The Effect of Nutrient Limitation on Hydrogen Production by Nitrogenase in Continuous Cultures of *Azotobacter chroococcum*

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H₂ production by nitrogenase was investigated in O₂-, N₂-, C- or SO₄²⁻-limited continuous cultures of *Azotobacter chroococcum*. H₂ evolution occurred under air and was augmented when Ar replaced N₂. Pretreatment of each culture with 40% acetylene in air or Ar/O₂ mixtures inhibited the H₂-uptake hydrogenase and increased H₂ evolution. H₂ production in each culture was O₂-dependent, and, like acetylene reduction by nitrogenase, it increased to a maximum at an optimum O₂ concentration and was inhibited by excess O₂. The molar ratio of H₂ produced to N₂ reduced was least at the optimum O₂ concentration and increased sharply under O₂-limiting or O₂-inhibiting conditions in vivo. The minimum value achieved was approximately 1 in O₂-, N₂- or C-limited cultures, or with purified nitrogenase components assayed in vitro, and 0.5 in SO₄²⁻-limited cultures. These differences may reflect different mechanisms of N₂ reduction in vivo. Estimates of the levels of nitrogenase component proteins indicated Ac1:Ac2 ratios of 1 or less. However, Ac2 activity may be inhibited under O₂-limiting or O₂-inhibiting conditions. The maximum ratio of the number of electrons transferred to nitrogenase and to O₂ was 0.1 in assays with O₂-, C- or SO₄²⁻-limited cultures; thus, recycling by the H₂-uptake hydrogenase of H₂ produced by nitrogenase could contribute up to 7% of the total energy produced by respiration.

INTRODUCTION

H₂ production by nitrogenase was first demonstrated in vivo by Hoch *et al.* (1957, 1960) using soybean root nodules. Later studies indicated that nitrogenase catalysed ATP-dependent H₂ evolution in vitro (Burns & Bulen, 1965) and that this occurred in the presence of the reducible substrate N₂ but increased when N₂ was replaced by Ar. Despite reasonable doubts that this H₂-evolving activity of nitrogenase occurred in vivo (Postgate, 1974) or was an artefact of purification procedures (Burns & Hardy, 1975), H₂ production by nitrogenase in vivo is now clearly established (Schubert & Evans, 1976; Smith *et al.*, 1976). It represents a substantial proportion of nitrogenase activity and, presumably, a waste of biological energy (Evans *et al.*, 1977; Walker & Yates, 1978).

Factors which affect the distribution of electrons between H₂ production and N₂ reduction within nitrogenase in vitro include temperature, pH, the ratio of the two component proteins of nitrogenase (Ac1, the MoFe component, and Ac2, the Fe component) and the ATP concentration (for review, see Mortenson & Thorneley, 1979). This communication describes attempts to measure electron distribution within nitrogenase in vivo and the effect of different nutrient limitations on this distribution.

METHODS

Organism and growth. A non-gummy strain of *Azotobacter chroococcum* (NCIB 8003), developed in this laboratory, was maintained at room temperature in air on 2% (w/v) nutrient agar or agar slopes of medium

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'mannitol B' and subcultured monthly. Continuous cultures (650 or 220 ml; Baker, 1968) were maintained at 30 °C under air on modified Burk's medium at pH 6.7 ± 0.1 and $D = 0.1$ or 0.05 h^{-1} , as described by Walker & Yates (1978). The medium for carbon-limited continuous cultures contained $1.5 \text{ g mannitol l}^{-1}$. Sulphate-limited cultures contained $50 \mu\text{M-Na}_2\text{SO}_4$; all other sulphates were replaced by chlorides. Ammonia-grown cultures contained $1.5 \text{ g NH}_4\text{Cl l}^{-1}$. The dissolved oxygen concentration was measured using a sterilizable oxygen electrode (type 50; Uniprobe Instruments, Cardiff) connected to the culture by a side port and to an FL3 oxygen meter (Western Biological Equipment, Sherborne, Dorset).

