for factor IXaβ. A similar set of facts applies to a comparison of the $K_{m,app}$ of NPGB binding to factor Xa and APC, which leads to similarity in the $K_{m,app}$ for NPGB binding to each enzyme.

Registry No. APC, 42617-41-4; NPGB, 21658-26-4; Na, 7440-23-5; Cs, 7440-46-2.

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Effect of High pN_2 and High pD_2 on NH_3 Production, H_2 Evolution, and HD Formation by Nitrogenases†

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ABSTRACT: We have investigated the effect of the partial pressure of N_2 and D_2 on HD formation, H_2 evolution, and NH_3 production by nitrogenases from *Klebsiella pneumoniae* and *Clostridium pasteurianum*. By using pressures up to 4 atm, we have been able to extend the concentration range of N_2 and D_2 in our investigations beyond that used in previous studies. The pN_2 dependence of HD formation with constant pD_2 ideally shows no HD formation under zero pN_2, reaches a peak which depends on the pD_2, and then decreases to zero at very high pN_2. *K. pneumoniae* and *C. pasteurianum* nitrogenases differ in their $K_i(D_2)$ for nitrogen fixation. *C. pasteurianum* nitrogenase had the lower activity for formation of HD. With *K. pneumoniae* nitrogenase, D_2 enhanced H_2 evolution from 31% of the electron flux partitioned to H_2 in the absence of D_2 to 51% of the electron flux partitioned to H_2 at 400 kPa of D_2. With *C. pasteurianum* nitrogenase, the equivalent values were 33% and 48% of the total electron flux. Our results support the mechanism for nitrogenase-catalyzed reductions proposed by W. W. Cleland [Guth, J., & Burris, R. H. (1983) *Biochemistry 22*, 5111–5122].

Since Hoch et al. (1960) showed that soybean nodules produced HD when they were incubated with D_2 and N_2, HD formation has been used as a tool to provide information about the mechanism of nitrogen fixation. Hoch et al. proposed that HD is formed when D_2 reacts with an enzyme intermediate in N_2 reduction.

In the absence of other substrates, nitrogenases reduce protons to H_2 (Bulen et al., 1965); this H_2 evolution cannot be completely suppressed by N_2 although substrates such as C_2H_2 and HCN can completely suppress H_2 evolution at high substrate concentrations (Rivera-Ortiz & Burris, 1975; Simpson & Burris, 1984). Not only is H_2 a product of nitrogenase but it also is a specific inhibitor of N_2 reduction by nitrogenase (Wilson & Umbreit, 1937). Bulen (1976) combined these observations and proposed 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. As pointed out by Guth & Burris (1983) there is

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convincing experimental support for Bulen's proposal.

HD production is believed to require a single electron per molecule. Burgess et al. (1981a,b) demonstrated by following the rate of dithionite oxidation in the presence and absence of H_2 that H_2 does not alter the total electron flux under N_2. A constant electron allocation holds in the presence of D_2 only if the production of HD is allotted one electron per HD (Bulen, 1976; Newton et al., 1977). Burgess et al. (1981a,b) 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 water phase was only 2% of the rate of HD production in a parallel reaction under D_2. Jackson et al. (1968) had demonstrated no isotope effect on the inhibition of NH_3 formation by D_2 or H_2. The investigations of HD formation have led to several proposals for the mechanism of dinitrogen fixation (Bulen, 1976; Newton et al., 1977; Wherland et al., 1981; Burgess et al., 1981a,b; Li & Burris, 1983; Guth & Burris, 1983).

The model proposed by Wherland et al. (1981) and Burgess et al. (1981a,b) involves an N_2-independent route for the production of HD by nitrogenase. The work of Li & Burris (1983) and by Guth & Burris (1983) showed that N_2-independent HD formation is minimal if it exists at all. Guth & Burris (1983) also pointed out that the model proposed by Wherland et al. (1981) and Burgess et al. (1981a,b) incorrectly predicts that H_2 is a noncompetitive inhibitor of NH_3 formation, although several reports have shown that H_2 (D_2) is a competitive inhibitor of N_2 fixation (Wilson & Umbreit, 1937; Jackson et al., 1968; Hwang, et al., 1973; Rivera-Ortiz & Burris, 1975; Guth & Burris, 1983).

This work by Guth & Burris (1983) led to the proposal of two mechanisms of HD formation by nitrogenase, one suggested by the authors and another suggested by W. W. Cleland (Scheme I of this paper). Both models fit most of the results obtained, but one set of observations differed from the prediction from Cleland's mechanism. According to Cleland's mechanism, at least one H_2 must be produced for every two HD produced. Thus, the V_{max} for HD formation under saturating D_2 and N_2 should be 50% of the total electron flux, with the remaining 50% going to H_2 production (N_2 reduction blocked by D_2). D_2 should therefore enhance H_2 evolution from 25% of the total electron flux (at saturating pN_2 and zero pD_2) to 50% of the total electron flux (at saturating pN_2 and saturating pD_2). Note that only the *partitioning* of the electron flux is assumed to change with pD_2, whereas the total electron flux remains invariant. In contrast, the model of Guth and Burris envisions HD formation to be at the expense of NH_3 formation and to have the same V_{max} as NH_3 formation. Accordingly, in their model HD formation should reach 75% of the total electron flux under saturating N_2 and D_2, and H_2 evolution always should require 25% of the total electron flux, independent of the pD_2.

