mechanism for HD production, predicts that H_2 (D_2) should be a noncompetitive inhibitor of NH_3 formation. The data (Wilson & Umbreit, 1937; Wyss & Wilson, 1941; Strandberg & Wilson, 1967; Jackson et al., 1968; Hwang et al., 1973; Rivera-Ortiz & Burris, 1975; Figure 7 of this paper) clearly show that H_2 (D_2) is competitive vs. N_2.

A second clear distinction exists between the two models with respect to the expected effect on the HD/NH_3 ratio of varying the pN_2 at a fixed pD_2. The Kettering model predicts that this ratio should be independent of pN_2, except at low pN_2 where the postulated N_2-independent mechanism might contribute substantially to the total HD formation, thereby causing the HD/NH_3 ratio to rise at low pN_2. Our model predicts that high pN_2 should suppress the HD/NH_3 ratio to 0 in the extrapolated limit of very high pN_2, because at high pressures N_2 can successfully compete for all of E thereby blocking formation of ED_2 and HD. In the extrapolated limit of very low pN_2, our model suggests that the partitioning of enzyme between E and ED_2 (between NH_3 and HD formation) is a function solely of the pD_2, and the HD/NH_3 ratio should be independent of pN_2. The data (Figure 9) conform to the predictions of our model. The data of Newton et al. (1977), when plotted in this manner, give results identical with ours. In our view, the statement that the HD/NH_3 ratio tends to be 0 in the extrapolated limit of very high pN_2 is equivalent to the statement that H_2 (D_2) is a competitive inhibitor of N_2 fixation.

As described, the apparent $K_m(N_2)$ for NH_3 formation is substantially higher than the apparent $K_m(N_2)$ for HD formation. The Kettering group's model *requires* the existence of N_2-independent HD formation to explain this observation. Our model predicts that the apparent $K_m(N_2)$ for formation of HD and NH_3 may differ without invoking an N_2-independent mechanism for HD formation. Thus, in the presence of a fixed pD_2, HD formation is enhanced as N_2 saturates ED_2 but is simultaneously inhibited as N_2 effectively competes for E. We contend that at any pD_2, extremely high pN_2s will completely inhibit HD formation (Figure 9) by preventing creation of ED_2. The pN_2 dependence of HD formation, with constant pD_2, ideally would show no HD formation under 0 pN_2, reach a peak at a pN_2 which depends on the pD_2, and then decrease to 0 at a very high pN_2. The apparent $K_m(N_2)$ for HD formation in experiments such as those reported in Figure 3 is much lower than the apparent $K_m(N_2)$ for NH_3 formation because the former reaction is strongly inhibited by high pN_2.

Though our model clearly differs from the Kettering group's model in several respects, some of our analyses cannot clearly distinguish between the two models. For HD formation, our model predicts ordered, sequential kinetics (D_2 adding first) with competitive substrate inhibition by N_2 (Figure 8). The Kettering model predicts a sequential-type pattern and could account for a slight inhibition of HD formation under high pN_2 (by inhibition of the N_2-independent reaction). If the pN_2/pD_2 ratio were made arbitrarily high, however, our model predicts complete inhibition by N_2 while the Kettering model predicts only partial inhibition.

Similarly, the expected relationships between the kinetic constants do not provide an unambiguous distinction between the two models. For example, our model predicts that the V_{max} values for HD formation and NH_3 formation (in terms of total electron flux) should be identical; the Kettering groups's model predicts either the same V_{max} (no N_2-independent HD formation) or a slightly higher V_{max} for HD formation (due to an N_2-independent pathway). Also, our model predicts that the dissociation constant for D_2 calculated from the HD formation data should be the same as the inhibition constant

for H_2 (D_2) measured by the NH_2 formation data; the Kettering model predicts that the two constants either must be the same (no N_2-independent HD formation) or can be the same if there are two reactions that produce HD which have the same dependence on pD_2.

In summation, our model accounts for competitive inhibition by H_2 (D_2) of NH_3 formation and the consequent complete inhibition of HD formation by high pN_2; the Kettering model cannot account for these observations. Our model can account for the apparent difference of the $K_m(N_2)$ for formation of HD and of NH_3 and for the slight inhibition of HD formation often observed at high pN_2; the Kettering model could only account for these observations if there existed substantial N_2-independent HD formation. Both models predict that the V_{max} values for HD formation and for NH_3 formation should be nearly the same, and both models can explain why the dissociation constant for H_2 (D_2) calculated from the HD formation data is nearly the same as the inhibition constant for H_2 (D_2) for NH_3 formation.

