BBA 3830

THE INHIBITION BY HYDROGEN OF NITROGEN FIXATION IN AZOTOBACTER—A REAPPRAISAL

C. A. PARKER AND M. J. DILWORTH*
Institute of Agriculture, University of Western Australia, Nedlands (Western Australia)

(Received July 10th, 1962)

SUMMARY

The hydrogen inhibition of nitrogen fixation in cultures of *Azotobacter vinelandii* has been re-examined. Hydrogen caused a lag in the rate of nitrogen increase of the cultures at low pN_2, but not at high pN_2. When the cells were adapted to H_2, the lag at low pN_2 was eliminated, and nitrogen increase was exponential.

H_2-adapted cells were grown at varying pN_2 with and without H_2 and the cell nitrogen was measured. Kinetic analysis of the rate constants so obtained showed that the inhibition was not of the competitive type previously reported.

The reasons for differences between this and earlier work have been discussed, with particular reference to the possible production of differential oxygen concentrations at different pN_2, and to the effects of H_2 in inducing lags in nitrogen increase and in altering the $Q_{O_2}(N)$.

INTRODUCTION

Hydrogen was originally identified as an inhibitor of nitrogen fixation in nodulated legumes[1,2]. Further experiments also with legumes suggested a competitive relationship between H_2 and N_2[3,4]. Confirmation of the competitive nature of the inhibition was obtained using Azotobacter cultures in place of the more complex symbiotic system[5,6]. Whilst the methods used with Azotobacter included some direct estimations of nitrogen fixed, they were principally indirect and used respiration as a measure of nitrogen fixation. Hydrogen has since been shown to inhibit the fixation of nitrogen by a number of different organisms: *Clostridium pasteurianum*[7], *Nostoc muscorum*[8], *Rhodospirillum rubrum*[9], *Aerobacter aerogenes*[10], *Achromobacter* spp.[11] and a Clostridium spp.[12] In Aerobacter[10] and in Clostridium[7,12] the inhibition has been reported to be competitive. The evidence with the latter two organisms is less convincing than that with Azotobacter because (a) their experimental growth rates were very slow and (b) these organisms themselves evolve H_2 in their anaerobic metabolism.

In the autotroph *Desulphovibrio desulphuricans*, no inhibition of nitrogen fixation by H_2 was detected[13]. *Methanobacterium omelianskii* fixes N_2 while reducing CO_2 with H_2 in the synthesis of carbohydrates and, in the absence of organic sub-

* Present address: Microbiology Unit, Department of Biochemistry, University of Oxford, (Great Britain).

strates, the requirement for H_2 is absolute[14]. It therefore seems unlikely that H_2 will inhibit nitrogen fixation in this organism. Further doubt is cast on the role of H_2 as a consistent inhibitor of N_2 fixation by its lack of inhibition in cell-free extracts of Clostridium[15,16].

The reduction of N_2 to ammonia appears to be the most probable pathway by which nitrogen fixation occurs[17]. BAYLISS[18] has shown that the reduction of N_2 to ammonia has a favorable free energy change, and that nitrogen can therefore be regarded as an oxidant in this process. Competition between O_2 and N_2 reported by PARKER AND SCUTT[19] supports this view, and suggests that the enzyme "nitrogenase" may have closer affinities with an oxidase than with hydrogenase.

The view that hydrogenase and nitrogenase may be identical has been criticised[20] on the following grounds: (a) other non-nitrogen fixing organisms contain hydrogenase, (b) hydrogenase in Azotobacter and Clostridium is equally active with molybdenum or with vanadium, whereas nitrogen fixation is inhibited by vanadium, (c) iron deficiency causes inhibition of both nitrogen fixation and hydrogenase activity, whereas molybdenum deficiency reduces nitrogen fixation but not hydrogenase action.

A re-investigation of the hydrogen effect on nitrogen fixation therefore seemed warranted. *Azotobacter vinelandii* was used in this work because under the conditions of our experiments it oxidises carbohydrate quantitatively and therefore lends itself to studies in Warburg flasks where the composition of the gas phase can be controlled. Data obtained from direct measurement of the bacterial nitrogen under these conditions suggested that H_2 is not a competitive inhibitor of nitrogen fixation.