Newton et al. (1977) and Guth & Burris (1983) found H_2 formation to be independent of pD_2 in support of the model of Guth and Burris. On the other hand, Li & Burris (1983) found that H_2 evolution was enhanced by D_2; in contrast to others they observed an enhancement of the total electron flux by D_2. A careful verification is warranted to determine whether or not the total electron flux actually varies with the pD_2.

In this paper we have examined further the effect of the partial pressure of N_2 and D_2 on H_2 evolution, HD formation, and NH_3 production. By using pressures up to 4 atm, we have been able to extend the concentration range of N_2 and D_2 in our investigations beyond concentrations used in previous studies and have found rather unexpected results.

Materials and Methods

Growth of Bacteria and Purification of Dinitrogenase. *Klebsiella pneumoniae* M5aL was grown on the medium described by Eady et al. (1972) at 30 °C in 300-L batches in a nitrogen-sparged fermentor. The cells were harvested in the exponential phase by continuous centrifugation and were frozen and stored in liquid nitrogen. Nitrogenase was prepared from frozen cell paste as described by Li & Burris (1983). *Clostridium pasteurianum* nitrogenase was purified by Ji-lun Li as described by Li & Burris (1983).

The specific activity of Kp1[1] was 1600–2000 nmol of C_2H_4 formed min^{-1} (mg of Kp1)$^{-1}$. The specific activity of Kp2 was 1100–1400 nmol of C_2H_4 formed min^{-1} (mg of Kp2)$^{-1}$. The specific activity of Cp1 and Cp2 was 1100–1200 nmol of C_2H_4 formed min^{-1} (mg of Cp1)$^{-1}$ and 1900–2100 nmol of C_2H_4 formed min^{-1} (mg of Cp2)$^{-1}$, respectively. No hydrogenase activity (measured as ATP-independent H_2 evolution from dithionite and methylviologen) could be detected in the purified Kp nitrogenase preparations. Both Cp1 and Cp2 contained trace amounts of hydrogenase (about one part in 10^5 of the nitrogenase by weight, Li and Burris, 1983), which gave specific activities of 5 nmol of H_2 evolved min^{-1} (mg of Cp1)$^{-1}$ and 3 nmol of H_2 min^{-1} (mg Cp2)$^{-1}$, respectively. Reaction mixtures with enzymes but without ATP were used as controls in studying HD formation by Cp nitrogenase.

Unless otherwise stated, all experiments were performed with a fourfold molar excess of dinitrogenase reductase (Fe-protein; Kp2, Cp2) to dinitrogenase (MoFe-protein; Kp1, Cp1). We assumed the molecular weights of Kp1 (219 000), Kp2 (66 800), Cp1 (212 000), and Cp2 (57 650) given by Eady (1980).

Protein was measured by the microbiuret method as described by Goa (1953).

Nitrogenase Assays. Unless otherwise stated all reactions were carried out at 30 °C in 9-mL serum bottles fitted with serum stoppers. These standard serum bottles are heavy walled and retain several atmospheres pressure without difficulty. The 1-mL reaction mixture contained 5 μmol of ATP, 10 μmol of $MgCl_2$, 40 μmol of creatine phosphate, 0.1 mg of creatine phosphokinase (EC 2.7.3.2), 20 μmol of $Na_2S_2O_4$, 50 μmol of MOPS adjusted to pH 7.0 with KOH, and nitrogenase proteins as indicated.

In our experiments, three products were formed: H_2, HD, and NH_3. All three products were measured in each reaction vessel. The volume of the gas phase was 8.2 mL. Air was added through a glass manifold fitted with 22-gauge needles. N_2 and D_2 were removed from storage vessels and were added to the serum bottles with plastic syringes. Gas pressures were expressed as kilopascals (100 kPa = 750 mmHg or 750 torr). Pressures of gases were calculated from the volume of gases added to the 8.2-mL gas space of the reaction vessel. These pressures were corrected from room temperature to the reaction temperature of 303 K and from observed atmospheric pressure to pressure in the reaction vessel in kilopascals (e.g., 15 mL of N_2 at 295 K and corrected barometric pressure of 730 mmHg was added to the 8.2-mL gas phase [(15/8.2)(303/295)(730/750) 100 = 182.9 kPa of N_2]. pAr was

[1] Abbreviations: Kp1 and Cp1, dinitrogenase from *Klebsiella pneumoniae* and *Clostridium pasteurianum*, respectively; Kp2 and Cp2, dinitrogenase reductase from *K. pneumoniae* and *C. pasteurianum*, respectively; kPa, kilopascal(s) (100 kPa is equivalent to 750 torr); MOPS, 3-(*N*-morpholino)propanesulfonic acid.