Chemostat cultures were considered limited by a given nutrient if (1) reduction in concentration of the nutrient by 50% decreased bacterial concentrations proportionally, and (2) a doubling in concentration of all other nutrients in the medium had no effect on bacterial concentration. Apparent O_2 limitation was achieved when conditions (1) and (2) did not apply, no O_2 was detectable in solution and an increase in the stirring rate caused an increase in yield. If dissolved O_2 was detectable, apparent N_2 limitation was assumed (Dalton & Postgate, 1969).

Protein purification. *Azotobacter chroococcum* was grown in 400 l all-glass fermenters as described by Baker (1978). The nitrogenase proteins were isolated and purified by the methods of Yates & Planqu (1975). Purified Ac1 and Ac2 were inactive in isolation but had specific activities of 1730 and 1580 nmol ethylene produced $\text{min}^{-1} (\text{mg protein})^{-1}$, respectively, when assayed with the optimum concentration of the complementary protein. Ac1 and Ac2 yielded two and one protein bands, respectively, on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. They were stored in liquid nitrogen and handled anaerobically in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ under Ar or N_2 .

Protein estimations. All protein concentrations were estimated by the Lowry method, with bovine serum albumin, Fraction V (Sigma) as the standard.

Acetylene reduction by nitrogenase in vitro. The assay system was as described by Eady *et al.* (1972). Ethylene was determined in a Pye 104 gas chromatograph fitted with a flame ionization detector.

Hydrogen production by nitrogenase in vitro. The assay system was as above without added acetylene. Gas samples (0.5 ml) for H_2 detection were injected into a column of molecular sieve 5A, mesh 80–100 (1.5 mm $\times$ 45 cm) fitted to a Pye 204 thermal conductivity detector with Ar as the carrier gas.

Acetylene reduction in vivo. Air plus acetylene (10%, v/v) was passed over the chemostat culture for 25 min, after which the gas flow was stopped; the gas space was isolated by clamping the inlet and outlet tubes without stopping the flow of medium. Gas samples (0.5 ml) were removed aseptically through a 'Suba-seal'; ethylene was determined as described above. Alternatively, culture samples (5 ml) were removed without acetylene pretreatment and assayed for ethylene reduction in 25 ml flasks at 30 °C under Ar/ O_2 mixtures containing 10% (v/v) acetylene over a range of O_2 concentrations.

Hydrogen production and O_2 consumption in vivo. Hydrogen production by *A. chroococcum* was measured in clamped off continuous cultures (see above) at 5 min intervals for 30 min under four sets of conditions: (1) under air; (2) the air was flushed out with Ar/ O_2 / CO_2 (78.87:21.1:0.03, by vol.) for 30 min and H_2 evolution was measured under Ar/ O_2 / CO_2 ; (3) the culture was flushed with Ar/ O_2 / CO_2 + 40% (v/v) acetylene for 20 min to inhibit the H_2 -uptake hydrogenase activity (Fig. 4) and then with Ar/ O_2 / CO_2 for 1–1.5 h to remove acetylene before measuring the rate of H_2 production; (4) the culture was flushed with air to remove Ar/ O_2 / CO_2 and H_2 production was measured again. Alternatively, samples (5 ml) from the continuous cultures were assayed for H_2 production under Ar/ O_2 or N_2 / O_2 over a range of O_2 concentrations after pretreatment (2) or (3) as described above. Ethylene produced from residual acetylene after treatment (3) was usually less than 1% of H_2 produced. O_2 concentrations before and after each experiment were measured by thermal conductivity.

The H_2 : N_2 ratio. This was the molar ratio of H_2 produced to N_2 reduced by nitrogenase and was calculated from the following formulae:

$$\text{for experiments in vivo} \quad \frac{\text{H}_2 \text{ evolved under air}}{\frac{1}{3}[(\text{H}_2 \text{ evolved under Ar/O}_2) - (\text{H}_2 \text{ evolved under N}_2/\text{O}_2)]}$$

$$\text{for experiments in vitro} \quad \frac{\text{H}_2 \text{ evolved under N}_2}{\frac{1}{3}[(\text{H}_2 \text{ evolved under Ar}) - (\text{H}_2 \text{ evolved under N}_2)]}$$

Values for NH_4^+ levels produced by nitrogenase in vitro agreed to within 10% of the value to be expected from this calculation. Nitrogen analysis of cultures indicated that N_2 fixation during growth was less (60–80%) than N_2 fixation calculated from the difference in H_2 evolved under Ar/ O_2 and N_2 / O_2 . Whether this was due to a systematic error in analysis or a stimulation of nitrogenase activity by pretreatment with acetylene (see later) is not known.