METHODS

The organism used was *A. vinelandii* strain O, kindly made available by Professor P. W. WILSON. A vigorous culture was preserved in ampoules by vacuum-drying and was reconstituted at frequent intervals to reduce physiological variability during the experiments. The experimental cultures were aerated overnight in M22 medium, centrifuged at 600 × *g* for 5 min, and the supernatant and non-flocculating cells removed[19]. (This procedure prevented bacterial agglutination in the Warburg flasks during the experiments.) The cultures were then standardised to a known density at 600 mμ and aerated again for 2 h before use[19].

Gases

The hydrogen was prepared commercially by electrolysis of hot NaOH solution and was guaranteed free of all impurities except traces of oxygen. Argon was from the British Oxygen Co. Nitrogen was dry hospital nitrogen containing less than 0.5 % O_2. The pO_2 used throughout was 0.15 atm. to provide adequate oxygen during the course of the experiments, whilst allowing a reasonable rate of growth and respiration[19,21]. The pressure of H_2 (when used) was 0.3 atm.; the balance of the gas to atmospheric pressure was argon. Gases were stored over saturated NaCl solution.

Manometric technique

2 ml of bacterial culture was shaken in each 25-ml flask. Gases were introduced into the flasks after each of three evacuations to 6 cm Hg pressure, using saturated NaCl solution to displace the gas from the containers. Oxygen uptake was measured by conventional techniques at 30° (see ref. 22).

Nitrogen analysis

Nitrogen determinations were made by an ultra-micro Kjeldahl method[23], modified from a method described by CONWAY[24].

RESULTS

Experiment 1: Type of bacterial growth with various gas mixtures

The growth rate constant k (defined by $k = \frac{1}{t} \ln \frac{\text{nitrogen at time } t}{\text{initial nitrogen}}$) can only be used as a measure of the rate of nitrogen fixation under conditions where the nitrogen increase is exponential. The rate of increase of bacterial nitrogen was therefore examined.

After the growth and centrifugation described in METHODS, the organism was exposed to gas mixtures containing 0.03 atm. N_2 with and without 0.3 atm. H_2. Sixteen flasks received 1 ml of Azotobacter culture each. Twelve were evacuated and filled with the experimental gases, placed in the bath, shaken for 15 min and equilibrated. The remaining four flasks were killed at this point, to provide values for the initial bacterial nitrogen. The shaken flasks, two from each gas treatment, were removed each hour and killed immediately. After 3 h, all sixteen cultures were analysed for total nitrogen, and the data plotted in semi-logarithmic form. A typical plot is shown in Fig. 1, where it is seen that a lag phase was induced in the rate of increase of cell nitrogen at the low pN_2 in the presence of H_2, but not in its absence.

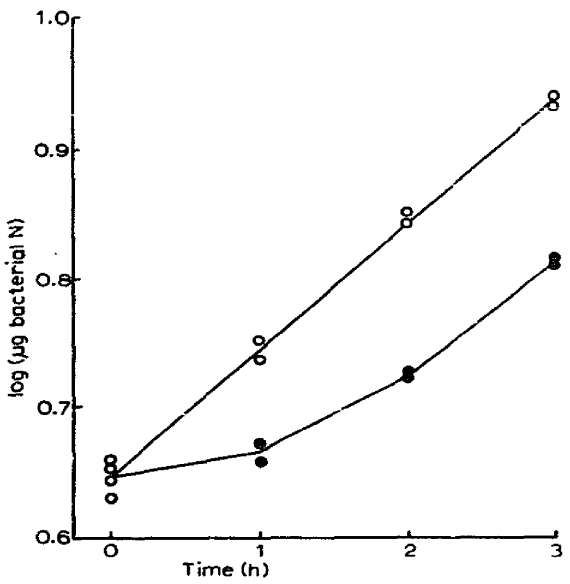

Fig. 1. Induction of a lag phase by H_2 in the nitrogen increase of *A. vinelandii* cultures at 0.03 atm N_2. Gases: 0.03 atm. N_2, 0.15 atm. O_2 with: 0.82 atm. A (O—O); 0.3 atm. H_2 and 0.52 atm. A (●—●).

