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The Relationship between Nitrogen Fixation and the Production of HD from D_2 by Cell-Free Extracts of Soya-Bean Nodule Bacteroids

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1. Cell-free extracts prepared from soya-bean nodule bacteroids produced HD from D_2 in the presence of dithionite, an ATP-generating system and nitrogen. 2. Crude extracts of bacteroids or of *Azotobacter vinelandii* showed some background D_2 exchange when any one of these was omitted. 3. Partial purification of bacteroid extracts diminished this background activity and gave increased D_2 exchange and nitrogen fixation. 4. Although increasing pN_2 stimulated both reactions, the apparent K_m (N_2) for nitrogen fixation was much higher than the apparent K_m (N_2) for D_2 exchange when partially purified bacteroid extracts were used. 5. Carbon monoxide was a competitive inhibitor of nitrogen fixation by partially purified bacteroid extracts, but D_2 exchange was inhibited in a non-competitive fashion. 6. These results are discussed in relation to the possible existence of enzyme-bound intermediates of nitrogen fixation.

Nitrogen-dependent formation of HD from D_2 by intact detached nodules of soya bean was reported by Hoch, Schneider & Burris (1960) and it was suggested that this may have been due to an exchange reaction with enzyme-bound intermediates between nitrogen and ammonia; Bergersen (1963) showed that HD formation and nitrogen fixation by soya-bean nodules responded to oxygen and carbon monoxide in similar ways, but the two reactions were quantitatively distinguishable.

Jackson, Parshall & Hardy (1968) reported that the formation of HD from D_2 catalysed by cell-free preparations of *Azotobacter vinelandii* was enhanced in the presence of nitrogen. It was suggested that this nitrogen-enhanced exchange reaction was analogous to the exchange occurring when di-imide or hydrazine complexes of platinum were incubated with D_2. This could constitute evidence that enzyme-bound intermediates occurred in the reaction:

$$N_2 + 6H \rightarrow (HN{:}NH \rightarrow H_2N \cdot NH_2) \rightarrow 2NH_3$$

Kelly (1968) confirmed nitrogen-enhanced HD formation by root nodules of *Medicago lupulina* and *Alnus glutinosa*; however, he found that extracts of *A. vinelandii* and *Azotobacter chroococcum* catalysed HD formation from $D_2 + H_2O$ or from $H_2 + D_2O$ in a reaction that was inhibited and not enhanced by nitrogen. This HD formation was similar to nitrogen fixation in that it was dependent on ATP and dithionite and was inhibited by carbon monoxide. HD formation by these preparations did not occur when alternative substrates for nitrogenase, such as acetylene and methyl isocyanide, were used instead of nitrogen. From these results the author concluded that the HD formation that he observed was due to exchange at the nitrogen-binding site and was inhibited by any substrate that was bound at the same site, in proportion to the K_m value and concentration of that substrate. The additional HD formation that was observed to be nitrogen-enhanced in the nodule systems might be due to exchange at some stage in an electron-transport pathway that was activated only in the presence of nitrogen.

If the issue of the occurrence of enzyme-bound intermediates in nitrogen fixation is to be resolved, the nature of the D_2 exchange reaction must be studied further. We have conducted experiments with the soya-bean nodule system in which the relationships between the nitrogen-fixing and D_2-exchange reactions have been studied in cell-free preparations. Kinetic studies with carbon monoxide as a specific competitive inhibitor of nitrogen fixation (Bergersen & Turner, 1968) were made because, if the exchange reaction was indeed catalysed by bound intermediates of the nitrogen-fixation reaction, then the kinetics of the inhibition of both reactions by carbon monoxide might be similar. Some results of work with extracts of *A. vinelandii* are included for comparison.