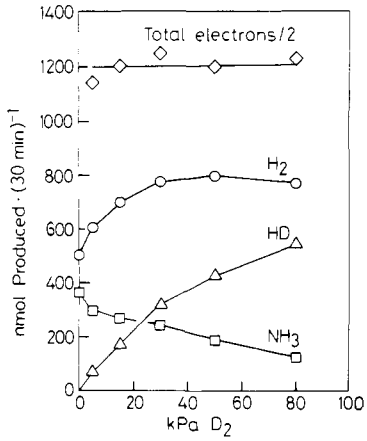


FIGURE 1: Effect of pD_2 on HD formation, H_2 evolution, NH_3 production, and total electron flux by nitrogenase from *Clostridium pasteurianum*. Total electron flux is expressed in electron pairs and was calculated as $HD/2 + 3NH_3/2 + H_2$. Reactions were performed in 9.5-mL serum bottles at 30 °C as described under Materials and Methods. Argon was used as diluent gas if the total pressure of D_2 and N_2 was below 100 kPa. Reactions were begun by addition of 100 μg each of Cp1 and Cp2 and terminated after 30 min by addition of trichloroacetic acid.

calculated by applying these pressure and temperature corrections to the pAr as measured by a Hg manometer when the vessels were filled with Ar on the manifold. If the total pressure was above 150 kPa, the stoppers were wired onto the bottles. The systems were gas tight, and there was no appreciable loss of gas by diffusion through the rubber stoppers during the rather short exposure times used. After addition of gases, $Na_2S_2O_4$ was added, and the bottles were incubated at 30 °C on a shaker for 5–10 min to equilibrate the temperature and to scavenge traces of O_2 from the gas phase. Reactions were initiated by addition of enzyme mixture and terminated by addition of 0.3 mL of 25% (w/v) trichloroacetic acid (TCA). Unless otherwise stated the reaction time was 30 min. Results with these techniques were reproducible.

If D_2 was not present in the gas phase, H_2 was determined in a 0.5-mL gas sample by gas chromatography. A thermal conductivity detector, a column of molecular sieve 5A, and Ar as carrier gas were used; the column temperature was 50 °C. The gas chromatograph was calibrated with cylinder H_2 diluted with argon. If D_2 was present, H_2, HD, and D_2 were determined by injection of 0.1–0.3 mL of gas into a MAT 250 isotope ratio mass spectrometer, and the gases were analyzed and calculated as described by Guth & Burris (1983). NH_3 then was determined in each reaction vessel as described below.

C_2H_2 Reduction. C_2H_2 reduction assays were performed in either 9- or 22-mL bottles fitted with serum and vaccine stoppers, respectively. The gas phase was 10% C_2H_2 in argon. C_2H_2 was produced by addition of CaC_2 to H_2O in a simple generator bottle as described by Burris (1974). Assays were initiated by addition of enzyme and terminated with 0.3 mL of 25% TCA. Gas samples (0.5 mL) were removed with 1.0-mL plastic syringes, and ethylene was measured on a Varian 600 D gas chromatographic unit equipped with flame ionization detector and a column of Porapak N, operated at 50 °C with N_2 as carrier gas.

Ammonia Determination. One milliliter of 4.5 M K_2CO_3 was added to the reaction mixtures to initiate the microdiffusion of NH_3 to glass rods previously dipped in 1 M H_2SO_4 (Burris, 1972). After overnight microdiffusion, NH_3 was assayed by the indophenol method of Chaykin (1969). A_{625} was measured after incubation at room temperature for 1 h. NH_4Cl was used as standard.

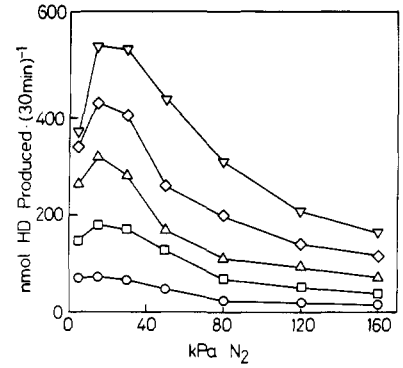


FIGURE 2: Dependence of HD formation by *Clostridium pasteurianum* nitrogenase on pN_2 at various pD_2. The results were obtained from experiments similar to that shown in Figure 1. D_2 pressures were 5 (○), 15 (□), 30 (△), 50 (◇), and 80 kPa (▽).