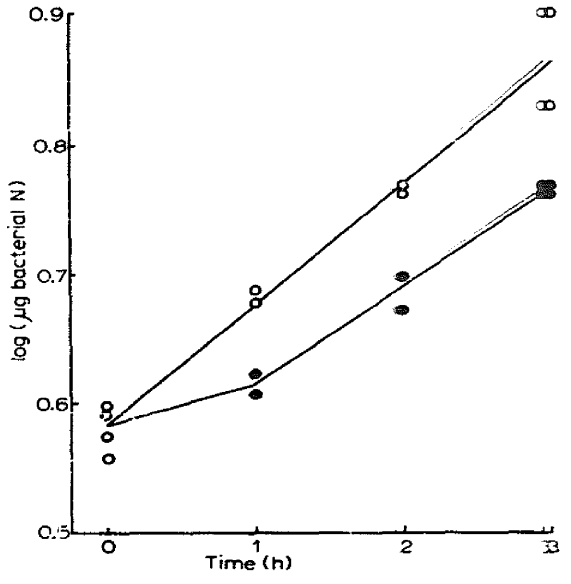

Fig. 2. The effect of pN_2 on the H_2-induced lag. Gases: c.15 atm. O_2, 0.3 atm. H_2 with 0.15 atm. N_2 and 0.4 atm. A (O—O); 0.03 atm. N_2 and 0.52 atm. A (●—●).

Experiment 2: Effect of pN_2 on H_2-induced lag

The effect of H_2 on the rate of nitrogen increase in Azotobacter cultures under both high (0.15 atm.) and low (0.03 atm.) pN_2 was then tested. Fig. 2 is derived from the results of an experiment in which it is shown that at the higher pN_2 the lag due to H_2 did not occur. The lag phase induced by H_2 at 0.03 atm. N_2 is again apparent.

Experiment 3: Adaptation to H_2

The induction of lag by H_2 at low pN_2 precludes analysis of the rate constants by the LINEWEAVER AND BURK[25] method unless this lag can be overcome. To this end a modification was introduced in the pre-experimental aeration procedure. The diluted cultures were grown in 10-ml aliquots in two 100-ml flasks shaken for 2.5 h at 30° under a gas mixture of the following composition: N_2, 0.05 atm.; O_2, 0.15 atm.; H_2, 0.3 atm. and A, 0.5 atm. The two cultures were combined and 1-ml aliquots were taken into the 25-ml flasks, which were then gassed with the following mixture: N_2, 0.03 atm.; O_2, 0.15 atm.; H_2, 0.3 atm. and A, 0.52 atm. Four aliquots of the initial culture were killed as in Expt. 1, and two of the shaken cultures were killed every half hour.

Nitrogen analyses were carried out and the data plotted semilogarithmically (Fig. 3). The plot is linear and, as could be expected from the shape of the later parts of the curves in the low nitrogen treatments of Expts. 1 and 2, the lag phase was overcome.

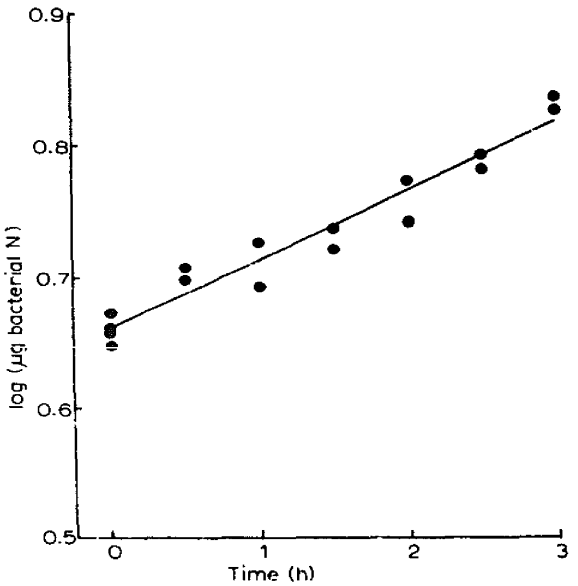

Fig. 3. Elimination of the lag by pre-incubation of cells with H_2. Gas mixtures are detailed in the text.

Experiment 4: Hydrogen–nitrogen interactions

Cultures were grown by aeration overnight and centrifuged as in Expt. 1. From this, two 10-ml cultures were adapted to H_2 at 30° for 2.5 h as in Expt. 3. These two cultures were then mixed and sixteen 1-ml aliquots were pipetted into the 25-ml

flasks. Twelve of these were gassed, each with a different mixture (containing O_2, 0.15 atm.; N_2, 0.03, 0.0375, 0.0475, 0.0625, 0.0875 or 0.16 atm.; H_2, 0 or 0.3 atm. and A, to 1 atm.), and shaken for 15 min to permit equilibration. The four remaining flasks were killed at this time and analysed later with the rest to give the initial bacterial nitrogen. The 12 shaken cultures were killed after 3 h growth, and analysed for nitrogen. The rate constant, k, was calculated from the nitrogen data assuming exponential increase.