MATERIALS AND METHODS

Preparation of extracts of bacteria and bacteroids. Soya beans (cultivar Shelby) were grown in pots of sand–vermiculite mixture in a glasshouse as previously described (Bergersen, 1958). Seeds were inoculated at sowing with strain CC711 of *Rhizobium japonicum*. Nodules were detached after 30–32 days and used immediately. Bacteroid suspensions and cell-free extracts were prepared as described previously (Bergersen & Turner, 1968) and the supernatant from centrifugation at 100000*g* for 30min. was used as the source of crude bacteroid nitrogenase. *Azotobacter vinelandii* strain OP (P. W. Wilson, Madison, Wis., U.S.A.) was grown at 30° with high aeration in Burk's medium in a 7l. fermentor. The cells were harvested during the late exponential phase and cell-free extracts were prepared in the same manner as for bacteroids. The extract was centrifuged at 144000*g* for 30min. and the supernatant used as the source of crude nitrogenase.

In experiments with partially purified bacteroid extracts, the following procedure was used. Washed bacteroids were prepared from 100g. fresh wt. of nodules from soya beans (cultivar Lincoln) inoculated with strain CB1809 of *R. japonicum*. The bacteroids were suspended in 25mM-tris–HCl buffer, pH7·4, and broken as before. The broken bacteroids were then centrifuged under argon at 66000*g* for 30min. The supernatant was transferred by syringe to capped argon-filled tubes and heated at 60° for 10min. Precipitated protein was removed by centrifugation at 13000*g* for 10min. and the supernatant (18ml.) was transferred by syringe to an argon-filled Diaflo cell (Amicon Corp., Lexington, Mass., U.S.A.) fitted with a UM20E dialysis membrane. The volume was decreased to 5ml. at 10° under argon at 50lb./in.^{2}; 13ml. of buffer was added and the volume again decreased to 5ml. This procedure removed 92% of solutes passing the membrane (cut-off 20000 mol.wt.), gave a 16-fold increase in specific activity (measured as C_2H_2 reduction) in some experiments, was consistently superior to anaerobic gel filtration with Sephadex G-25 for the removal of solutes of low molecular weight and had the advantage of concentrating the extract.

Gas mixtures and analysis. Gas mixtures were prepared from good-quality commercial gases and the concentrations expressed as partial pressures measured in mm.Hg. $^{15}N_2$ prepared from $(^{15}NH_4)_2SO_4$ (Isocommerz, Berlin-Adlershof, East Germany) and D_2 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) were included in mixtures as needed. In some experiments C_2H_2 (Commonwealth Industrial Gases, Sydney, N.S.W., Australia) was scrubbed through conc. H_2SO_4 and water and used as an alternative substrate to N_2. Argon was used to bring mixtures to 1 atm. Gas mixtures, excluding those containing C_2H_2, were stored over $CrSO_4$ solution to remove traces of O_2; mixtures containing C_2H_2, which is reduced by $CrSO_4$ solutions, were stored over argon-saturated water.

Mass-spectrometer analyses were used to check gas composition before and after incubation and to measure the evolution of H_2 and the formation of HD from D_2. For HD-formation studies, initial gas samples were taken for analysis from each vessel before incubation because of variations between vessels even when filled from the same gas reservoir and because of the relatively small changes in HD partial pressures that occurred during incubation.