Role of the Component Ratio on the Distribution of Products. Wherland et al. (1981) reported that when the Av1/Av2 molar ratio was increased from 0.5 to 10 under a 50% D_2/40% N_2/10% Ar atmosphere, nitrogenase was diverted to H_2 evolution from HD formation (which decreased about 60%) and NH_3 formation (which decreased about 90%). They observed that the HD/NH_3 ratio rose at high Av1/Av2 ratios, thus indicating that formation of HD and formation of NH_3 are not affected identically by the component ratio. We observed very similar results with nitrogenase from *Klebsiella pneumoniae* (Figure 10b). In our experiment, increasing the Kp1/Kp2 ratio to about 11 caused the electron allocation to HD to decrease by 50%, the allocation to NH_3 to decrease by 95%, and the HD/NH_3 ratio to increase 9-fold to 18.

Wherland et al. (1981) also concluded that under a 50% D_2/50% Ar atmosphere the allocation of electrons to HD formation was unchanged by varying the component ratio (the data show a 50% increase at high Av1/Av2 ratios). This was taken to indicate that HD formation under a 50% D_2/50% Ar atmosphere (N_2-independent HD formation) responds differently to the component ratio than does N_2-dependent HD formation. If the HD formation observed by the Kettering group under a 50% D_2/50% Ar atmosphere was in fact due to the presence of trace amounts of N_2 in their reaction vials, we would expect the allocation of electrons to HD formation to decrease at high Av1/Av2 ratios rather than change very little (increase about 50%) as they observed (Wherland et al., 1981). We have no simple explanation for this apparent contradiction. The design of our experiments differed from those reported by Wherland et al. (1981) in several respects which may be important. In our experiment, the Kp2 concentration was kept constant, and Kp1 was varied. The total electron flux did not change drastically (Figure 10a); thus, a slight error in the determination of contaminating HD and H_2 in the D_2 would have had little effect on the apparent percentage of electron flux allocated to HD. This may be preferable to the method of Wherland et al. (1981) in which the total protein concentration was kept constant while the component ratio was varied. This forced the electron flux to decrease about 95% when Av1 was made to predominate in the reaction mixture, and so the amount of H_2 and HD formed may have been quite small compared to the contaminating H_2 and HD. Also, the Kettering group found that under conditions of a high Av1/Av2 ratio, the specific activity of Av2 decreased about 60%, indicating that substantial inhibition by Av1 of the total electron flux occurred in their experiments, while inhibition by high Kp1 was limited to 25% in our experiment (Figure 10a). Such inhibition is an extreme case of the electron allocation effects and should be minimized where possible by inclusion of excess reductant (Hageman & Burris, 1978).

Despite these contradictory results, the increase of the HD/NH_3 ratio at high dinitrogenase to dinitrogenase reductase ratios is a very interesting observation. We ruled out the possibility that the observed increase in the HD/NH_3 ratio could be accounted for by a much longer lag for NH_3 formation than for HD formation. We found that the electron flux to HD plus NH_3 is unchanged by varying the pD_2 at a Kp1/Kp2 ratio of 5.5 as well as at a Kp1/Kp2 ratio of 0.25 (Figure 4a,b). Thus, the increase of the HD/NH_3 ratio means that H_2 (D_2) is a stronger inhibitor of NH_3 formation at higher Kp1/Kp2 ratios. This is shown directly in Figure 11.

There are several ways to imagine that the component ratio could affect the strength of inhibition by H_2 (D_2). Perhaps at low flux rates (high dinitrogenase to dinitrogenase reductase ratios), a substantial amount of nitrogenase is in a form (oxidized?) to which H_2 (D_2) can bind but N_2 cannot. Or, perhaps the enzyme-bound intermediate with which H_2 (D_2) is postulated to react becomes long-lived enough that free H_2 (D_2) can react with it. Either of these possibilities would lead to stronger, and noncompetitive, inhibition by H_2 (D_2) of NH_3 formation at high dinitrogenase to dinitrogenase reductase ratios.

