THE RELATIONSHIP BETWEEN HYDROGEN EVOLUTION, HYDROGEN EXCHANGE, NITROGEN FIXATION, AND APPLIED OXYGEN TENSION IN SOYBEAN ROOT NODULES

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[*Manuscript received March* 14, 1963]

Summary

Hydrogen evolution and exchange and nitrogen fixation by detached soybean root nodules were studied at various external oxygen tensions (pO_2). The main findings were:

(1) Increasing pO_2 stimulated hydrogen evolution up to about 50% oxygen, above which it was inhibited in the absence but not in the presence of nitrogen.

(2) 10% deuterium inhibited hydrogen evolution by decreasing the effects of rising pO_2.

(3) Nitrogen inhibited hydrogen evolution competitively when two different pO_2 values were used to produce different H-donor concentrations. When nitrogen fixation was not limited by lack of nitrogen (10% nitrogen, 20% oxygen), about 5 μmoles hydrogen/g/hr were evolved, suggesting that a metabolic pool of molecular hydrogen exists in these nodules under natural physiological conditions. At high pO_2 up to 25 moles of hydrogen were evolved for every mole of nitrogen fixed.

(4) The exchange reaction (HD formation from deuterium) was affected by pO_2 in a similar way to nitrogen fixation, an average of 2–3 moles of HD being formed for every mole of nitrogen fixed.

(5) Carbon monoxide inhibited hydrogen evolution only at high external pO_2 while HD formation was inhibited at all pO_2 values. This inhibitor reduced nitrogen fixation more than either hydrogen evolution or HD formation.

These results are discussed in terms of current theories of the pathways of nitrogen fixation in legume root nodules.

I. Introduction

All known agents of biological nitrogen fixation have been found to be active in one or more phases of the metabolism of molecular hydrogen (Wilson and Burris 1947; Kamen and Gest 1949; Hoch, Schneider, and Burris 1960). Although this apparently functional association of ability to metabolize both molecular nitrogen and hydrogen has been the subject of theoretical discussion (Winfield 1955; Pratt 1962) comparatively little experimental information is available. This is especially true of the legume root nodule system. Soybean nodules have been shown to evolve hydrogen and to catalyse an exchange reaction between deuterium and endogenous H-donors which resulted in HD formation in the gas phase (Hoch, Schneider, and Burris 1960): nitrogen inhibited hydrogen evolution but was necessary for the exchange reaction, while carbon monoxide, although erratic in its effect, was more inhibitory to the exchange reaction than it was to hydrogen evolution; nitrous

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oxide, a specific competitive inhibitor of nitrogen fixation in this system, inhibited both evolution and exchange. Hoch, Schneider, and Burris (1960) submitted, in explanation of their results, a scheme in which H_2, HD, and D_2 were in equilibrium with partially reduced nitrogen bound to nitrogenase, while the same enzyme was also able to release hydrogen from donors of H when nitrogen was not bound to it.

Bergersen (1962*a*) showed that the bacteroids of soybean nodules were separated from the external atmosphere by a barrier or barriers which shielded them from oxygen and that, although increased oxygen tension (pO_2) up to an external value of about 50% oxygen increased nitrogen fixation, higher values gave a rise in pO_2 within the barriers which led to competitive inhibition of nitrogen fixation. These results, which suggested that under natural conditions the pO_2 adjacent to the bacteroids was very low, were confirmed by the finding (Bergersen 1962*b*) that the leghaemoglobin of these nodules was almost completely reduced until the external pO_2 was raised above about 50%, when oxygenation of the pigment proceeded with increasing pO_2.

The purpose of the experiments described in this paper was to examine the interrelations of hydrogen metabolism (as expressed by hydrogen evolution and HD formation from deuterium), nitrogen fixation, and external oxygen tension when soybean root nodules were used as the agents of fixation.

II. Materials and Methods

(*a*) *Nodules*

Nodules of known age were obtained from glasshouse-grown soybeans which had been inoculated with strain CC711 of *Rhizobium japonicum*, as previously described (Bergersen 1958). However, in this work the soybean variety used was Shelby, cv. Lincoln being no longer available. Nodules were detached from the plants into beakers immersed in ice and experiments were commenced within 1 hr of picking the first nodule.