Experimental procedures. Cell-free extracts of bacteroids and *A. vinelandii* were shaken in 15ml. side-arm flasks (total liquid volume 2ml.) with the required gas mixtures at 25° and 30° respectively, and with the following additions: the ATP-generating system consisted of ATP (sodium salt, 10μmoles) (Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.), creatine phosphate (sodium salt, 50–150μmoles according to optimum concentrations determined for each batch) and creatine kinase (1mg.) (Sigma Chemical Co., St Louis, Mo., U.S.A.), all dissolved in 25mM-potassium phosphate buffer, pH7·0, or 25mM-tris–HCl buffer, pH7·4, and neutralized to pH7·0. $MgSO_4$ (10μmoles) was added separately and $Na_2S_2O_4$ was made up daily in argon-saturated 25mM-potassium phosphate or tris–HCl buffer, pH7·4, and stored under argon at 0° until used. The reaction vessels containing the ATP, creatine phosphate, $MgSO_4$ and buffer were flushed three times with argon and then filled with appropriate gas mixtures to a predetermined pressure so that the final pressure, after an initial gas sample had been taken and the other constituents had been added, would be 700mm.Hg. When CO was used, this gas was added by gas-chromatography syringe through a rubber-capped side-arm port and the contents were allowed to come to equilibrium for 10–15min.; the extract was added by syringe, followed by creatine kinase and finally $Na_2S_2O_4$ (29μmoles) to start the reaction. Reactions were stopped by the injection of 0·2ml. of 0·1M-H_2SO_4 or 10% (w/v) trichloroacetic acid.

The nitrogen fixed was calculated as before, from the NH_3 N of the flask contents and its ^{15}N enrichment, with 50μg. of unlabelled carrier NH_3 N per vessel (Bergersen & Turner, 1968). The reduction of C_2H_2 to C_2H_4 was measured from the increase in C_2H_4 content of the gas in reaction vessels by means of a Philips PV4000 gas chromatograph equipped with 2m.×2mm. columns of Poropak R at 50°. C_2H_4 was retained for 1·8min. and C_2H_2 for 2·4min., and peak height was directly proportional to concentration when 200μl. gas samples were injected.

Protein contents of the cell-free extracts were determined by the Folin phenol reagent method (Lowry, Rosebrough, Farr & Randall, 1951).

RESULTS

Crude extracts. The formation of HD from D_2 was consistently enhanced by nitrogen when either *A. vinelandii* or bacteroid extracts were used with an ATP-generating system and dithionite. Omission of either dithionite or ATP-generating system diminished both nitrogen fixation and HD formation. However, there was some HD formation in the absence of nitrogen and with *A. vinelandii* extracts it was considerable. Thus, in one experiment, an *A. vinelandii* extract (38·7mg. of protein) fixed 1·35μmoles of ammonia in a 30min. assay, produced 0·61μmole of HD in the presence of nitrogen (149mm.Hg) and 0·35μmole of HD with no added nitrogen. With bacteroid extracts the background HD formation was similar when nitrogen or the ATP-generating system was omitted, and accounted for 25–30% of the HD formed by extracts of high nitrogen-fixing ability when both ATP and nitrogen were supplied (Table

Table 1. *ATP, dithionite and nitrogen requirement of* HD *formation from* D_2 *by CC*711 *bacteroid extracts*

Reaction vessels contained 11·7mg. of bacteroid extract protein, and were incubated for 30min. at 25° with $^{15}N_2$ as shown and D_2 (43mm.Hg). Other components are described in the text.

Treatment	$^{15}N_2$ pressure (mm.Hg)	N_2 fixed (nmoles of NH_3)	HD formed (nmoles)
Complete	0	—	98·6
	164	214·9	332·0
ATP and ATP-generating system omitted	0	—	87·1
	164	0·8	54·1*
ATP, ATP-generating system and dithionite omitted	0	—	35·8*
	164	0·3	52·4*

* HD formation below 80nmoles/vessel is too low to be measured with any degree of accuracy.

Table 2. *Effects of nitrogen, acetylene and* CN^- *ion on* HD *formation and* H_2 *evolution by CC*711 *bacteroid extracts*

Reaction vessels contained 6·2mg. of bacteroid protein and were incubated for 30min. at 25°. Gas mixtures contained $^{15}N_2$ (175mm.Hg) or C_2H_2 (140mm.Hg) and CN^- ion was used at a concentration of 5·2mM. Other details are described in the text.