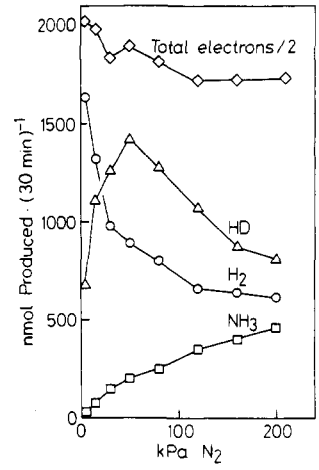


FIGURE 3: Effect of pN_2 on HD formation, H_2 evolution, NH_3 production, and total electron flux by nitrogenase from *Klebsiella pneumoniae*. Conditions were as described in the legend to Figure 1 and under Materials and Methods; 75 μg each of Kp1 and Kp2 was used.

RESULTS

Effect of pN_2 on HD Formation. Figure 1 shows a typical experiment in which the rates of H_2 evolution, HD formation, and NH_3 production by *C. pasteurianum* nitrogenase were measured as a function of pD_2 at a fixed value of pN_2 (15 kPa). D_2 had no effect on the total electron flux but diverted nitrogenase from production of NH_3 to formation of HD and H_2. The assumption is made in our calculations of the total electron flux that production of one molecule of HD requires one electron, whereas one molecule of NH_3 and H_2 require three and two electrons, respectively; this is logical, and evidence has been presented in its support (Burgess et al., 1981a,b; Guth & Burris, 1983).

Similar experiments to that shown in Figure 1 were made with values of pN_2 of 5, 15, 30, 50, 80, 120, and 160 kPa and with five different pD_2 values. The HD production rates are summarized in Figure 2. As the pN_2 increased at constant pD_2, HD formation increased, reached a peak, and then decreased again in response to further increase in the pN_2. Although the pattern of HD formation as a function of pN_2 seemed to the identical, the absolute amount of HD produced increased with increasing pD_2. The pN_2 at which the HD production reached its maximum also appeared to increase with increasing pD_2. To test this, further experiments were performed similar to the experiment illustrated by Figure 3. However, nitrogenase from *Klebsiella pneumoniae* was used, rather than nitrogenase from *C. pasteurianum*. the $K_i(H_2)$ for inhibition of NH_3 formation is smaller for Kp nitrogenase

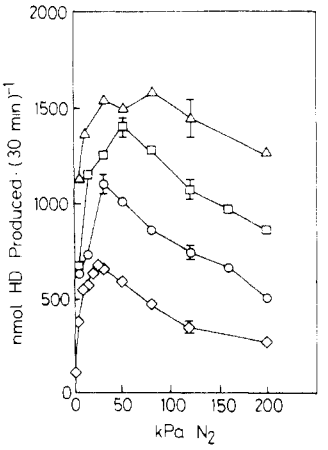


FIGURE 4: Dependence of HD formation by *Klebsiella pneumoniae* nitrogenase on pN_2 at various pD_2. The results were obtained from experiments similar to that shown in Figure 3. D_2 pressures were 25 (◇), 50 (○), 100 (□), and 200 kPa (△). Bars represent standard deviations.

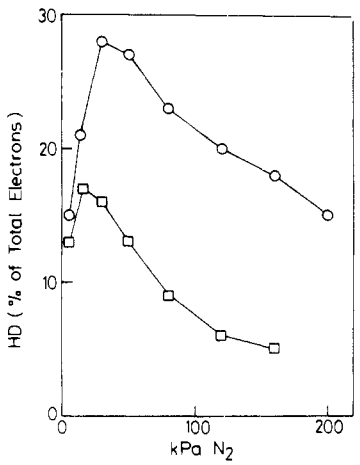


FIGURE 5: Effect of pN_2 on the percentage of the total electrons partitioned to HD by *Clostridium pasteurianum* (□) and *Klebsiella pneumoniae* (○) nitrogenase at 50 kPa of D_2. Results were obtained from experiments similar to those described in Figures 1 and 3.

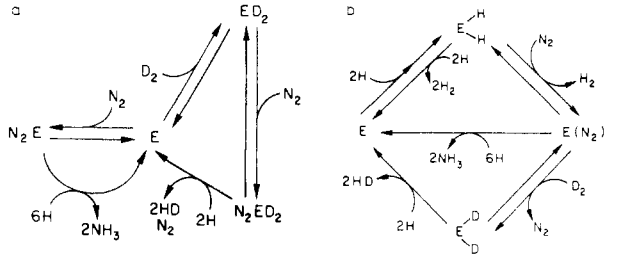
(10–15 kPa of D_2) than for Cp nitrogenase (30–35 kPa of D_2) (Li & Burris, 1983). This means that the pN_2 at which HD formation is expected to reach its maximum rate is greater for Kp nitrogenase than for Cp nitrogenase. The results of these experiments are shown in Figure 4. Although HD formation showed the same pattern of dependence on pN_2 as did Cp nitrogenase, at a fixed pD_2 the pN_2 at which HD formation reached its maximum rate was higher than the pN_2 found for Cp nitrogenase, as predicted.