H_2 -uptake hydrogenase activity. This was measured manometrically with methylene blue as the electron acceptor, as described by Walker & Yates (1978).

O₂-dependent H₂ uptake. This was measured by O₂-dependent uptake of H³H. H³H was prepared by treating sodium boro[³H]hydride (from The Radiochemical Centre, Amersham) with water and then suitably diluted with H₂ to use as a stock. Bacterial samples (5 ml) were taken directly from the chemostat before or after pretreatment with acetylene and incubated with 10% (v/v) H₂ + H³H and a range of O₂ concentrations under the same conditions (temperature, shaking rate etc.) as those used for determining H₂ evolution by nitrogenase. Samples (0.2 ml) of the aqueous phase were tested for H³H incorporation (H³HO production) in a Nuclear Enterprises scintillation counter with NEN 250 as the scintillant. Scintillations per ml of H₂ + H³H in the stock mixture were determined by conversion to H₂O in a sealed vessel (Schoeniger flask) with a glowing platinum wire.

Ac1:Ac2 molar ratios in vivo. (1) *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).* Continuous culture samples were subjected to SDS-PAGE (Laemmli, 1970; Robson, 1979*a*) using SDS from Serva (Heidelberg, F.R.G.) to separate the subunits of Ac1 (Kennedy *et al.*, 1976). The gels were scanned at 600 nm using a microdensitometer attachment to the Pye Unicam SP1800. From such traces the peaks corresponding to nitrogenase polypeptides were cut out and weighed. Ac1:Ac2 molar ratios were calculated with reference to standard curves prepared in a similar manner.

(2) *Activity measurements.* Samples from continuous cultures were centrifuged at 40 000 g for 20 min, resuspended in 25 mM-Tris/HCl buffer pH 7.4 and disrupted by passage through a French press at 26 MPa. The crude extract or supernatant, after centrifuging at 40 000 g for 30 min, was assayed with increasing concentrations of purified Ac1 or Ac2 using acetylene as the reducible substrate.

NH₄⁺ production. NH₄⁺ produced by nitrogenase in vitro was assayed by the method of Maryan & Vorley (1979).

RESULTS

H₂ was evolved by O₂-limited, N₂-fixing chemostat cultures in air and evolution increased substantially when Ar replaced N₂ or when the H₂-uptake hydrogenase was inhibited by pretreatment with acetylene (Fig. 1*a*). Acetylene-treated cells also showed augmented H₂ production under Ar/O₂. O₂ was required for H₂ evolution (Fig. 2*a*). NH₄⁺-grown cells did not evolve H₂.

Similar results were obtained with O₂-, N₂-, C- and SO₄²⁻-limited (Fig. 1*b*, 2*b*) populations although the rate of H₂ evolution varied according to the physiological status. The data agree with our earlier evidence (Walker & Yates, 1978) that H₂ was produced by nitrogenase and that it was partly recycled by the H₂-uptake hydrogenase. Our results can be summarized as follows.

1. H₂ was evolved under air in all cases except one SO₄²⁻-limited culture.

Fig. 1. H₂ evolution by O₂-limited (*a*) and SO₄²⁻-limited (*b*) continuous cultures under air (●), under Ar/O₂/CO₂ (78.87:21.1:0.03, by vol.) (■), under air after pretreatment with acetylene (○) and under Ar/O₂/CO₂ after pretreatment with acetylene (□). Pretreatment with acetylene (40%) was for 20 min; the cultures were then flushed for 1 h before measuring H₂ evolution.