Substrate	H_2 evolved (nmoles)	HD formed (nmoles)
$^{15}N_2$	628	164
C_2H_2	291	86
CN^-	283	72
Control	925	96

1). With less active extracts the background HD formation was relatively unchanged and thus constituted a higher percentage of the nitrogen-enhanced HD formation (Table 2).

The time-courses of nitrogen fixation and nitrogen-enhanced HD formation were similar. When the reciprocals of HD formation and nitrogen fixation by crude bacteroid extracts were plotted against the reciprocals of nitrogen pressures to give Lineweaver–Burk plots, the intercepts giving $1/K_m$ were similar (0·011 and 0·016mm.$^{-1}$ respectively). Both of these values are within the range for K_m (N_2) obtained previously for nitrogen fixation by bacteroid extracts (Bergersen & Turner, 1968). However, the HD-formation values used contained a proportion due to non-specific activity.

When acetylene or CN^- ion replaced nitrogen as the substrate for bacteroid nitrogenase, there was no enhancement of HD formation over control treatments, whereas with nitrogen HD formation was almost doubled. Acetylene was reduced to ethylene and, although the products of the reduction of CN^- ion were not measured, the suppression of H_2 evolution in the presence of CN^- ion suggested that it also was reduced (Table 2).

The inhibition of nitrogen-enhanced HD formation by carbon monoxide proved to be complex, and the effects of D_2 and carbon monoxide on nitrogen fixation were unexpected. Although both nitrogen fixation and HD formation were completely inhibited by carbon monoxide at a partial pressure of 4mm.Hg, the kinetics of the inhibition of the two reactions were different. Even with concentrations of carbon monoxide as low as 0·6mm.Hg, the nitrogen enhancement of HD formation was reversed and the reaction was most inhibited at the higher nitrogen concentration. This effect prevented the determination of the K_i (CO) for HD formation and the assessment of the form of the inhibition. Further, when the results for nitrogen fixation from the same experiments were used to determine K_i (CO), it was found that the inhibition in the presence of D_2 was non-competitive in form, whereas previous work in the absence of H_2 had shown carbon monoxide to be a competitive inhibitor of nitrogen fixation (Bergersen & Turner, 1968). The K_i (CO) was 0·3mm.Hg, similar to the value obtained previously.

Partially purified extracts. To clarify the results obtained with crude extracts, some experiments were repeated with partially purified extracts. One objective was to decrease the background HD formation that occurred in the absence of nitrogen and in the absence of the ATP-generating system. The examples given in Table 3 show that the crude extracts of strain CB1809 bacteroids were more active than the strain CC711 extracts used previously, but the extent of the nitrogen-independent HD formation was again about 25–30% of that obtained in the presence of nitrogen.

It was found that there were three components to the background activity: (*a*) HD formation, in the presence of the complete system but with no added nitrogen in the gas phase, persisted at about 25% of the total activity through all purification steps. These nominally zero-nitrogen treatments actually contained a pN_2 of 4–6mm.Hg due to contamination of the gas mixtures during storage over chromous sulphate solutions and the presence of small concentrations of nitrogen in the argon

Table 3. *Properties of crude, heated, and heated and dialysed extracts of CB1809 bacteroids*

Reaction vessels contained the constituents shown and D_2 (40mm.Hg). They were shaken for 30min. at 25°. The results were obtained from separate experiments. Complete treatments contained tris buffer, ATP, creatine phosphate, creatine kinase and Mg^{2+} as described in the text.

Preparation	Bacteroid protein (mg./vessel)	Treatment	pN_2 (mm.Hg)	N_2 fixed (nmoles of NH_3)	HD formed (nmoles)
Crude	6·96	Complete	5·0*	—	276·8
			153·9	951·6	853·7
Heated	6·63	Complete	4·5*	—	254·7
			112·0	1465·3	1183·0
		ATP and ATP-generating system omitted	4·5*	—	0·0
			112·0	4·5	262·0
Heated and dialysed	8·44	Complete	5·5*	—	245·2
			154·3	1155·5	990·3
		ATP and ATP-generating system omitted	6·4*	—	99·6
			155·5	16·4	87·0

* Contaminating N_2 in nominally zero-N_2 treatments.