Figure 5 shows that the percentage of the total electrons partitioned to HD by Cp nitrogenase at 50 kPa of D_2 was only about half of that found for Kp nitrogenase under the same conditions. The figure also shows that the pN_2 at which HD production by Cp nitrogenase reached its maximum was only half of that observed for Kp nitrogenase (15 and 30 kPa, respectively).

Replotting as a double-reciprocal (Lineweaver–Burk) plot, the data from the part of Figure 4 in which HD formation was decreased by a further increase in pN_2 showed that N_2 is a competitive substrate inhibitor of HD formation (data not shown).

A double-reciprocal plot of the results for NH_3 production from the experiments with Cp and Kp nitrogenases showed that D_2 in each case was a competitive inhibitor of NH_3 formation (data not shown). A plot of the slope of each

Scheme I: Models for Dinitrogenase-Catalyzed Reactions[a]



[a] (a) Mechanism of nitrogenase-catalyzed reductions as suggested by Guth and Burris. (b) Mechanism of dinitrogenase-catalyzed reductions as suggested by W. W. Cleland. Reprinted with permission from Guth & Burris (1983). Copyright 1983 American Chemical Society.

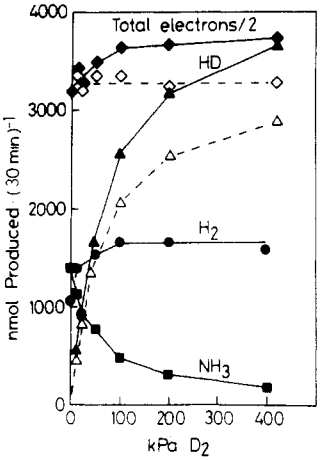


FIGURE 6: Effect of pD_2 on HD formation, H_2 evolution, NH_3 production, and total electron flux by nitrogenase from *Klebsiella pneumoniae*. Total electron flux is expressed as electron pairs and was calculated as $HD/2 + 3NH_3/2 + H_2$. Reactions were performed in 9.5-mL serum bottles at 30 °C as described under Materials and Methods. Argon was used as diluent gas if the total pressure of D_2 and N_2 was below 100 kPa. Reactions were initiated by addition of 100 μg of Kp1 and 200 μg of Kp2 and terminated after 30 min by addition of trichloroacetic acid. Since our mass spectrometer does not give identical signal output for each hydrogen isotope combination and since pure HD is not available, a calibration for this dihydrogen species could not be performed. The results are expressed in two ways; first, the HD measurements were plotted by assuming that the HD signal output was identical with that for D_2 (solid symbols) and, second, assuming that the HD signal output was identical with that for H_2 (open symbols). See the text for further details.

reciprocal plot vs. the corresponding inhibitor concentration at which it was obtained gave a straight line (data not shown). From these data the following constants were obtained: Cp nitrogenase, $K_m(N_2)$ = 7 kPa and $K_i(D_2)$ = 17 kPa; Kp nitrogenase, $K_m(N_2)$ = 10 kPa and $K_i(D_2)$ = 11 kPa.

Effect of pD_2 on H_2 Evolution. All of the data so far presented can be explained by either of the mechanisms (Scheme I) for nitrogenase-catalyzed reactions recorded by Guth & Burris (1983). But, as pointed out by Guth & Burris (1983), the two models differ with respect to the predicted effect of pD_2 on nitrogenase-catalyzed H_2 evolution. According to the mechanism suggested by the authors, changing the pD_2 should cause no change in the H_2 produced. On the other hand, the mechanism suggested by W. W. Cleland predicts that D_2 should enhance H_2 evolution from 25% of the electron flux in the absence of D_2 (at saturating pN_2, 75% of electron flux to NH_3 and 25% to H_2) to 50% of the electron flux at infinitely high pD_2 (no electron flux to NH_3, 50% to H_2 and 50% to HD). To distinguish between the two proposed models, the experiments shown in Figure 6 were performed. In these

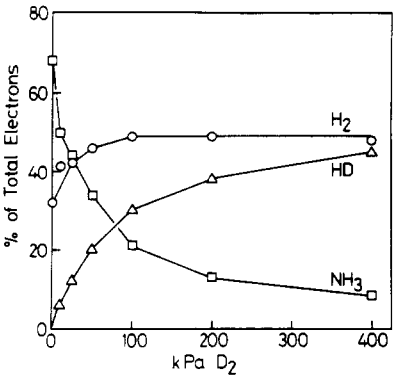


FIGURE 7: Dependence on pD_2 of the percentage of the total electron flux allocated to HD (△), H_2 (○), or NH_3 (□) by nitrogenase from *Klebsiella pneumoniae*.