The results from six such experiments were graphed (see Fig. 4) by the LINEWEAVER AND BURK method[25], where the reciprocal of the rate constant k is plotted against the reciprocal of the substrate concentration (pN_2).

The inhibition by hydrogen is significant (p less than 0.001), while the slopes of the "plus hydrogen" and "no hydrogen" lines do not differ significantly. In none of the six individual experiments were the pairs of lines such as to suggest competitive inhibition, despite the high variability inherent in the system. These results therefore indicate that H_2 is not a competitive inhibitor of N_2 fixation in *A. vinelandii*.

Oxygen uptake and cell nitrogen

The rates of increase of oxygen uptake did not always appear to parallel those of nitrogen content, both with respect to pN_2 and to the presence or absence of H_2. As a test of the validity of these observations, $Q_{O_2}(N)$ values were calculated from the oxygen uptakes for the last hour of the experiment and the final nitrogen content.

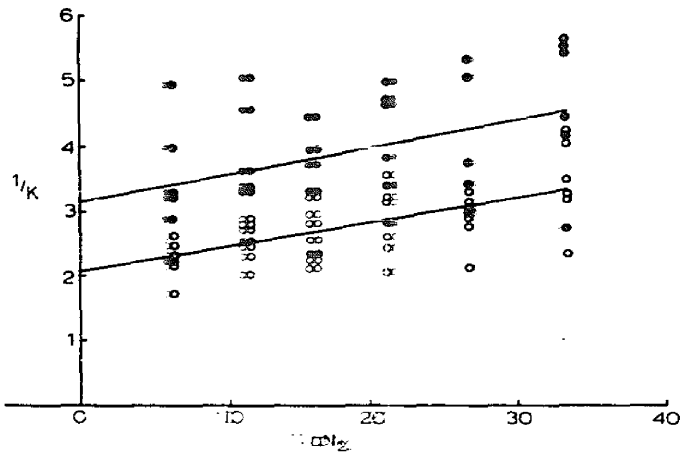

Fig. 4. LINEWEAVER–BURK double-reciprocal plot of velocity constant of nitrogen increase against gaseous nitrogen pressure: ●—●, in the presence of 0.3 atm. H_2 (equation: $1/k = 3.177 + 0.0408 \times 1/pN_2$); ○—○, in the absence of H_2 (equation: $1/k = 2.097 + 0.0364 \times 1/pN_2$).

Statistical analysis of the data for the six experiments of Fig. 4 showed that the Q_{O_2} (N) was significantly higher (p less than 0.02) under H_2 than in its absence, and that the pN_2 also affected the $Q_{O_2}(N)$ (p less than 0.05). Such results are not in accord with the usual assumption that oxygen uptake is a linear function of cell nitrogen, and emphasize the need for direct measurement of nitrogen fixation.

DISCUSSION

The differences between our results and previous findings are probably due to differences in technique. Earlier workers[5,6] have used the following methods for studies of H_2 inhibition of N_2 fixation by Azotobacter: (a) macro-nitrogen experiments on large volumes of either shaken or still cultures; (b) micro-respiration experiments on smaller (25–30 ml) volumes of either shaken or still cultures; (c) micro-respiration experiments in which oxygen uptake is assumed to be closely and consistently correlated with the nitrogen content of the cells.

In all these experiments it was assumed that H_2 did not cause deviations from exponential growth (or nitrogen fixation).

Techniques (a) and (b) have the serious disadvantage that there is inadequate control of the oxygen concentration in solution. Oxygen has been implicated as an active and competitive inhibitor of nitrogen fixation in Azotobacter, with which it was found that great care was needed if oxygen demand from the cells were not to exceed the dissolved oxygen supply[19]. If standard cultures are incubated under different partial pressures of nitrogen those cultures under the highest pN_2 will grow fastest and the greatest oxygen demands will be made by them. If oxygen diffusion becomes limiting, the pN_2 functions will become distorted, since the cultures under the highest pN_2 will be fixing nitrogen at lower concentrations of dissolved O_2 than those given less nitrogen gas. Under these conditions the true effect of H_2 on N_2 fixation would not be apparent.

The assumption that oxygen uptake is a constant function of cell nitrogen under all conditions is not supported by our results since (a) Q_{O_2} (N) was altered significantly in the presence of H_2 (b) Q_{O_2} (N) changed with pN_2 in the presence or absence of hydrogen.