Fig. 2. Effect of O_2 on H_2 evolution by cells from O_2 -limited (a) and SO_4^{2-} -limited (b) continuous cultures. Cultures were treated with Ar/O_2 /acetylene (48:12:40, by vol.) for 20 min and then flushed with $Ar/O_2/CO_2$ for 1 to 2 h to remove acetylene. Samples (5 ml) in 25 ml flasks were shaken at 120 strokes min^{-1} and an amplitude of 3 cm at 30 °C for 1 h under atmospheres of Ar or N_2 containing a range of O_2 concentrations; the reaction was stopped with 7 M-KOH (0.05 ml). $\square$, H_2 evolution under Ar/CO_2 ; $\circ$, H_2 evolution under N_2/O_2 ; ∇ , $H_2:N_2$ molar ratio, i.e. ratio of mol H_2 produced to mol N_2 reduced.

2. Pretreatment with acetylene increased the rate of H_2 evolution in every case. Pretreatment with acetylene also enhanced the rate of acetylene reduction, but this increase (10%) was small compared with the increase in the rate of H_2 evolution (>300%) by the same culture under Ar/O_2 (4:1) after an identical pretreatment.

3. H_2 evolution under air by O_2 -, C- or N_2 -limited cultures was enhanced 4- to 12-fold by pretreatment with acetylene, which suggests that hydrogenase normally recycled 75–92% of the H_2 produced by nitrogenase in these cultures. However, in one SO_4^{2-} -limited culture, pretreatment with acetylene only enhanced the rate of H_2 evolution under air by 7% (Fig. 1b).

4. The $H_2:N_2$ ratio after pretreatment with acetylene was always greater than 1 in O_2 -, N_2 - or C-limited cultures (Fig. 2a), but less than 1 in SO_4^{2-} -limited cultures (Fig. 2b, Table 1). Significant differences in specific activities of H_2 production occurred within each type of nutrient limitation even at similar turbidities, e.g. O_2 -limited cultures of A_{540} 0.29, 0.30 and 0.33 (EEL colorimeter) produced 0.8, 8.2 and 12.5 nmol H_2 min^{-1} (mg protein) $^{-1}$ under air without acetylene pretreatment. These differences may reflect small differences in the O_2 solution rate because of fluctuation in the stirring or air flow rates, which could affect both uptake hydrogenase and nitrogenase activity. Uptake hydrogenase activity (synthesis) also varied significantly between cultures under the same nutrient limitation (Partridge *et al.*, 1980). After pretreatment with acetylene the values for the percentage of nitrogenase activity

Table 1. H₂ evolution by continuous cultures of *A. chroococcum*

Results indicate the midpoint and extremes of the range of values obtained. H₂:N₂ molar ratios were calculated as described in Methods.

Nutrient limitation	Percentage of nitrogenase activity expended in H ₂ production		H ₂ :N ₂ molar ratio	
	Before acetylene treatment	After acetylene treatment	Before acetylene treatment	After acetylene treatment
O ₂	22 ± 16	34 ± 6	1.5 ± 0.4	1.9 ± 0.1
N ₂	30 ± 13	40	1.5 ± 0.8	2.0
C	14 ± 3	33 ± 3	0.5 ± 0.1	1.5 ± 0.2
SO ₄ ²⁻	6 ± 6	10.5 ± 0.5	0.2 ± 0.2	0.35 ± 0.05

Fig. 3. O₂ uptake and H₂ evolution by cells from C-limited continuous cultures amended with sucrose (1 mg ml⁻¹). Pretreatment with acetylene and other experimental conditions were as described for Fig. 2. O₂ consumption was measured by analysing the gas mixture by thermal conductivity at the beginning and end of the experiment. □, H₂ evolution; ◆, O₂ consumption.

expended in H₂ production and hence the values of H₂:N₂ ratios were more consistent within each nutrient limitation (Table 1).

O₂-dependent H₂ production

These experiments were undertaken to show the effect of changing O₂ concentrations on (a) H₂ production, (b) the H₂:N₂ ratio and (c) the numbers of electrons transferred to O₂ and nitrogenase, in bacteria grown under different nutrient limitations. These experiments, done in shaking flasks, also overcame the difficulties, mentioned above, of fluctuating stirring or air flow rates in cultures. Figure 2 shows that H₂ production increased to a maximum and then declined with increasing O₂ concentration. This bell-shaped curve coincided with that for acetylene reduction by nitrogenase in samples from identical cultures. The H₂:N₂ ratio was lowest at O₂ concentrations at or just below the optimum for maximum nitrogenase activity in all cases after hydrogenase activity had been inhibited by pretreatment with acetylene. This minimum ratio was usually approximately equal to 1 in cell samples from O₂- or C-limited cultures (Fig. 2a), but was significantly less than 1 in cell samples from SO₄²⁻-limited cultures (Fig. 2b).