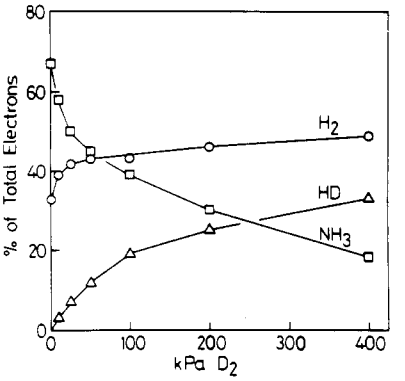


FIGURE 8: Dependence on pD_2 of the percentage of the total electron flux allocated to HD (△), H_2 (○), or NH_3 (□) by nitrogenase from *Clostridium pasteurianum*.

experiments nitrogenase-catalyzed H_2 evolution, HD formation, and NH_3 production were measured under optimal conditions with Kp nitrogenase. The experiments were performed under 50 kPa of N_2 and different pD_2's (0–200 kPa). To be certain that the experiments were performed under optimal conditions, an eightfold molar excess of Fe-protein over MoFe-protein was used. A pN_2 of 50 kPa was chosen, because a higher pN_2 inhibits HD formation at low pD_2 (see Figure 2). An experiment similar to that shown for Kp nitrogenase was performed with Cp nitrogenase; the results were similar but are not shown. Both in the experiment with Kp nitrogenase (Figure 6) and in that of Cp nitrogenase, there was a clear increase in the H_2 production with increasing pD_2. In each case there also seemed to be an increase in the total electron flux with increasing pD_2; this increase could come from an overestimation of HD production. As pointed out by Guth & Burris (1983), our mass spectrometer does not give identical signal output for each molecular species of dihydrogen. The apparatus was calibrated for H_2 by injecting a known amount of H_2 into a measured volume of D_2. Simple comparison of the isotope ratios obtained showed the H_2 was overestimated by 17.5% compared to D_2. This correction has been incorporated into our calculations. Since pure HD is not available, a similar calibration could not be performed for HD; hence, our HD measurements may be overestimated, and this may explain the observed increase in the total electron flux found with increasing pD_2. As shown in Figure 6 (dashed line) the calculated total electron flux is constant if it is assumed that the magnitude of the HD signal is identical with that for H_2 rather than that for D_2.

Figures 7 and 8 show the percentage of the electron flux allocated to the various products as a function of pD_2. In the experiment with Kp nitrogenase (Figure 7) D_2 enhanced H_2 evolution from 31% of the electron flux partitioned to H_2 in the absence of D_2 to 51% of the electron flux partitioned to H_2 at 400 kPa of D_2. With Cp nitrogenase the equivalent values were 33% and 48% of the total electron flux (Figure 8).

The mechanism proposed by Guth & Burris (1983) suggests that, under saturating N_2 and D_2, nitrogenase should form one H_2 per six HD (and no NH_3). In contrast, Cleland's mechanism predicts that, under saturating N_2 and D_2, only two HD (two electrons used) should be formed for every H_2 (two electrons used) formed (and no NH_3 formed). A replot (double-reciprocal plot) of our results from Figures 7 and 8 as $2H_2/HD$ vs. $1/pD_2$ (Figure 9) shows that $2H_2/HD$, at saturating pN_2 and infinitely high pD_2 (intercept at y axis), is very close to one for each nitrogenase. The reciprocal ($2HD/H_2$) again supports the conclusion that two HD are

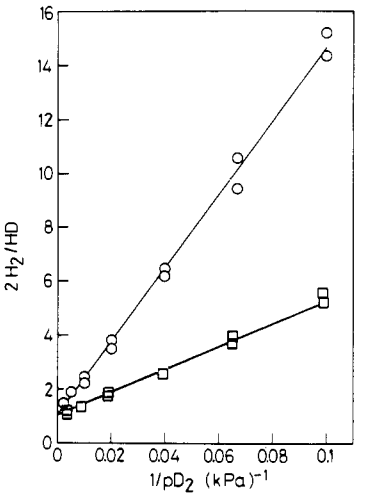


FIGURE 9: $2H_2/HD$ vs. $(pD_2)^{-1}$. $2H_2/HD$ ratios were calculated from the results presented in Figures 7 and 8 obtained with nitrogenase from *Klebsiella pneumoniae* and *Clostridium pasteurianum*, respectively.

formed for each H_2 evolved at infinitely high pD_2.

Discussion

HD formation by nitrogenase has offered valuable insight into the mechanism of biological nitrogen fixation. Several schemes have been proposed to explain the mechanism of HD formation by nitrogenase (Hoch et al., 1960; Bergersen, 1963; Jackson et al., 1968; Turner & Bergersen, 1969; Schrauzer, 1976; Newton et al., 1976, 1977; Chatt, 1980; Wherland et al., 1981; Burgess et al., 1981a,b; Li & Burris, 1983; Guth & Burris, 1983; Eady & Thorneley, 1984).