In any one experiment equal fresh weights of nodules were used in each flask of 50 ml volume (Fig. 1). All results are expressed on the basis of dry weights of nodules. Where the nodules were extracted for ^{15}N analyses, dry weights of a number of similar nodule samples were obtained and the mean value used as the basis for expression of the results. Incubations were done at 25°C with shaking.

(*b*) *Mass Spectrometer Determinations*

Isotope measurements and gas mixture analyses were made with an M86 (Atlas-Werke, Bremen) mass spectrometer. Partial pressures of gases were determined from the magnitudes of the peaks of the masses present, corrected by the source calibration for each component.

(*c*) *Gas Mixtures*

These were prepared from good quality commercial gases by means of manifolds equipped with mercury manometers. The composition of the mixtures was checked before and after incubations by mass spectrometric analyses. The deuterium used

(Bio-Rad Laboratories, California) contained 5% impurities and the composition of the hydrogen species was 96·98% deuterium and 3·02% hydrogen. $^{15}N_2$ was generated from $^{15}NH_4NO_3$ as previously described (Bergersen 1962*a*). In all gases the impurities present were due to traces of residual air.

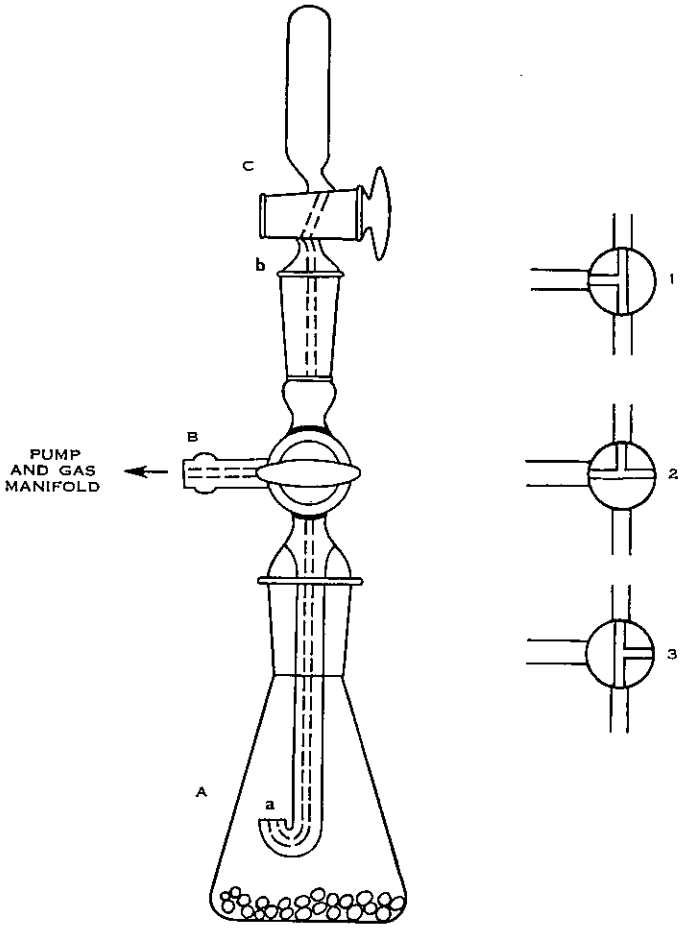

Fig. 1.—Incubation flask *A*, and gas sampling attachments. The flask is evacuated and filled with gas mixtures with the three-way stopcock *B* in position 1 at right. During incubation this stopcock is at position 2. When taking a sample the bulb *C* is evacuated (position 2) and fil'ed from the flask by turning stopcock *B* to position 3 in a clockwise direction. This sample contains some gas from the dead space *a–b* and is pumped out by turning *B* anticlockwise to position 2. *B* is then returned to position 3 thus again filling *C* with gas from the flask. The sample bulb stopcock is then closed and stopcock *B* returned to position 2 before detaching the sample bulb.

