The Number of Hydrogenases in Cyanobacteria

By G. EISBRENNER, P. ROOS AND H. BOTHE*

*Botanisches Institut der Universität Köln, II. Lehrstuhl, Gyrhofstrasse 15, D-5000 Köln 41,
Federal Republic of Germany*

(Received 3 November 1980; revised 27 January 1981)

Cyanobacteria consume H_2 by two different pathways: the oxyhydrogen reaction and anaerobic, light-dependent H_2 utilization. The two pathways are shown here to be induced differently by incubating cyanobacteria anaerobically under H_2 . In the unicellular *Anacystis nidulans* and in N_2 - and NH_4^+ -grown *Anabaena cylindrica* and *Nostoc muscorum*, such treatment greatly enhances the activity of the oxyhydrogen reaction in all cell types. In contrast, the light-dependent pathway, determined by the H_2 -dependent photoreduction of $NADP^+$, is demonstrable with higher activity only in heterocysts.

Whereas the activity of the oxyhydrogen reaction is directly correlated to the structural integrity of membranes, there is an inverse correlation between membrane integrity and H_2 formation catalysed by hydrogenase. These findings, together with physiological considerations, suggest that a 'reversible' soluble hydrogenase does not exist in photoautotrophic cyanobacteria. No definite conclusions about the existence of two membrane-bound uptake hydrogenases are possible at present.

INTRODUCTION

The relationship between H_2 metabolism and N_2 fixation in cyanobacteria has been studied extensively (for reviews, see Bothe *et al.*, 1978; Hallenbeck & Benemann, 1979; Bothe & Eisebrenner, 1981). Cyanobacteria can evolve molecular H_2 in a nitrogenase-dependent reaction, as do other aerobic N_2 -fixing micro-organisms. The extent of such H_2 formation is still a matter of dispute, although most investigators now agree that maximal activity depends mainly on the culture and assay conditions employed, and less on the strain used. H_2 formation is not simply a waste of energy, since cyanobacteria re-utilize the gas evolved, by means of a hydrogenase. This enzyme is membrane-bound in all aerobic N_2 -fixing organisms and is unidirectional, catalysing only H_2 uptake in the intact cells. Recent evidence (Bothe *et al.*, 1977a; Eisebrenner *et al.*, 1978; Bothe & Eisebrenner, 1978; Eisebrenner & Bothe, 1979; see also Peterson & Burris, 1978; Peschek, 1979a, b, 1980; Tetley & Bishop, 1979) suggests that H_2 utilization proceeds by two different pathways in cyanobacteria. In the main pathway, the gas is consumed in an O_2 -dependent reaction, being coupled to electron flow and to ATP formation of respiration. This oxyhydrogen reaction is likely to minimize the loss of energy caused by nitrogenase-dependent H_2 formation. It may also remove O_2 from the nitrogenase site, thereby protecting the enzyme from damage by this gas. The second pathway of H_2 utilization strictly requires exclusion of O_2 and activation by light in cyanobacteria. This pathway is particularly seen in the H_2 -supported C_2H_2 reduction of carbon-limited cultures (Benemann & Weare, 1974; Bothe *et al.*, 1977a) or of isolated heterocysts (Eisebrenner *et al.*, 1978; Peterson & Wolk, 1978). Anaerobic, light-dependent H_2 utilization is also demonstrable with low activity in reactions independent of N_2 fixation (Bothe *et al.*, 1978; Eisebrenner & Bothe, 1979; Peschek, 1979a).

A controversy has recently arisen about the number of hydrogenases in cyanobacteria. The pathways may or may not be catalysed by two different uptake hydrogenases. In addition, it has been suggested that cyanobacteria contain a soluble 'reversible' hydrogenase, catalysing H_2 formation under physiological conditions (Tel-Or *et al.*, 1978; Daday *et al.*, 1979; Hallenbeck & Benemann, 1978, 1979). The existence of such an enzyme has been mainly deduced from the observation that the reversible, $Na_2S_2O_4$ - and methyl viologen-dependent activity is demonstrable in extracts of both heterocysts and vegetative cells, with essentially the same specific activity in the two cell types (Tel-Or *et al.*, 1978; Bothe *et al.*, 1978). In contrast, uptake hydrogenase(s) appear(s) to be restricted to N_2 -fixing cells (Tel-Or *et al.*, 1978; Peterson & Wolk, 1978).