Although HD formation from D_2 is a general property of nitrogenase preparations (Li & Burris, 1983), nitrogenases from different bacteria differ in their $K_i(D_2)$ values for nitrogen fixation and in their rates of catalyzing HD formation. Among the nitrogenases tested, preparations from *C. pasteurianum* had the highest $K_i(D_2)$ values for nitrogen fixation and the highest rates of catalyzing HD formation. Our estimated values of the $K_m(N_2)$ and $K_i(D_2)$ for nitrogen fixation show that the $K_m(N_2)$ for nitrogen fixation is very similar for *C. pasteurianum* and *K. pneumoniae* nitrogenases (7 and 10 kPa of N_2, respectively), whereas the $K_i(D_2)$ as predicted is higher for *C. pasteurianum* nitrogenase than for *K. pneumoniae* dinitrogenase (17 and 11 kPa of D_2, respectively) although this difference is not as great as had been reported previously for H_2. It was particularly the estimated $K_i(D_2)$ for *C. pasteurianum* nitrogenase of 17 kPa of D_2 which was smaller than the values of $K_i(H_2)$ which had been reported previously for

this organism [32 kPa of H_2 by Lockshin & Burris (1965) and 55 kPa of H_2 by Dilworth et al. (1965)].

The larger $K_i(D_2)$ for *C. pasteurianum* nitrogenase indicates that *C. pasteurianum* nitrogenase is less sensitive to D_2 (H_2) inhibition than is *K. pneumoniae* nitrogenase; hence, as shown in Figures 1 and 3 relatively less HD is formed and more NH_3 is produced by *C. pasteurianum* nitrogenase at a given pD_2 (note that scales differ on x axes). Figure 5 clearly shows that the *C. pasteurianum* enzyme supports a lower rate of HD formation than the *K. pneumoniae* enzyme and that HD formation is most noticeably inhibited by a high pN_2 with the clostridial enzyme. The experiment with the clostridial enzyme (Figure 2) and that with the enzyme from *K. pneumoniae* (Figure 4) each indicates that, at any pD_2, an extremely high pN_2 will completely inhibit HD formation. The experiment with the *K. pneumoniae* enzyme also shows that the pN_2 at which the HD formation reaches its maximum rate increases with increasing pD_2. This together with the finding by Li & Burris (1983) that little if any HD formation takes place in the absence of N_2 suggests the following pN_2 dependence of HD formation: no HD is formed from D_2 at zero pN_2; with constant pD_2 and increasing pN_2 the HD formation reaches a peak (depends on the pD_2), and with further increase in pN_2 the formation of HD decreases; this decreasing curve extrapolates to zero HD at infinite pN_2. The above finding and suggestions are in agreement with the prediction made by Guth & Burris (1983) after computer simulation of their results.

Our finding that D_2 enhances H_2 evolution by nitrogenase from 31% of the electron flux partitioned to H_2 in the absence of D_2 to 51% of the electron flux partitioned to H_2 at 400 kPa of D_2 (*K. pneumoniae* nitrogenase) and from 33% of the electron flux partitioned to H_2 in the absence of D_2 to 48% of the electron flux partitioned to H_2 at 400 kPa of D_2 (*C. pasteurianum* nitrogenase) contradicts the reports by Guth & Burris (1983) and by Newton et al. (1977), who reported that H_2 evolution was independent of pD_2. Our results agree with those of Li & Burris (1983), who observed that H_2 evolution increased with increasing pD_2. The observations are crucial to the distinction between the two proposed models for nitrogenase-catalyzed reductions. The results of Guth & Burris (1983) and of Newton et al. (1977) suffer from the fact that the effect of pD_2 on H_2 evolution was investigated only over a relatively narrow range of pD_2 (15–70 kPa of D_2), in contrast to the present measurements which were made over the range 0–400 kPa of D_2. The range between 0 and 15 kPa of D_2 is in fact the most interesting part of the curve, because the increase in H_2 evolution as a function of an increase in pD_2 is greatest in the range below the $K_i(D_2)$ for nitrogen fixation, i.e., below about 10 kPa of D_2 for both the *K. pneumoniae* nitrogenase used by Guth & Burris (1983) and for the *Azotobacter vinelandii* nitrogenase used by Newton et al. (1977).

Our results on the effect of pD_2 on H_2 evolution support Cleland's mechanism, as they clearly question the only criticism Guth & Burris (1983) had concerning this mechanism.

Guth & Burris (1983) left the possibility open that H_2 evolution from nitrogenase under saturating N_2 constitutes an unavoidable "leak" of low potential reducing equivalents. They reported 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 allocations of electrons to H_2 are changed. On this basis they concluded that the V_{max} for HD formation is the same as the V_{max} for NH_3 formation which means that nitrogenase theoretically can be completely diverted from NH_3 production to HD formation even under saturating N_2. They concluded that under such conditions (saturating N_2 and D_2) 3/4 of the total electron flux will go to HD production and 1/4 to H_2 evolution, the same ratios as between NH_3 and H_2 at saturating N_2. This again means that under optimal conditions and saturating N_2 nitrogenase forms one H_2 per two NH_3 (in the absence of D_2) or one H_2 per six HD (in the presence of very high pD_2). According to Guth & Burris (1983), H_2 evolved under either of these limiting conditions either could arise from an "unavoidable leak" of electrons, or could occur during the early stages of N_2 fixation before N_2 reduction is aborted by the reaction of D_2 (H_2) with the putative intermediate in nitrogen fixation.