(*d*) *Gas Sampling*

The incubation flasks were equipped with sampling attachments (Fig. 1) which permitted the extraction of 2-ml samples of gas into evacuated sample bulbs which could be closed and transferred to the mass spectrometer for analysis. The connec-

tions were flushed by taking a sample and pumping it out before collecting the sample for analysis.

(e) *Measurement of Hydrogen Evolution and HD Formation*

Samples of gas from the incubation flasks were admitted to the mass spectrometer after freezing in dry ice or liquid air to remove water vapour. The magnitudes of the mass 2, mass 3, and mass 4 peaks were measured, corrected for background, and the results were expressed for hydrogen and HD as a percentage of masses 2+3+4 (percentage hydrogen species). Where helium was used as the mass 4

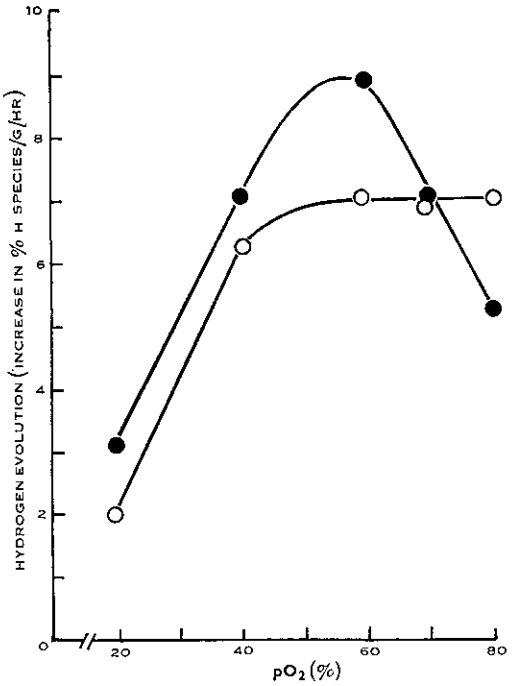

Fig. 2.—Relationship between hydrogen evolution and oxygen tension (pO_2), with and without nitrogen. Flasks contained nodules 29 days old, oxygen as shown, 10% helium, argon to 1 atm. ○ 10% nitrogen. ● No nitrogen.

component the value of the mass 4 peak was multiplied by 2·41 (the ratio of the deuterium/helium peaks when equal volumes of the two gases were admitted at the same inlet pressure), and the value obtained was regarded as being equivalent to the deuterium mass 4 peaks in the calculation of percentage hydrogen species: in this way all experiments were comparable, whether deuterium or helium was used as the mass 4 component. In most experiments the 50-ml flasks contained 10% deuterium or helium. The actual volume of hydrogen evolved or HD formed could thus be calculated from the percentage hydrogen species value and, after correction for temperature and pressure, could be expressed as number of μmoles.

(*f*) *Measurement of Nitrogen Fixation*

For this purpose 10% $^{15}N_2$ was included in the gas mixtures and, after incubation, the nodules were crushed and extracted with 3N HCl, the soluble portion being used for the determination of the atoms % excess ^{15}N as previously described (Bergersen 1962*a*). The total nitrogen of the hydrochloric acid extract was determined in the course of the analysis and, since with the short incubations used in this work (1·5 hr) all the newly fixed nitrogen remained in the acid-soluble portion of the nodules, the actual amount of nitrogen fixed could be calculated from the total nitrogen of the extract, the atoms % excess ^{15}N of the incubation gas, and the atoms % excess ^{15}N of the extract.

