Biochem. J. (1968) **109**, 322
Printed in Great Britain

Hydrogen–Deuterium Exchange Reactions Catalysed by Nitrogenase

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(*Received* 18 *June* 1968)

Hoch, Schneider & Burris (1960) reported that whole nodules of soya beans catalysed the exchange between D_2 and H_2O to produce HD, which was detected by mass spectroscopy. More exchange was observed in the presence of N_2, and the authors suggested that some of the exchange was being catalysed by an enzyme-bound intermediate formed during reduction of N_2 to NH_3. The apparent enhancement by N_2 of exchange in nodules was confirmed by Bergersen (1963).

The N_2-fixing system of *Azotobacter vinelandii* catalyses H_2 evolution if supplied with ATP and $Na_2S_2O_4$ (Bulen, Burns & LeComte, 1965), but the bacterial hydrogenase, though able to catalyse H_2 uptake in the presence of a suitable acceptor, does not evolve H_2 (Hyndman, Burris & Wilson, 1953). Jackson & Hardy (1967) reported that $D_2+H_2O \rightarrow HD$ exchange was observed only in the presence of N_2; no exchange was observed under A or if an alternative substrate of the nitrogenase such as acetylene (Dilworth, 1966; Schöllhorn & Burris, 1966) or cyanide (Hardy & Knight, 1966) was present. Both ATP and $Na_2S_2O_4$ were necessary, and CO, which does not inhibit H_2 evolution (Burns & Bulen, 1965), inhibited the exchange reaction. Hardy & Burns (1968) have suggested that enzyme-bound intermediates in the N_2-fixation pathway catalysed the exchange and considered a model platinum complex that showed exchange dependent on bound di-imide or hydrazine. Other substrates of nitrogenase were assumed to form intermediates unsuitable or too transient to catalyse significant exchange. In this paper evidence is presented that nitrogenases from various systems differ in ability to catalyse exchange.

Methods and materials. Purified nitrogenase of *Azotobacter chroococcum* was prepared by using a procedure similar to that of Kelly, Klucas & Burris (1967). For experiments in D_2O the two fractions of nitrogenase were freeze-dried separately and resuspended in D_2O. Some loss of activity was observed, but the dried material was stable under A at $-20°$ for several weeks. Crude nitrogenase of *A. vinelandii* was prepared in a way similar to that used for *A. chroococcum* (Kelly, 1968). Nitrogenase of *Clostridium pasteurianum* was prepared from dried material by the procedure of Carnahan, Mortenson, Mower & Castle (1960). Nodules of alder (*Alnus glutinosa*) and the legume *Medicago lupulina* were obtained from field-grown plants and washed thoroughly in water. Nodules of alder were broken into convenient pieces to introduce them into reaction flasks. Nodules were removed from *M. lupulina* with scissors without any special effort to exclude small roots.

Exchange reactions were carried out in double-side-armed Warburg flasks attached to glass tubes of capacity about 5ml., which could be closed at either end by taps and attached directly to the mass spectrometer. One arm of each flask was sealed with a rubber cap, the other with the usual gassing stopper. After introduction of material into the flask, these were attached to the tubes and were well flushed with the appropriate gas, which passed into the flasks, through the mass-spectrometer tube and out to the atmosphere. The upper tap of the spectrometer tube and side-arm stopper were closed simultaneously. Other gases, substrates and compounds were introduced into the system through the rubber cap by using hypodermic syringes. The nodules, blotted dry on tissue, were tested under an atmosphere of 25% D_2+65% N_2 or A+10% O_2; O_2 was added last and assumed to initiate reactions. Control flasks were included that contained acetylene, and samples were removed from these at intervals to determine the extent of acetylene reduction, which was assumed to parallel N_2 fixation (R. W. F. Hardy, R. D. Holsten, E. K. Jackson & R. C. Burns, unpublished work). A reaction time of about 1hr. at 20° was adequate for a detectable amount of HD and D_2 to be formed from *M. lupulina* nodules, and acetylene reduction over this period was linear. A longer period of incubation was necessary for the alder nodules, and acetylene reduction was not linear over the period. The reactions were stopped by addition of trichloroacetic acid and the system was allowed to stand for 15min. to ensure adequate mixing of the gas throughout the systems; only then was the lower tap of the mass-spectrometer tube closed so that analysis for HD, H_2 and D_2 could be made on the M.S.3 mass spectrometer. Essentially similar procedures were used for the cell-free nitrogenase systems, though O_2 was omitted and instead an ATP-generating system and $Na_2S_2O_4$ were added and anaerobic handling procedures were used. Reactions were run at 30°. In experiments in D_2O,