In contrast, the mechanism for nitrogenase-catalyzed reductions proposed by W. W. Cleland envisions that H_2 evolution is an obligatory part of N_2 reduction and occurs when N_2 binds to nitrogenase. Thus, Cleland's model is consistent with the observation that 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 (Rivera-Ortiz & Burris, 1975; Simpson & Burris, 1984). Cleland's mechanism predicts further that two HD are formed for every H_2 and that, at saturating pD_2 and pN_2, 50% of the total electron flux goes to H_2 and 50% to HD formation. Thus, the fact that extrapolation of our experimental observations to infinitely high pD_2 indicates two HD formed per H_2 for both *K. pneumoniae* and *C. pasteurianum* nitrogenases (Figure 9) lends support to Cleland's mechanism [in Guth & Burris (1983)] rather than to the mechanism suggested by Guth and Burris.

Guth & Burris (1983) also explored the effect of changing the ratio of dinitrogenase to dinitrogenase reductase on the inhibition of NH_3 formation by D_2. They found that, at high Kp1/Kp2 ratios, D_2 was a stronger inhibitor than at low Kp1/Kp2 ratios. They pointed out that D_2 could become a stronger competitive inhibitor of NH_3 formation if the affinity of nitrogenase for N_2 was decreased relative to its affinity for D_2 as the Kp1/Kp2 ratio increased. Cleland's model presents an opportunity for a different rationalization of the observations: $E(N_2)$, with which H_2 (D_2) must react to inhibit NH_3 formation, may merely become longer lived at lower electron flux rates, thereby allowing more time for reaction with H_2 (D_2). If, in fact, the lifetime of E (N_2) is a factor in the strength of inhibition by H_2 (D_2) at low electron flux, then the rate of reaction of H_2 (D_2) with E (N_2) must become at least partially rate limiting. This in turn suggests that there may be an H_2/D_2 isotope effect on the inhibition of NH_3 formation by H_2 and D_2 at low electron flux. H_2/D_2 isotope effects have not been observed, but perhaps this is because observations always have been made at high electron flux when the reaction of H_2 (D_2) with E (N_2) has not been rate limiting.

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

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Inactivation of Myoglobin by Ortho-Substituted Arylhydrazines. Formation of Prosthetic Heme Aryl–Iron but Not *N*-Aryl Adducts†

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ABSTRACT: Stable phenyl–iron complexes are known to form in the reactions of myoglobin, hemoglobin, and catalase with phenylhydrazine. The phenyl moiety in these complexes migrates from the iron to a nitrogen of the porphyrin upon denaturation of the hemoproteins. Complexes obtained from myoglobin and ortho-substituted phenylhydrazines, however, are much less stable, have distinct chromophores, and do not yield *N*-arylporphyrins. These abnormal properties imply that the complexes differ in structure (e.g., they are aryldiazenyl- rather than aryl–iron complexes) or that ortho substitution strongly alters the chemistry of aryl–iron complexes. The present NMR studies unambiguously demonstrate that ortho-substituted phenylhydrazines give normal aryl–iron complexes but that the aryl group in these complexes is conformationally locked and is unable to shift from iron to nitrogen.

The reaction of phenylhydrazine with hemoglobin, which irreversibly alters the hemoprotein chromophore (Itano & Robinson, 1961; Jandl et al., 1960), gives rise to superoxide and phenyl radicals (Goldberg et al., 1976, 1979; Hill & Thornalley, 1981; Augusto et al., 1982) and hydrogen peroxide (Rostorfer & Cormier, 1957; Cohen & Hochstein, 1964). Myoglobin reacts similarly with phenylhydrazine except that hemoglobin precipitates from the solution whereas myoglobin does not (French et al., 1978; Augusto et al., 1982). *N*-Phenylprotoporphyrin IX is obtained when the prosthetic group is extracted aerobically from the inactivated hemoproteins (Ortiz de Montellano & Kunze, 1981; Saito & Itano, 1981), but the phenyl group is bound to the iron rather than to one of the nitrogens when the prosthetic moiety is extracted anaerobically (Kunze & Ortiz de Montellano, 1983; Ortiz de Montellano & Kerr, 1983). Coordination of the phenyl to the iron in the intact myoglobin complex has been confirmed by an NMR study (Kunze & Ortiz de Montellano, 1983) and by X-ray crystallography (Ringe et al., 1984). The phenyl group thus shifts from the heme iron to one of its nitrogens as the heme complex separates from the protein matrix (Au-

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