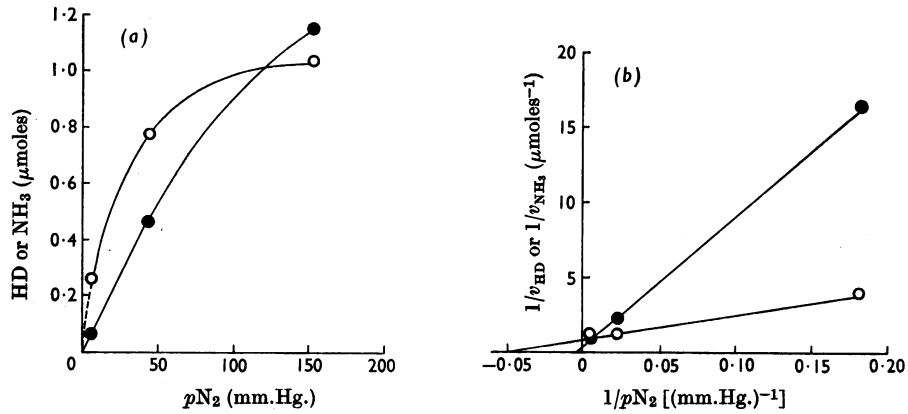


Fig. 1. (*a*) Effect of pN_2 on N_2 fixation (●), measured by the amount of NH_3 produced, and HD formation (○) by partially purified CB1809 bacteroid extracts. (*b*) Results given in (*a*) plotted by the method of Dixon (1953) to show the differing K_m (N_2) values of the two reactions. Reaction vessels, shaken at 25° for 30min., contained 8·44mg. of bacteroid protein, ATP-generating system, dithionite, Mg^{2+} and tris buffer, pH 7·4, as described in the text. Gas mixtures contained N_2 as shown and D_2 (40mm.Hg).

and D_2. From Fig. 1(*a*), which shows the effects of pN_2 on HD formation, it is clear that these low nitrogen concentrations were sufficient to account for the effect. (*b*) Table 1 shows that there was some HD formation in the absence of added ATP when nitrogen was absent. This was believed to be due to bacteroid hydrogenase (Dixon, 1967) in the extract. It was diminished to 10% of the total activity, or less, by the heating step (0nmole in the heated extract and 99·6nmoles in the heated and dialysed extract; Table 3). (*c*) After heating and removal of precipitated protein from the extract, HD formation, in the presence of nitrogen but without the ATP-generating system, remained at about 25% of the total activity. This could have been due to the presence of endogenous ATP or factors giving rise to ATP during incubation. It was diminished to less than 10% of the total activity by the dialysis step (Table 3).

The effects of pN_2 on nitrogen fixation and HD formation are shown in Fig. 1. Nitrogen stimulated HD formation, reaching a maximum near 150 mm.Hg, at which pN_2, nitrogen fixation was still increasing sharply. Thus, in these experiments, the apparent K_m (N_2) was much higher for nitrogen fixation than it was for HD formation (Fig. 1*b*).

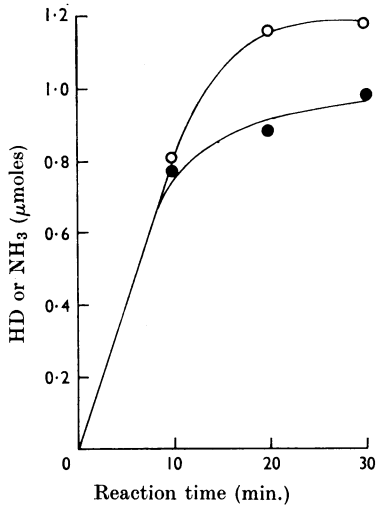


Fig. 2. Time-course of N_2 fixation (●) and HD formation (○) by partially purified CB1809 bacteroid extracts. Reaction vessels, shaken at 25°, contained 11·1mg. of bacteroid protein, ATP-generating system, dithionite, Mg^{2+} and tris buffer, pH7·4, as described in the text. Gas mixtures contained D_2 (41·9mm.Hg) and N_2 (146mm.Hg). Reactions were terminated by the injection of 0·2ml. of 10% (w/v) trichloroacetic acid.