Finally, there is the evidence presented here in Figs. 1 and 2 that H_2 can induce a lag phase in Azotobacter cultures at low pN_2 but not at high pN_2. The reason for this effect at low pN_2, which seems to resemble the well-known tendency for cultures to lag in poor media, is unknown. The slopes of the curves in Figs. 1 and 2 with H_2 and low pN_2 show that the organism does adapt to H_2 after a period of 1–2 h, and this conclusion is supported by the data of Fig. 3.

Should the LINEWEAVER AND BURK plot be used with rate constants which hide a differential lag of the type shown in Expt. 2, there would be a strong tendency towards a competitive plot. At low pN_2 nitrogen fixation would be unduly depressed by H_2 and, were the resultant rate constants plotted as reciprocals, the line would be tilted upward at the distal end.

With cultures adapted to H_2 and under conditions such that nitrogen increase was exponential, our results do not show a competitive relationship between H_2 and N_2 in the process of nitrogen fixation.

ACKNOWLEDGEMENTS

We are indebted to the University of Western Australia for research grants, to Professor E. J. UNDERWOOD for his support, and one of us (M.J.D.) to the Commonwealth Scientific and Industrial Research Organization for a Studentship.

REFERENCES

1 P. W. WILSON AND W. W. UMBREIT, *Arch. Mikrobiol.*, 8 (1937) 440.
2 P. W. WILSON, W. W. UMBREIT AND S. B. LEE, *Biochem. J.*, 32 (1938) 2084.
3 P. W. WILSON, *Ergeb. Enzymforsch.*, 8 (1939) 13.
4 P. W. WILSON, S. B. LEE AND O. WYSS, *J. Biol. Chem.*, 139 (1941) 91.
5 O. WYSS AND P. W. WILSON, *Proc. Natl. Acad. Sci. U.S.*, 27 (1941) 162.
6 O. WYSS, C. J. LIND, J. B. WILSON AND P. W. WILSON, *Biochem. J.*, 35 (1941) 845.
7 D. W. S. WESTLAKE AND P. W. WILSON, *Can. J. Microbiol.*, 5 (1959) 617.
8 R. H. BURRIS AND P. W. WILSON, *Botan. Gaz.*, 108 (1946) 254.
9 D. C. PRATT AND A. W. FRENKEL, *Bacteriol. Proc.*, (1958) 110.
10 R. M. PENGRA AND P. W. WILSON, *J. Bacteriol.*, 75 (1958) 21.
11 R. D. GOERZ AND R. M. PENGRA, *J. Bacteriol.*, 81 (1961) 568.
12 S. HIAI, T. MORI, S. HINO AND T. MORI, *J. Biochem. (Tokyo)*, 44 (1957) 839.
13 J. LE GALL, J. C. SENEZ AND F. PICHINOTY, *Ann. inst. Pasteur*, 96 (1959) 223.
14 M. J. PINE AND H. A. BARKER, *J. Bacteriol.*, 68 (1954) 589.
15 G. E. HOCH AND D. W. S. WESTLAKE, *Federation Proc.*, 17 (1958) 243.
16 J. E. CARNAHAN, L. E. MORTENSON, H. F. MOWER AND J. E. CASTLE, *Biochim. Biophys. Acta*, 44 (1960) 520.
17 P. W. WILSON, in W. RUHLAND, *Handbuch der Pflanzenphysiologie*, Vol. 8, Springer-Verlag, Berlin, 1958, p. 9.
18 N. S. BAYLISS, *Australian J. Biol. Sci.*, 9 (1956) 364.
19 C. A. PARKER AND P. B. SCUTT, *Biochim. Biophys. Acta*, 38 (1960) 230.
20 D. J. D. NICHOLAS, D. J. FISHER, W. J. REDMOND AND M. A. WRIGHT, *J. Gen. Microbiol.*, 22 (1960) 191.
21 M. J. DILWORTH AND C. A. PARKER, *Nature*, 191 (1961) 520.
22 W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques and Tissue Metabolism*, Burgess Publishing Co., Minneapolis, 1949, Chapter 1.
23 C. A. PARKER, *Australian J. Exptl. Biol. Med.*, 39 (1961) 515.
24 E. J. CONWAY, *Microdiffusion Analysis and Volumetric Error*, Crosby-Lockwood & Son, London, 1950, p. 96.
25 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.