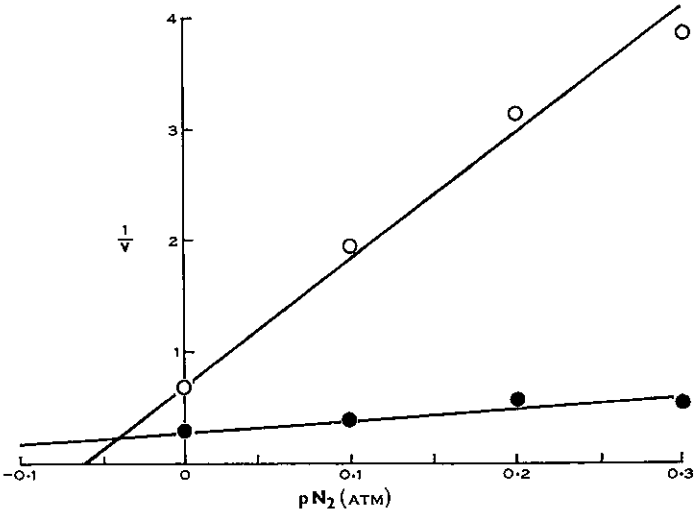

Fig. 3.—Competitive inhibition of hydrogen evolution by nitrogen. Flasks contained nodules 34 days old, 10% helium, nitrogen as shown, and argon to 1 atm. *v*, hydrogen evolution as increase in % H species/g/hr. ○ 20% oxygen. ● 60% oxygen.

III. Results

Under the conditions used, the nodules evolved hydrogen, converted deuterium to HD, and fixed nitrogen as a linear function of time for at least 2 hr after the gas mixtures were admitted and incubations were commenced. The rates of hydrogen evolution and HD formation were affected by oxygen tension; the maximum evolution of hydrogen was about 8–9% H species/g/hr or about 16–18 μmoles/g/hr, while the maximum HD formation was 1·5–2·0% H species/g/hr or about 3–4 μmoles/g/hr.

(*a*) *Effects of* pO_2 *and Nitrogen on Hydrogen Evolution*

The effects of 10% nitrogen upon evolution of hydrogen are shown in Figure 2. The inhibition of hydrogen evolution above 60% oxygen was not always so great as to produce intersection of the graphs: on some occasions the values of the plus and minus nitrogen treatments merely became equal at 80% oxygen. In all other respects results of different experiments were consistent.

Figure 3 expresses the results of an experiment in which hydrogen evolution was measured at different pN_2 values and at two pO_2 values. The straight-line relationship between 1/(rate of hydrogen evolution) and pN_2 confirms the competitive nature of the inhibition of hydrogen evolution by nitrogen (Dixon 1953) if it is assumed that the two pO_2 values represent two different concentrations of the endogenous H-donors. However, exact determination of the constants involved is difficult because of the unknown nature and concentration of these donors.

(*b*) *Relationship between Hydrogen Evolution and HD Formation*

Three types of response to pO_2 were found. The most common was that shown in Figure 4(*a*), in which hydrogen evolution continued to increase with pO_2 up to 80% oxygen while HD formation reached a maximum at about 50% oxygen. On some occasions this type of response was replaced by that illustrated in Figure 4(*b*)

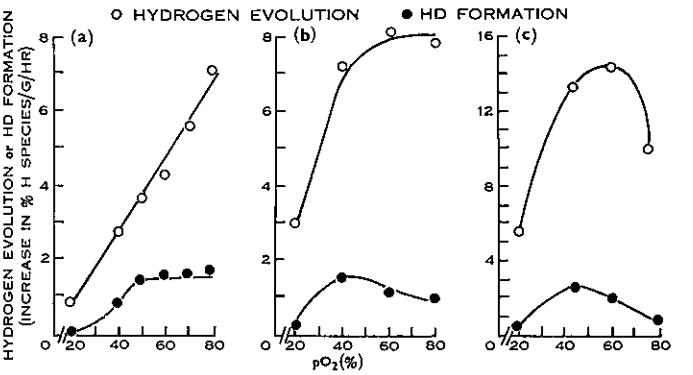

Fig. 4.—Relationship between hydrogen evolution and exchange at various pO_2 values. Flasks contained 10% nitrogen, 10% deuterium, oxygen as shown, and argon to 1 atm. (*a*) The most common type of response to pO_2, nodules aged 29 days. (*b*) Type of response encountered twice during 19 experiments; nodules aged 24 days. (*c*) The response of a sample of nodules 16 days old.

in which hydrogen evolution reached a maximum at about 50% oxygen and remained steady while HD formation declined above 50% oxygen. No reason could be found for the differences between these two types of response, that of Figure 4(*b*) appearing twice during the course of the experiments independently of nodule age or glasshouse conditions. The third type of response (Fig. 4(*c*)) was obtained only once with young nodules (16 days old) which had just commenced to fix nitrogen. In all cases the response to pO_2 was characterized by the ratio hydrogen evolved/HD formed increasing sharply at pO_2 values higher than 50%.