The apparent K_m (N_2) value for nitrogen fixation was 120mm.Hg, which was similar to the upper end of the range given by Bergersen & Turner (1968).

The time-courses for HD formation and nitrogen fixation by partially purified extracts were similar (Fig. 2), but, with the increased activity, they were rather shorter than those obtained with crude extracts.

The effects of carbon monoxide on nitrogen fixation and HD formation were re-examined with partially purified extracts. In these experiments the anomalies found repeatedly with crude extracts were removed. Nitrogen fixation was competitively inhibited by carbon monoxide with K_i 0·17mm.Hg (cf. 0·23 for crude extracts; Bergersen & Turner, 1968), even when D_2 was present. HD formation was also inhibited by carbon monoxide with 50% inhibition at about 0·5mm.Hg pCO. However, the kinetics of the inhibition showed it to be non-competitive. These results are illustrated in Fig. 3, which also shows results obtained earlier with crude extracts for comparison.

DISCUSSION

The results presented show that the nitrogen-enhanced formation of HD from D_2 by nitrogen-fixing cell-free extracts of soya-bean nodule bacteroids resembles the reduction of nitrogen to ammonia in requiring both ATP and reductant, being stimulated by nitrogen and inhibited by carbon monoxide. These results are in agreement with the findings of Jackson *et al.* (1968) for *A. vinelandii*. Kelly (1968), although confirming with *A. chroococcum* and *Clostridium pasteurianum* extracts that HD formation required ATP and reductant, found that the reaction was inhibited by nitrogen and predicted that, although intact root nodules showed nitrogen-enhanced HD formation, cell-free systems would not do so. This has not been found in our experiments. Jackson *et al.* (1968) found that the HD-forming activity accompanied nitrogen-fixing activity during purification. This was also true with the limited amount of purification accomplished with the bacteroid extracts.

Although nitrogen fixation and HD formation by bacteroid extracts had similar experimental properties, they could be distinguished, in our experiments, by kinetic means. The apparent K_m (N_2) for the two reactions was different and, although carbon monoxide was a competitive inhibitor of nitrogen fixation, it was not a competitive inhibitor of HD formation. If it is conceded that HD formation results from an exchange between the H atom of bound di-imide or hydrazine occurring as intermediates in the reduction of nitrogen to ammonia, as proposed by Jackson *et al.* (1968), the reactions under consideration could be expressed as shown in Scheme 1. If the overall reaction rate is not modified by the rates of the non-reversible reduction steps (k_3 to k_5), the relationship between the nitrogen concentration and the rate of ammonia production would be a function of k_2/k_1, the Michaelis constant [K_m (N_2)]. In these circumstances it would be expected that the concentration of a D_2-exchanging intermediate would also be a function of the K_m (N_2) and that HD formation would have had a similar relationship to nitrogen concentration as did ammonia production. Although this appeared to be so with crude extracts of bacteroids, partially purified extracts did not show such a relationship between the two reactions. This could mean (*a*) that HD formation was not catalysed by an enzyme-bound intermediate, or (*b*) that one or more of the reduction steps modified the overall reaction rate. Similar reasoning can be applied to the different kinetic effects of carbon monoxide on nitrogen fixation and HD formation. Competitive inhibition of nitrogen binding by carbon monoxide should similarly inhibit the production of D_2-exchanging intermediates, provided that carbon monoxide was not affecting preceding or succeeding reduction steps. In this case the non-competitive inhibition of HD formation may have been due to (*a*) or (*b*) above, or (*c*) to carbon