Alternatively, our model for inhibition of NH_3 formation by H_2 (D_2) suggests that the strength of inhibition by H_2 (D_2) could increase with increasing Kp1/Kp2 if the affinity of E for N_2 were to decrease relative to the affinity of E for D_2. The inhibition would remain competitive. There is no consensus on how the component ratio affects the affinity of nitrogenase for N_2. We found that the $K_m(N_2)$ for NH_3 formation increases from 12 kPa at a Kp1/Kp2 ratio of 0.25 to 27 kPa at a Kp1/Kp2 ratio of 5.5. Wherland et al. (1981) concluded indirectly that the $K_m(N_2)$ for NH_3 formation increases at higher Av1/Av2 ratios. In contrast, Hageman & Burris (1980) found that the $K_m(N_2)$ was variable with the Av1/Av2 ratio, and Bergersen & Turner (1973) found that the $K_m(N_2)$ of nitrogenase from soybean nodules was *decreased* by increasing the ratio of dinitrogenase to dinitrogenase reductase. Further study of the effect of the component ratio on the kinetic parameters of HD formation and the inhibition of NH_3 formation by H_2 (D_2) should prove valuable.

A comparative study of nitrogenase from different sources would prove interesting. For example, nitrogenase from *Clostridium pasteurianum*, when compared to the enzymes from *K. pneumoniae* and *A. vinelandii*, is weakly inhibited by H_2. Lockshin & Burris (1965) reported a $K_i(H_2)$ of about 0.5 atm for the *C. pasteurianum* enzyme whereas the $K_i(H_2)$ for the *A. vinelandii* enzyme is 0.2 atm (Jackson et al., 1968) and the $K_i(D_2)$ for the *K. pneumoniae* enzyme is about 11 kPa (Table I). In addition, Li & Burris (1983) reported that the *C. pasteurianum* enzyme supports a lower rate of HD formation than the other two enzymes and that HD formation was inhibited by high pN_2 most noticeably with the clostridial enyzme. Because the affinity for N_2 does not differ markedly between these nitrogenases (Strandberg & Wilson, 1967), our model for the inhibition of NH_3 formation by H_2 (D_2) suggests that the unusual behavior of the clostridial enzyme could occur if the affinity of clostridial nitrogenase for H_2 (D_2) is lower than that of the nitrogenases from *K. pneumoniae* and *A. vinelandii*. Elucidation of the reason for these differences

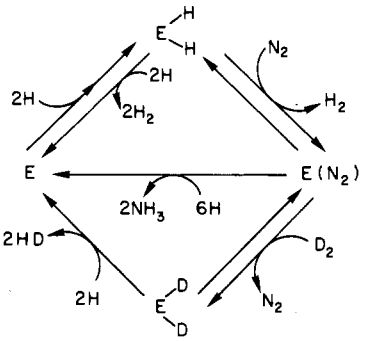

FIGURE 12: Mechanism of nitrogenase-catalyzed H_2 evolution, N_2 reduction, and inhibition of NH_3 formation by H_2 (D_2) as suggested by W. W. Cleland.

between the various nitrogenases should prove challenging and interesting.

It may be interesting to compare HD formation by intact *A. vinelandii* with HD formation supported by purified enzyme. The $K_m(N_2)$ for nitrogenase in vivo has been reported (Strandberg & Wilson, 1967) to be 10-fold lower than the $K_m(N_2)$ for NH_3 production by the purified enzyme (Hardy & Knight, 1967), which suggests that D_2 may be a weak inhibitor of the enzyme in vivo.

Is H_2 Evolution an Obligatory Part of the Chemical Mechanism of N_2 Fixation? ATP-dependent H_2 evolution from protons is catalyzed by nitrogenase in the absence of other reducible substrates but can be completely blocked by the substrates C_2H_2 and HCN (Rivera-Ortiz and Burris, 1975). In contrast, N_2 can only partially suppress H_2 evolution; at the extrapolated maximum of very high pN_2 the minimum ratio of H_2 evolved per N_2 fixed is one (Rivera-Ortiz & Burris, 1975). This has led to many proposals that H_2 evolution is an obligatory part of the chemical mechanism of N_2 reduction, thereby explaining why H_2 evolution decreases from 100% of the total electron flux in the absence of N_2 to 25% of the total electron flux under saturating N_2. Yet, we have observed that the extrapolated minimum ratio of H_2 evolved to N_2 fixed is dependent on the component ratio, and Hageman & Burris (1980) showed that the minimum extrapolated ratio is pH dependent. Thus, the ratio of H_2 evolved per N_2 fixed under saturating N_2 is variable, and the possibility remains that H_2 evolution from nitrogenase in the presence of saturating N_2 is essentially an unavoidable "leak" of low potential reducing equivalents into the aqueous environment and is not an obligatory part of the chemical mechanism of dinitrogen fixation.