(*c*) *Inhibitory Effect of Deuterium upon Hydrogen Evolution and Nitrogen Fixation*

The measurement of HD formation required the inclusion of 10% deuterium in the gas mixture. This level of deuterium inhibited the evolution of hydrogen at the lower pO_2 values and the maximum rate of evolution was attained at 10–20% oxygen higher than the pO_2 necessary for the maximum rate with helium as the mass 4 component (Fig. 5). Deuterium also inhibited nitrogen fixation when this was

measured at various pO_2 values (Fig. 6). The relationship between these effects is shown in the data of Table 1, in which it is seen from the ratios hydrogen evolved/

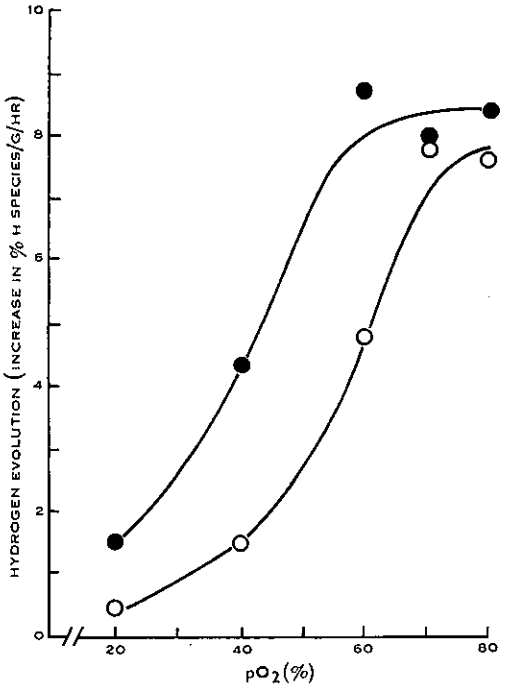

Fig. 5.—Inhibition of hydrogen evolution by deuterium. Flasks contained nodules aged 25 days, 10% helium or deuterium, 10% nitrogen, oxygen as shown, and argon to 1 atm. ● Helium. ○ Deuterium.

TABLE 1

INHIBITORY EFFECT OF DEUTERIUM UPON NITROGEN FIXATION AND HYDROGEN EVOLUTION AT VARIOUS pO_2 VALUES

50-ml flasks contained 2·0 g (fresh wt.) nodules aged 22 days, oxygen as shown, 10% $^{15}N_2$ (90 atoms %), 10% deuterium or helium, and argon to 1 atm

pO_2 (%)	Mass 4 Component: Deuterium			Mass 4 Component: Helium		
	Nitrogen Fixed (μmoles/g/hr)	Hydrogen Evolved (μmoles/g/hr)	Moles Hydrogen / Moles Nitrogen	Nitrogen Fixed (μmoles/g/hr)	Hydrogen Evolved (μmoles/g/hr)	Moles Hydrogen / Moles Nitrogen
20	0·66	4·84	7·30	1·59	5·14	3·23
40	1·46	8·60	5·89	2·52	12·50	4·96
50	1·59	11·05	6·95	2·65	18·52	6·98
60	1·53	17·64	11·57	2·19	18·19	8·31
80	1·33	16·44	12·40	2·05	18·15	8·83

nitrogen fixed that 10% deuterium inhibited fixation more than hydrogen evolution, and that this ratio increased in a regular manner with helium but showed a sharp

rise at 50–60% oxygen when deuterium was present. The results also indicate that deuterium renders the nitrogen-fixing system less susceptible to the inhibitory effect of oxygen. This was shown by the pO_2 for maximum fixation being about 10% higher in the presence of 10% deuterium than in its absence (Fig. 6).