all components of the system were prepared in D_2O. When acetylene, methyl isocyanide or cyanide was present as substrate, gas samples were removed for analysis by gas–liquid chromatography. Corrections were made for the small amounts of HD, D_2 or H_2 present as impurities in the various gases. The D_2 (99·5% pure), H_2 (99·9% pure) and A (99·99% pure) were obtained from Air Products, Hythe, Southampton; for other materials and techniques, see Kelly (1968).

Results. *M. lupulina* nodules catalysed some exchange to form HD under argon; under N_2 half as much H_2 but twice as much HD was formed. Thus the HD/H_2 ratio was about 1·3 under A but about 4·8 under N_2. The decreased amount of H_2 evolved under N_2 was presumably due to the reaction:

$$N_2 + 3H_2 \rightarrow 2\,NH_3$$

The results with alder nodules were less conclusive; exchange to form HD and evolution of H_2 were detected, and there was only slight difference in the amount formed under N_2 and A; the ratio was 1·6 under A and 2·2 under N_2.

With the crude nitrogenase of *Cl. pasteurianum* less H_2 evolution was observed under N_2 than under A. Considerable exchange was observed, the amount of HD formed being greater when the reaction was carried out under 50% A than under 50% N_2. These extracts had an active reversible hydrogenase and therefore some of the exchange was unrelated to nitrogenase. Non-specific exchange was confirmed by repeating the experiment without the ATP-generating system.

Since hydrogenase of *Azotobacter* is not reversible, non-specific exchange was not observed in corresponding experiments with crude extracts of *A. vinelandii* or *A. chroococcum*. In both cases, no significant exchange was observed in the absence of ATP-generating system or $Na_2S_2O_4$. With both systems, less H_2 was evolved under N_2 and the amount of HD formed was also less than under A. Thus N_2 inhibited H_2 evolution by 25% in *A. vinelandii* and by 29% in *A. chroococcum*. The corresponding values for HD were about 35% and 40%.

More extensive tests were carried out with the purified nitrogenase of *A. chroococcum*. As reported by Jackson & Hardy (1967), no exchange was detected with 50% A or 50% N_2 in the absence of ATP-generating system or $Na_2S_2O_4$, and the exchange was proportional to the partial pressure of D_2. The exchange was completely inhibited by 10% CO, which did not affect H_2 evolution. However, in contrast with the report by Jackson & Hardy (1967), the exchange observed under 50% A was slightly greater than under 50% N_2. No exchange was observed with either gas mixture in the presence of acetylene or 5 mM-methyl isocyanide, though acetylene did not completely inhibit H_2 evolution. When cyanide was added as substrate at 2 mM, exchange was inhibited 40%, but the reduction of cyanide was not linear throughout the incubation period; higher concentrations of cyanide inhibited nitrogenase activity. In corresponding experiments with nitrogenase in D_2O and gas mixtures of H_2+A or H_2+N_2, essentially similar results were obtained, though the amounts of exchange were greater. The HD/H_2 ratio with the $A+D_2+H_2O$ system was about 0·3, but with the $A+H_2+D_2O$ system the HD/D_2 ratio was 0·75. The inhibitory effect of the N_2 was more marked in D_2O, being 20% compared with only 10% in the H_2O system. The inhibitory effect of cyanide on exchange (70%) was also more noticeable. This was probably because its rate of reduction was lower in D_2O than H_2O (Kelly, Postgate & Richards, 1967) and therefore its concentration declined more slowly during the reaction.

Discussion. The observation reported here, that exchange between D_2 and H_2O catalysed by N_2-fixing plant root nodules is enhanced by N_2, agrees with Hoch *et al.* (1960) and Bergersen (1963). However, in the cell-free systems of free-living *Azotobacter*, no evidence was obtained for N_2 enhancement of the exchange; indeed N_2 slightly inhibited the exchange. The observations that the exchange required an ATP-generating system and $Na_2S_2O_4$, and that CO or acetylene inhibits the exchange, agree with those of Jackson & Hardy (1967), and make it very probable that nitrogenase was catalysing the exchange. However, the observation that exchange occurred in the absence of N_2 does not support the hypothesis that the D_2 exchanges with an enzyme-bound di-imide or hydrazine complex.