The results of Newton et al. (1977), which we have confirmed in this report, are important in this regard. They found that as nitrogenase is diverted from production of NH_3 to production of HD by increasing the pD_2, neither the total electron flux to HD plus NH_3 nor the allocation of electrons to products (H_2 or HD plus NH_3) is changed. Since the V_{max} for HD formation is therefore the same as the V_{max} for NH_3 formation (Table I), nitrogenase theoretically can be completely diverted from NH_3 formation to HD formation, even under saturating N_2. Under such conditions (E is saturated by D_2, and ED_2 is saturated by N_2), 75% of the total electron flux will go to HD production and 25% to H_2 evolution. Thus, under optimal conditions and saturating N_2, nitrogenase forms one H_2 per two NH_3's (in the absence of D_2) or one H_2 per six HD's (in the presence of very high pD_2's). Therefore, any schemes for the mechanism of nitrogenase which envision that one H_2 is obligatorily formed during the transfer of six electrons to N_2 must also explain how one H_2 is obligatorily formed during the transfer of six electrons to HD. The possibility that each molecule of dinitrogenase produces either two molecules of NH_3 or six molecules of HD *at a time* must now be considered.

Further details on how electrons are distributed between production of NH_3 and HD may be obtained by pursuing the observations of Davis et al. (1975). They found that the presence of H_2 diverted nitrogenase from production of NH_3 to increased production of C_2H_4. If this experiment were performed with D_2, rather than with H_2, one could determine whether the increased C_2H_4 production is at the expense of the formation of NH_3, HD, or both. It may be, for example, that once N_2 reacts with ED_2 (in our model, Figure 6), an electron pool can be used to produce C_2H_4 as well as HD, but once N_2 reacts with E, C_2H_2 cannot be reduced. If so, the increased C_2H_2 reduction in the presence of D_2 would be at the expense of some HD formation. It is quite possible that further study of HD formation by nitrogenase will lead to new insights on the mechanism of nitrogenase action.

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Appendix

W. W. Cleland has suggested to us a very interesting proposal for the mechanism of HD formation by nitrogenase, which we present in Figure 12. In Cleland's model, H_2 is obligatorily released from nitrogenase upon the binding of N_2, thus accounting for the observed limiting stoichiometry of one H_2 evolved per N_2 fixed. This reaction is reversible in the presence of H_2 (and D_2). The presence of H_2 shifts the equilibrium between H–E–H and E(N_2) toward the former species and consequently shifts nitrogenase to the production of H_2 at the expense of N_2 fixation. Similarly, the presence of D_2 leads to the formation of D–E–D and to the production of HD, at the expense of N_2 fixation. This mechanism proposes that H_2 evolution, which is a mandatory feature of N_2 fixation, inhibition of N_2 fixation by H_2 (D_2), and HD formation under D_2 are all caused by the same reaction (or its reversal). It incorporates the ideas that HD formation is a one-electron-requiring process, is N_2 dependent, and is inhibited by high pN_2 (N_2 can react with D–E–D). The mechanism predicts that H_2 (D_2) is competitive vs. N_2 and that the apparent $K_m(N_2)$ values for production of HD and NH_3 may differ, and can account for the observed relationships between the kinetic constants (Table I).

In spite of these successful features of the model, there is a set of observations that the mechanism contradicts. According to this mechanism, as presented in Figure 12, one H_2 must be produced for every two HD's produced. Thus, the V_{max} for HD formation should be 50% of the total electron flux, which is only two-thirds that of the V_{max} for NH_3 formation. H_2 (D_2) therefore should enhance H_2 evolution and reduce the percentage of electron flux partitioned to HD plus NH_3. The data of Hadfield & Bulen (1969) and Newton et al. (1977) and Figures 4 and 5 of this paper contradict these expectations. Li & Burris (1983) did find that H_2 evolution was enhanced by D_2 though this was not at the expense of HD plus NH_3. There is, therefore, some disagreement over an observation which is crucial to the distribution between the model of Cleland and other models which have been presented. Cleland's model provides an interesting alternative to those

proposals which envision HD formation to be the direct result of the reaction of D_2 with an enyzme-bound intermediate in N_2 fixation and points out that there are several key features of nitrogenase action which may be accessible by the continued investigation of the mechanism of inhibition of N_2 fixation by H_2 (D_2).

Registry No. H_2, 1333-74-0; D_2, 7782-39-0; N_2, 7727-37-9; CO, 630-08-0; NH_3, 7664-41-7; HD, 13983-20-5; nitrogenase, 9013-04-1.

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