The present investigation critically examines the experimental evidence for the occurrence of the soluble 'reversible' hydrogenase. It is concluded that such an enzyme is unlikely to exist in the photoautotrophic cyanobacteria. The distribution of the two pathways of H_2 utilization among vegetative cells and heterocysts is also described.

METHODS

Abbreviations. DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; MV, methyl viologen; PMS, phenazine methosulphate.

Organisms. The Sammlung von Algenkulturen of the Pflanzenphysiologisches Institut, University of Göttingen, F.R.G., supplied *Anabaena cylindrica* (no. 1403-2) and *Anacystis nidulans* (no. 1402-1). *Nostoc muscorum* strain 7119 was a kind gift of Dr D. I. Arnon, University of California, Berkeley, U.S.A. The cultures were grown either aerobically or under flushing $H_2/N_2/CO_2$ (20:75:5, by vol.) in the absence of combined nitrogen (Eisbrenner *et al.*, 1978). For NH_4^+ -grown cells the medium contained 40 mM- $NaNH_4HPO_4$; these cultures did not contain heterocysts.

Preparation of extracts. To prepare heterocysts from *Anabaena cylindrica* (see Eisbrenner *et al.*, 1978), the centrifuged cells were suspended in 30 mM-HEPES buffer pH 7.6 containing 5 mM- $MgCl_2$ and passed twice through a chilled French press at 1600 lbf in $^{-2}$ (1 lbf in $^{-2} \approx 7$ kPa). After centrifugation (1000 g, 5 min), the supernatant was used as the extract from vegetative cells. The pellet consisted almost exclusively of heterocysts (less than 2% of vegetative cells) and was suspended in the $MgCl_2$ /HEPES buffer. All manipulations were performed under Ar and in ice as far as possible. For enzyme determinations, the heterocysts were broken in the French press at 25 000 lbf in $^{-2}$.

Nostoc muscorum was suspended in $MgCl_2$ /HEPES buffer pH 7.6, passed twice through the French press at 1650 lbf in $^{-2}$ and centrifuged (1000 g, 5 min). The supernatant (vegetative cell extract) showed photosynthetic NADP $^+$ reduction with DCPIP/ascorbate as the electron donor (Table 2). Since this procedure left approximately 20% of the vegetative cells intact in the case of *Nostoc muscorum*, the pellet was suspended in the $MgCl_2$ /HEPES buffer, broken twice at 2400 lbf in $^{-2}$ and centrifuged (1000 g, 10 min). The pellet then consisted of heterocysts and less than 2% vegetative cells. This heterocyst preparation, suspended in $MgCl_2$ /HEPES buffer, was broken at 25 000 lbf in $^{-2}$, resulting in particles which showed both H_2 - and DCPIP/ascorbate-dependent NADP $^+$ reduction (Table 2).

Assays. NADP $^+$ reduction was performed in Fernbach flasks at 35 000 lx, 28 °C for 15 min. The assays (final vol. 2 ml) contained cyanobacterial particles (0.02 to 0.06 mg chlorophyll) and the following (in μ mol): HEPES buffer pH 7.6, 100; $MgCl_2$, 10; NADP $^+$, 3; and (where indicated) DCPIP, 0.3; sodium ascorbate, 20; DCMU (dissolved in dimethyl sulphoxide), 0.04. The gas phase consisted of Ar or (where indicated) Ar plus 10 mM- H_2 . The reactions were terminated by centrifugation (6000 g, 10 min), and the supernatant was diluted fivefold with water. Absorbing proteins were partly removed by adding an equal volume of a solution of saturated $(NH_4)_2SO_4$ followed by centrifugation (6000 g, 10 min). NADPH was then determined either by the difference in absorbance at 340 nm before and after the addition of 20 μ M-PMS, or in a Perkin-Elmer fluorescence spectrophotometer, using the emission band at 445 nm and excitation at 340 nm (Klingenberg, 1970).