Gaseous H_2 is a competitive inhibitor of N_2 fixation (Wilson, Umbreit & Lee, 1938; Wilson, 1966) in *Azotobacter* and therefore probably binds at the same site as N_2 itself. The nitrogenase system is able to reduce a variety of substrates and, though the mechanism of reduction is not known, the tendency is for the bound molecule to split and accept H_2. The same tendency could exist when D_2 is bound, and in water the product from 1 mol. of D_2 would then be 2 mol. of HD. Such exchange would show the same requirements as N_2 fixation and would be inhibited, not only by CO, but by all other substrates of the nitrogenase that compete for the same region of the enzyme. The amount of inhibition of exchange caused by various substrates would be in proportion to their K_m values and concentration; N_2 (K_m about 0·2 atm.) caused only slight inhibition of exchange; acetylene (K_m about 0·01 atm.) caused complete inhibition; the cyanide

ion inhibited by only 40% when its concentration fell appreciably below its K_m of 1·4mM.

Therefore H_2 and D_2 are substrates for the nitrogenase and the relative rates of exchange observed with purified nitrogenase of *A. chroococcum* indicate that H_2 is a better substrate than D_2. Inorganic metal complexes exist that show similar behaviour and exchange between a cobalt hydride and D_2 has been reported (Parshall, 1968). Unlike the platinum complex considered by Hardy & Burns (1968), this cobalt complex is able to bind an N_2 molecule.

Nodules do show exchange enhanced by N_2, but, in the absence of N_2, a control mechanism may operate to switch off, or divert, the supply of electrons normally passed to nitrogenase. In the absence of electrons or ATP, no HD exchange would be catalysed by the nitrogenase. Support for this hypothesis is provided by the observation that, at low partial pressures of O_2, bacteroids have higher Q_{O_2} values in the absence of N_2 (Bergersen, 1968). This is explained by assuming that, in the absence of N_2, electrons, which would otherwise be accepted by the nitrogenase and used for fixation, are diverted to O_2. Bacteroids should show the same N_2-enhanced exchange as whole nodules, but all cell-free nitrogenases should show exchange dependent on ATP, a supply of electrons not requiring N_2 but inhibited to differing extents by other substrates or CO.

Bergersen, F. J. (1963). *Aust. J. biol. Sci.* **16**, 669.

Bergersen, F. J. (1968). *J. gen. Microbiol.* (in the Press).

Bulen, W. A., Burns, R. C. & LeComte, J. R. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 532.

Burns, R. C. & Bulen, W. A. (1965). *Biochim. biophys. Acta*, **105**, 437.

Carnahan, J. E., Mortenson, L. E., Mower, N. F. & Castle, J. E. (1960). *Biochim. biophys. Acta*, **44**, 520.

Dilworth, M. J. (1966). *Biochim. biophys. Acta*, **127**, 285.

Hardy, R. W. F. & Burns, R. C. (1968). *Annu. Rev. Biochem.* (in the Press).

Hardy, R. W. F. & Knight, E., jun. (1966). *Biochim. biophys. Res. Commun.* **23**, 409.

Hoch, G. E., Schneider, K. C. & Burris, R. H. (1960). *Biochim. biophys. Acta*, **37**, 273.

Hyndman, L. A., Burris, R. H. & Wilson, P. W. (1953). *J. Bact.* **65**, 522.

Jackson, E. K. & Hardy, R. W. F. (1967). *Plant Physiol.* **42**, 538.

Kelly, M. (1968). *Biochem. J.* **107**, 1.

Kelly, M., Klucas, R. V. & Burris, R. H. (1967). *Biochem. J.* **105**, 3c.

Kelly, M., Postgate, J. R. & Richards, R. (1967). *Biochem. J.* **102**, 1c.

Parshall, G. (1968). *J. Amer. chem. Soc.* **90**, 1669.

Schöllhorn, R. & Burris, R. H. (1966). *Fed. Proc.* **25**, 710.

Wilson, P. W. (1966). In *Colloq. Biological Nitrogen Fixation*, Ed. by W. S. Silver; *Science* (1967), **157**, 100.

Wilson, P. W., Umbreit, W. W. & Lee, B. S. (1938). *Biochem. J.* **32**, 2084.