At extremes of O₂ concentration the H₂:N₂ ratio rose sharply to values as high as 25 at low O₂ or 5 at high O₂ concentrations. C-limited cultures were extremely sensitive to O₂ concentrations above the optimum for nitrogenase activity, so accurate H₂:N₂ ratios could not be measured at high O₂ concentrations.

Fig. 4. (a) Inhibition of uptake hydrogenase activity by pretreatment with acetylene. An O₂-limited culture was treated with 40% acetylene as described in Methods. After flushing to remove acetylene, cells were harvested from 200 ml culture by centrifugation at 40 000 *g* for 10 min, resuspended in 0.2 M-phosphate buffer pH 8.0 containing EDTA (3 mM) and NaF (100 mM) and assayed for H₂ uptake activity with methylene blue as the electron acceptor. ○, control (without acetylene pretreatment); □, acetylene-treated cells.

(b) Inhibition of O₂-dependent H³H uptake by pretreatment with acetylene. An SO₄²⁻-limited culture was pretreated with acetylene and assayed as described for Fig. 2 under Ar/O₂ mixtures containing 12% H₂ + H³H. H³H incorporation was determined by liquid scintillation counting. ●, control (without acetylene pretreatment); ○, acetylene-treated cells.

The maximum ratio of the number of electrons transferred to nitrogenase compared with the number consumed by O₂ uptake was approximately 0.1 in all cases and was obtained at O₂ concentrations at or below the optimum for nitrogenase activity. Above this optimum, nitrogenase activity declined while O₂ consumption increased (Fig. 3).

Inhibition of hydrogenase activity by acetylene

Pretreatment of continuous cultures with 40% acetylene inhibited hydrogenase activity and O₂-dependent H₂ uptake by 80–90% (Fig. 4). Preliminary experiments indicated that the hydrogenase recovered slightly (10%) 2 h after flushing to remove the acetylene (C. D. P. Partridge & M. G. Yates, unpublished observation).

H₂:N₂ molar ratios in vitro

Changes in either the ATP concentration or the creatine phosphokinase concentration had a profound effect on the H₂:N₂ ratio provided the Ac1:Ac2 ratio was greater than 1 (Fig. 5*a, b*). The H₂:N₂ ratio increased to infinity at progressively lower Ac1:Ac2 ratios as the ATP concentration was diminished. However, it approached an apparently limiting value of 1 at all ATP concentrations as the Ac1:Ac2 ratio approached 1. The H₂:N₂ ratio remained close to 1 at Ac1:Ac2 ratios of less than 1. Therefore, in these experiments the H₂:N₂ ratio was always 1 or more. It did not change significantly when the Ac1 purified from batch-grown cells was substituted by Ac1 purified from SO₄²⁻-limited *A. chroococcum*, as might have been expected if the low H₂:N₂ ratio in SO₄²⁻-limited cultures reflected a different type of Ac1.

Ac1:Ac2 molar ratios in vivo

SDS-PAGE. Densitometer traces of electrophoretograms indicated that O₂- and N₂-limited cells contained similar molar ratios of Ac1:Ac2 (Fig. 6*a*) which appear to be significantly different from that of C-limited cells in which Ac2 was particularly low (Fig. 6*b*). Peak areas indicated that Ac1:Ac2 molar ratios ranged from 0.3 to 0.5 in O₂-limited cells to 1.0 in C-limited cells. NH₄⁺-grown cells, in which nitrogenase was repressed, were analysed as

Fig. 5. (a) Effect of Ac1:Ac2 ratio and ATP concentration on the H₂:N₂ ratio in vitro. The details of the nitrogenase assay in vitro are given in Methods. The Ac1:Ac2 molar ratio was calculated from estimated molecular weights of 227 000 for Ac1 and 64 000 for Ac2 (Yates & Planqué, 1975). The Ac2 concentration was constant at 9.2 μg ml⁻¹, 0.14 μM. Initial ATP concentrations: O, 5 mM; □, 1 mM; Δ, 0.1 mM.