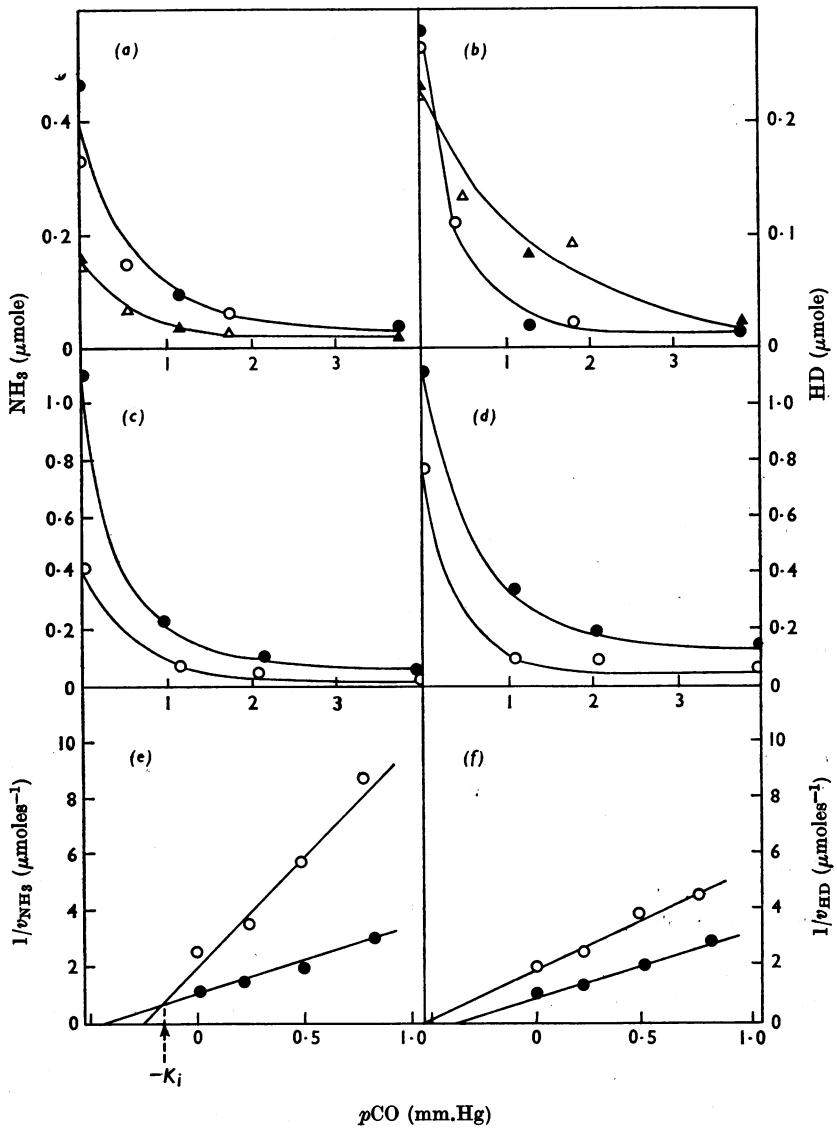
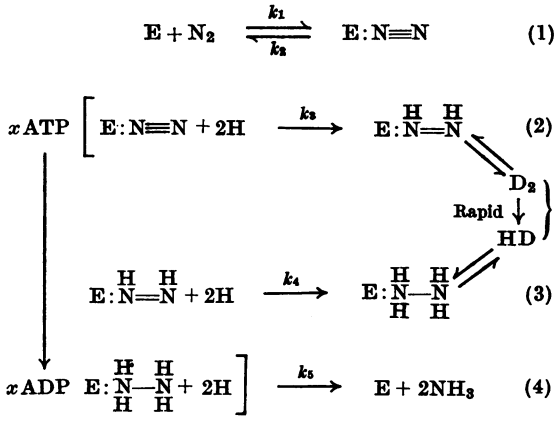