(*d*) *Effects of Carbon Monoxide*

Figure 7 shows the effect of carbon monoxide on hydrogen evolution and HD formation. Evolution of hydrogen was unaffected by carbon monoxide at low pO_2 but was inhibited at high pO_2. The exchange reaction, however, was inhibited by carbon monoxide at all pO_2 values tested. From the data of Table 2 it is clear that 1% carbon monoxide inhibited nitrogen fixation more than HD formation.

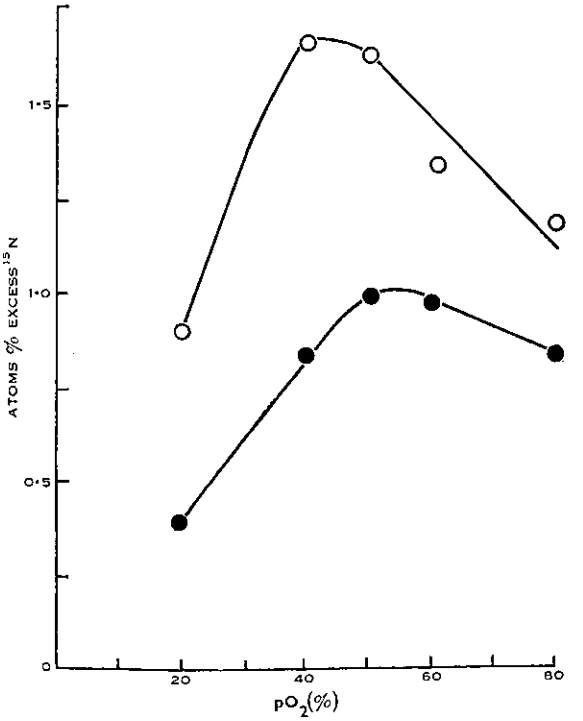

Fig. 6.—Inhibition of nitrogen fixation by hydrogen (as deuterium) at various pO_2 values. Flasks contained nodules aged 22 days, 10% $^{15}N_2$ (92 atoms %), oxygen as shown, and argon to 1 atm. ○ 10% helium. ● 10% deuterium.

(*e*) *Effects of Nodule Age*

Nodules of a range of ages were examined for hydrogen evolution, HD formation, and nitrogen fixation at 20, 40, 60, and 80% oxygen (Table 3). No great effects of age were found in the relationship between HD formation and nitrogen fixation, although there was a trend towards the ratio HD formed/nitrogen fixed rising with increasing age. Thus the mean ratio for all pO_2 values rose from about 2 at 16 days to about 3 at 31 and 42 days. No such trend was present in the ratios of hydrogen evolved/nitrogen fixed.

IV. Discussion

(a) General

Hoch, Schneider, and Burris (1960) found that the metabolism of hydrogen by nodules was unpredictable and in the present work the occasional appearance of atypical patterns of pO_2 response (Figs. 4(*b*) and 4(*c*)) was a source of difficulty. It is, however, possible to offer an explanation for one aspect of their data. These authors reported that, although the exchange reaction (HD formation) was clearly sensitive to carbon monoxide, the inhibition of the evolution of hydrogen was erratic. Examination of their data shows that variable pO_2 was used in their experiments and it is possible that they observed inhibition only when using the higher pO_2

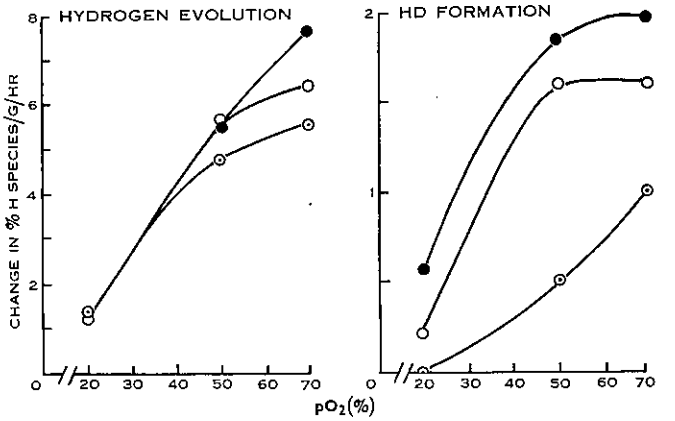

Fig. 7.—Effects of carbon monoxide on hydrogen evolution and exchange at various pO_2 values. Flasks contained nodules 29 days old, 10% deuterium, 10% nitrogen, oxygen as shown, and argon to 1 atm. ● No carbon monoxide. ○ 0·5% carbon monoxide. ⊙ 1·0% carbon monoxide.