(b) Effect of Ac1:Ac2 ratio and creatine phosphokinase concentration on the H₂:N₂ ratio in vitro. Conditions were as described for (a), with an initial MgATP concentration of 5 mM. Creatine phosphokinase additions: Δ, 5 μg; □, 100 μg; O, 300 μg.

Fig. 6. Densitometer traces after SDS-PAGE of cells from O₂-limited (a) and C-limited (b) continuous cultures. The positions of nitrogenase polypeptides were identified using purified proteins. The acrylamide concentration was 12.5% (w/v).

controls to test the possibility that other peptides may co-migrate with the nitrogenase proteins. No major bands co-migrated with Ac1 or Ac2 in O₂-, N₂- or C-limited cultures, but SO₄²⁻-limited cells contained a major peptide with a similar R_F to Ac2 (trace not shown).

Activity measurements. Titrations of crude extracts or supernatants from cultures grown under the different nutrient limitations against purified Ac1 or Ac2 showed little or no stimulation by Ac1 and usually a 3- to 7-fold stimulation by Ac2 (Fig. 7a). Titrations of purified Ac1 against Ac2 showed saturation of acetylene-reducing activity at between 10 and

Fig. 7. (a) Acetylene reduction by crude extracts with Ac1 or Ac2. Crude extracts of cells from an N₂-limited continuous culture were prepared and tested for acetylene reduction as described in Methods. The uninhibited extract contained 17.75 mg protein ml⁻¹. □, with increasing Ac2; ○, with increasing Ac1.

(b) Titration of inhibited extract or supernatant with Ac2. □, crude extract; ○, supernatant prepared by centrifuging the crude extract at 40 000 g for 20 min.

15 times the activity with equimolar Ac1 and Ac2 (M. G. Yates, unpublished data). From these observations we deduced that there was at least a 2-fold molar excess of Ac2 in the present *A. chroococcum* preparations.

Several crude extracts had very low intrinsic nitrogenase activities which were enhanced up to 50-fold by excess purified Ac2 (Fig. 7b). Supernatants derived by centrifuging these extracts had higher intrinsic activity but saturated at the same level with excess Ac2 (Fig. 7b). The results indicated that the crude extracts contained an easily sedimented or easily destroyed inhibitor of endogenous Ac2 activity. This inhibitory effect was usually destroyed by further disrupting the extract by passage through a French press.

DISCUSSION

The nitrogenase of *A. chroococcum* clearly diverts a substantial proportion of electrons to H₂ production rather than N₂ reduction. After inhibiting the uptake hydrogenase by pretreatment with acetylene, the proportion of total electron flux to nitrogenase which is diverted to H₂ production ranges from 11% in SO₄²⁻-limited cultures to between 80 and 90% in either O₂-limited or O₂-inhibited cultures.

Three separate mechanisms have been proposed to explain H₂ production by nitrogenase. (1) Nitrogenase will reduce protons to H₂ in the presence or absence of other reducible substrates. This proton-reducing site is distinguished from other substrate-reducing sites on nitrogenase by its insensitivity to CO. (2) H bound to a metal may act as a leaving group for the binding of N₂, e.g.

(Chatt, 1980; if N₂ or other reducible substrate were absent, then protons could also react with these H groups to give H₂). This mechanism could yield an overall reaction for N₂ reduction by nitrogenase as (Schrauzer, 1977):

(3) H₂ produced by nitrogenase may react with a diazene or di-imide-type intermediate of N₂ reduction to yield N₂ and a further molecule of H₂ (Schrauzer, 1977):

Evidence supporting mechanism (3) arises from the observations that (a) nitrogenase supports N₂-dependent H²H exchange (Bulen, 1976), (b) a dinitrogen hydride type intermediate occurs during N₂ fixation (Thorneley *et al.*, 1978) and (c) electron balance analysis shows that the 'H²H exchange' reaction yields a nett gain of H₂ (Newton *et al.*, 1977). All three H₂-producing reactions could occur simultaneously, but only mechanism (2) involves obligatory H₂ production to yield a minimum H₂:N₂ ratio of 1. H₂ production by mechanism (3) in aerobes would depend on mechanisms (1), (2) or exogenous H₂; in anaerobes the reversible, conventional hydrogenase produces H₂ which could react with a diazene-type intermediate of N₂ fixation. However, if mechanism (2) does not occur it is possible to achieve H₂:N₂ ratios of less than 1.