Fig. 3. Effects of CO on N_2 fixation and HD formation. (*a*) and (*b*) Inhibition of N_2 fixation (*a*) and HD formation (*b*) by crude CC 711 bacteroid extracts (two experiments): Expt. 1: ▲, pN_2 35·7, pD_2 27·3 (mm.Hg); ●, pN_2 153, pD_2 29·4 (mm.Hg); 11·8 mg. of bacteroid protein/vessel. Expt. 2: △, pN_2 50·4, pD_2 24·5 (mm.Hg); ○, pN_2 144, pD_2 24·5 (mm.Hg); 8·9 mg. of bacteroid protein/vessel. Reaction vessels contained ATP-generating system, dithionite, Mg^{2+} and potassium phosphate buffer, pH 7·0, as described in the text and were shaken at 25° for 30 min. (*c*) and (*d*) Inhibition by CO of N_2 fixation (*c*) and HD formation (*d*) by partially purified CB 1809 bacteroid extracts. ○, pN_2 37·1, pD_2 44·1 (mm.Hg); ●, pN_2 153·2, pD_2 44·1 (mm.Hg). Reaction vessels contained 11·1 mg. of bacteroid protein, ATP-generating system, dithionite, Mg^{2+} and tris buffer, pH 7·4, as described in the text, and were shaken at 25° for 20 min. (*e*) and (*f*) CO inhibition of N_2 fixation (*e*) and HD formation (*f*) over a range of low CO concentrations. The results are plotted as described by Dixon (1953). The lines were fitted and intercepts determined by using standard procedures. Reaction vessels contained 13·5 mg. of CB 1809 bacteroid protein (partially purified) and were shaken at 25° for 10 min. Other reactants were as described in (*c*) and (*d*). ○, pN_2 32·4, pD_2 33·6 (mm.Hg); ●, pN_2 129·8, pD_2 33·6 (mm.Hg).



Scheme 1.

monoxide affecting the exchange reaction itself. We have previously drawn attention to the possibility that the reducing steps modify rates of reactions in cell-free nitrogen-fixing systems (Bergersen & Turner, 1968). There have been conflicting results in studies of carbon monoxide inhibition of nitrogen fixation. Bergersen & Turner (1968) with nodule bacteroids and with extracts, and Lockshin & Burris (1965) with *C. pasteurianum* extracts reported that carbon monoxide was a competitive inhibitor, but Hwang & Burris (1968) found it to be non-competitive. Competitive kinetics may apply only over a range of low substrate concentrations in which the overall reaction is strictly governed by the true K_m. In the present work the attempts to resolve the existence of enzyme-bound D_2-exchanging intermediates by kinetic means has clearly not been successful. The inability of CN^- ion and acetylene to catalyse D_2 exchange, although they are alternative substrates to nitrogen for reduction by nitrogenase, has been shown in the bacteroid system as well as in the other systems studied (Jackson *et al.* 1968; Kelly, 1968). These alternative substrates would not be expected to have enzyme-bound D_2-exchanging intermediates. The proposition that intermediates of nitrogen reduction are responsible for the nitrogen-dependent D_2 exchange thus rests on its close relationship to nitrogen fixation in terms of the requirements for the reaction and on the failure of acetylene and CN^- ion to support the reaction.

There were some minor differences between the experiments described by Jackson *et al.* (1968) and Kelly (1968) and our own. First, the pD_2 values that we used were much lower than the others, so that nitrogen fixation was inhibited to only a small extent (about 10%) by the D_2. The temperatures at which the reactions were done were also different. It is not considered that these differences contributed to the differences between the results of our experiments and those of Kelly (1968), because our results are in substantial agreement with those of Jackson *et al.* (1968).

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