values, as was found in the experiments described in this paper. Because of the differential effects of nitrogen and carbon monoxide upon hydrogen evolution and HD formation, Hoch, Schneider, and Burris suggested that these two aspects of hydrogen metabolism expressed separate activities of the enzyme nitrogenase in order that their results and the known competitive inhibition of nitrogen fixation by nitrous oxide and hydrogen could be explained as substrate–inhibitor competition at a single enzyme site. The separation of the evolution of hydrogen from the site of the interconversion of hydrogen and deuterium to HD is supported in the present work by the effects of high external pO_2 which caused inhibition of the exchange reaction relative to hydrogen evolution. Recently it has been shown (Bergersen 1962*a*) that oxygen is also a competitive inhibitor of nitrogen fixation at high external pO_2. It is likely that this represents competition for reducing power between one electron transport system with oxygen as the terminal acceptor and another whose terminal acceptor is nitrogen, the common point being rate-limiting for both pathways. Such a common point could also be limiting for hydrogen evolution, thus

explaining the competitive effects of hydrogen and nitrogen with respect to both nitrogen fixation and hydrogen evolution.

TABLE 2

INHIBITORY EFFECT OF CARBON MONOXIDE UPON NITROGEN FIXATION, HYDROGEN EVOLUTION, AND HD FORMATION AT VARIOUS pO_2 VALUES

50-ml flasks contained 2·5 g (fresh wt.) nodules aged 31 days, oxygen and carbon monoxide as shown, 10% $^{15}N_2$ (82 atoms %), 10% deuterium, and argon to 1 atm

pO_2 (%)	Carbon Monoxide (1%)	Nitrogen Fixed (μmoles/g/hr)	Hydrogen Evolved (μmoles/g/hr)	HD Formed (μmoles/g/hr)	Moles Hydrogen / Moles Nitrogen	Moles HD / Moles Nitrogen
20	+	0·16	4·16	0·37	26·00	2·31
	—	1·15	4·80	0·90	4·17	0·78
50	+	0·94	15·76	2·41	16·77	2·56
	—	2·23	19·36	3·67	8·68	1·65
70	+	0·86	17·56	2·98	20·42	3·47
	—	2·17	24·72	4·25	11·39	1·96

TABLE 3

RELATIONSHIP BETWEEN HYDROGEN EVOLUTION AND EXCHANGE AND NITROGEN FIXATION FOR NODULES OF DIFFERENT AGES AT FOUR OXYGEN TENSIONS

50-ml flasks contained 1·4 g (fresh wt.) nodules, oxygen as shown, 10% deuterium, 10% $^{15}N_2$ (80 atoms %), and argon to 1 atm

pO_2 (%)	Age of Nodules (days):				
	16	24	31	31	42
	Moles HD Formed/Moles Nitrogen Fixed				
20	1·47	1·03	3·53	3·08	—
40	2·66	2·91	3·20	2·91	4·09
60	2·41	2·36	2·86	3·06	3·60
80	1·65	2·11	2·32	3·16	2·81
	Moles Hydrogen Evolved/Moles Nitrogen Fixed				
20	—	9·67	16·26	7·30	—
40	12·52	13·44	15·01	5·89	17·82
60	18·25	16·61	19·55	11·57	21·68
80	19·52	15·66	25·46	12·40	20·08

The results which have been presented are compatible with the working hypothesis proposed by the author (Bergersen 1960), but they do not add any information which enables further critical examination of it.