The evidence from O₂-, N₂- or C-limited cultures of *A. chroococcum* shows that on no occasion did the H₂:N₂ ratio drop below 1 after the cells were pretreated with acetylene to inhibit the H₂-uptake hydrogenase. On the other hand, SO₄²⁻-limited cells had H₂:N₂ ratios of 0.5, i.e. significantly below the minimum value of 1 necessary if mechanism (2) pertains. This pattern was observed both in cultures under specified nutrient limitations and in measurements of H₂ production over a range of O₂ concentrations by samples from these cultures. It follows that, if the evidence from the SO₄²⁻-limited bacteria reflects a general mechanism for N₂ fixation, then mechanism (2) does not occur in biological systems. However, H₂:N₂ ratios in vitro with Ac1 and Ac2 purified from SO₄²⁻-limited cells were significantly higher than those in vivo and H₂:N₂ ratios calculated from data present in the literature are always greater than 1 (Silverstein & Bulen, 1970; Shah *et al.*, 1975; Thorneley & Eady, 1977). N₂-fixing hydrogenase-negative mutants of *Klebsiella pneumoniae* also showed H₂:N₂ ratios greater than 1 (Andersen & Shanmugam, 1977) as did *A. chroococcum* grown under three of the four nutrient limitations tested here. Therefore the possibility of mechanism (2) prevailing in vitro and in many instances in vivo cannot be excluded.

The Ac1:Ac2 ratios in vivo, determined by activity and electrophoresis measurements, were similar and less than 1 under all four nutrient limitations. On the other hand, experiments in vitro showed that changes in ATP concentration affected the H₂:N₂ ratio only when the Ac1:Ac2 ratio was greater than 1. The marked increase in the H₂:N₂ ratio observed in all cases at low O₂ concentrations, when the ATP concentration limits nitrogenase activity (Haaker *et al.*, 1974), can be explained by assuming that Ac2 is partly inhibited to give an effective Ac1:Ac2 ratio of greater than 1 under these conditions. This inhibitor may be the sedimentable component present in crude extracts (Fig. 7*b*). Such inhibition must be relieved at the optimum O₂ concentration since H₂:N₂ ratios were close to 1 in samples from O₂- or C-limited cells and less than 1 in samples from SO₄²⁻-limited cells. The high H₂:N₂ ratio at high O₂ concentrations can be explained by assuming that either conformational protection partly inactivates Ac2 (Robson, 1979*b*; Scherings *et al.*, 1977) or that competition with O₂ limits electron flow to nitrogenase and effectively decreases the steady-state concentration of reduced Ac2.

There seems little doubt from this and other studies (Smith *et al.*, 1976; Walker & Yates, 1978) that the H₂-uptake hydrogenase recycles H₂ produced by nitrogenase in *A. chroococcum*. This enzyme is most active under C-limited conditions (Partridge *et al.*, 1980) but synthesis is also increased in O₂-limited cells by lowering the O₂ solution rate (C. C. Walker & M. G. Yates, unpublished data). Superficially, it would appear that these conditions are those in which recycling H₂ produced by nitrogenase would be of greatest advantage to the cells. However, the maximum ratio of electron flow to nitrogenase and O₂ was approximately 0.1 in C-, O₂- and SO₄²⁻-limited cells. Therefore, at best, recycled H₂ could

provide between 2.3 and 6.8% of the electrons for respiration in C- or O₂-limited cells and between 1.2 and 5% in SO₄²⁻-limited cells.

A more satisfactory approach to the evaluation of H₂ production by nitrogenase *in vivo* is to use uptake hydrogenase-negative mutants of *A. chroococcum* such as those obtained in *Rhizobium japonicum* (Maier *et al.*, 1978). Attempts to obtain such mutants in *A. chroococcum* are in progress.

Whether the observed differences in H₂:N₂ ratios relate to the molar growth yields of N₂-fixing bacteria is currently under investigation.

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