(b) *Effects of Oxygen Penetration*

External pO_2 values above about 50–60% cause penetration of oxygen into the vicinity of the bacteroids (Bergersen 1962*a*) and progressive oxygenation of the leghaemoglobin (Bergersen 1962*b*). These conditions also produced inhibition of hydrogen evolution in the absence of nitrogen (Fig. 2), but in the presence of nitrogen inhibition by high pO_2 occurred only in one sample of very young (16 days) nodules. The reason for the protective effect of nitrogen was not apparent from these experiments. The inhibitory effects of carbon monoxide on hydrogen evolution only became apparent at a pO_2 of 50% and higher. If hydrogen evolution occurs deep within the nodule cells at a site surrounded by leghaemoglobin and the primary effect of carbon monoxide is the formation of CO-leghaemoglobin, then penetration of oxygen could diminish formation of this complex and allow carbon monoxide to penetrate more deeply and inhibit hydrogen evolution.

(c) *Hydrogen Evolution and Nitrogen Fixation*

Deuterium inhibited both hydrogen evolution and nitrogen fixation. The oxygen-response curve for evolution was modified in such a way that the curve for $+D_2$ was equivalent to the $-D_2$ curve moved up by 10–20% oxygen. At low pO_2 values deuterium inhibited nitrogen fixation and at external pO_2 values which produced oxygen penetration, deuterium reduced the inhibitory effects of oxygen upon nitrogen fixation (Fig. 6). In these experiments deuterium and hydrogen inhibited nitrogen fixation in the same way and it is reasonable to conclude that in the reactions considered here D_2 and H_2 or D and H behaved in similar, if not completely identical, ways.

The nitrogen-fixing system of root nodules is almost saturated with respect to nitrogen at about 10% nitrogen and a pO_2 of 20% (Wilson 1940), yet these nodules evolve about 5 μmoles hydrogen/g/hr under these conditions (Table 1). This result is not in agreement with Pratt (1962), who concluded from an examination of the literature "that H_2 is neither evolved nor taken up during the fixation of N_2 and that the hydrogenase performs some function other than the catalysis of reactions involving H_2". The evolution of hydrogen even when the fixing system is saturated with nitrogen is indicative that functioning nodules normally have a metabolic pool of molecular hydrogen. The existence of such a pool makes it possible for hydrogen to be one of the reductants of nitrogen bound on the nitrogenase. Callander and Roberts (1961) have shown that reduction of the $-N = N-$ bond by hydrogenase is possible when the electron density between the two atoms is reduced by substitution. Binding of nitrogen on the nitrogenase may produce this effect, making reduction by hydrogenase possible at some stage between N_2 and NH_3.

(d) *HD Formation and Nitrogen Fixation*

A fairly close relationship was found between HD formation and nitrogen fixation, 2–3 moles of HD being formed for every mole of nitrogen fixed. This relationship was only slightly affected by nodule age and applied pO_2 (Tables 2 and 3). This finding supports the concept that some stage of the nitrogen-fixation pathway

between N_2 and assimilable N-compounds is in equilibrium with molecular hydrogen and that in this equilibrium, rearrangement between the atoms of the molecule occurs. However, the comparatively high value of HD formed/nitrogen fixed of 2–3 suggests that either this exchange does not occur through a simple equilibration or that, if it does, the equilibration step is prolonged enough for every molecule of bound nitrogen to exchange 2–3 molecules of deuterium. An alternative should also be considered in which several molecules of deuterium are bound to an intermediate N-compound and 2–3 are released, rearranged with hydrogen from donors other than deuterium later in the pathway, giving the following overall reactions:

$$N_2+2D_2+8H \rightarrow NH_3+ND_2H+2HD+H_2,$$

or

$$N_2+3D_2+6H \rightarrow NH_3+ND_3+3HD.$$

The effects of carbon monoxide in inhibiting the overall nitrogen fixation more than HD formation can be quite readily explained if a step in the pathway is in equilibrium with hydrogen and is followed by a step which is more sensitive to carbon monoxide, but if the exchange of hydrogen occurs through binding of hydrogen to an N-intermediate followed by a definite rearrangement and release from another stage in the process, other explanations of this effect must be sought.

V. Acknowledgment

The author is indebted to Mrs. M. Stiller for her very competent technical assistance.

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