Nitrogenase activity was determined by the C_2H_2 reduction method and H_2 evolution and uptake were followed in a gas chromatograph equipped with a thermal conductivity detector and a molecular sieve column (Bothe *et al.*, 1977b). O_2 consumption was measured in a Clark-type electrode either in the presence of NADPH or NADH or in the absence of any added electron donor. NADPH was generated by glucose and glucose-6-phosphate dehydrogenase and NADH by galactose and galactose dehydrogenase.

RESULTS

Distribution of uptake and 'reversible' hydrogenase among heterocysts and vegetative cells

Hydrogenase and nitrogenase activities were compared in heterocysts and vegetative cells of *Anabaena cylindrica* grown under H_2 (Table 1). Nitrogenase was almost exclusively confined to heterocysts. $Na_2S_2O_4$ - and MV-dependent H_2 evolution was independent of ATP and was, therefore, catalysed by hydrogenase. The latter reaction was observed with essentially the same specific activities in both cell types, in agreement with previous findings (Tel-Or *et al.*, 1978; Bothe *et al.*, 1978). Since heterocysts account for approximately 5% of the cells in the filaments, about 90% of the total H_2 evolution activity was associated with the extracts from vegetative cells. H_2 uptake occurred with much higher activity than evolution, also in agreement with Tel-Or *et al.* (1978). In contrast to their observations, however, it was strictly dependent on the addition of an electron acceptor, the most effective being PMS (see Bothe *et al.*, 1978). PMS-dependent H_2 consumption was readily demonstrated in extracts of both vegetative cells and heterocysts, with the major part of the overall activity associated consistently with the vegetative cells (Table 1). Per cell, the rate of the PMS-dependent H_2 uptake was about eight times higher in heterocysts than in vegetative cells. It should be noted, however, that the overall activities of both the $Na_2S_2O_4$ - and MV-dependent H_2 evolution and of the PMS-dependent uptake were subject to large variations from experiment to experiment, depending mostly on the procedure used to break the cells (see below). In contrast to PMS, O_2 hardly supported any H_2 uptake by extracts from vegetative cells of *A. cylindrica*.

Pathways of hydrogen utilization in heterocysts and vegetative cells

The oxyhydrogen reaction. O_2 -dependent H_2 uptake proceeds through the respiratory chain (Bothe *et al.*, 1977a; Peschek, 1979b) and has been claimed to be confined to heterocysts in *Nostoc muscorum* (Tel-Or *et al.*, 1978) and *Anabaena* species (Peterson & Wolk, 1978). However, breakage of vegetative cells could easily damage the respiratory particles and/or release essential components of the electron transport chain. Control experiments with intact filaments indicated that the respiratory endogenous O_2 uptake was inhibited 70% by 1 mM-KCN and that the oxyhydrogen reaction proceeded through the KCN-sensitive respiratory pathway (Bothe *et al.*, 1977a). Extracts from vegetative cells were able to consume only small amounts of O_2 , and this activity could not be enhanced by the addition of NADPH, NADH, or H_2 (results not shown). Moreover, this small O_2 uptake was not affected by KCN. Obviously the respiratory chain was, indeed, damaged in the extracts from vegetative cells. Therefore, experiments with cell extracts do not allow any conclusion on the occurrence of the oxyhydrogen reaction or of the uptake hydrogenase in vegetative cells.

The oxyhydrogen reaction could be demonstrated unequivocally, in cells other than heterocysts, in NH_4^+ -grown *Anabaena cylindrica* and *Nostoc muscorum* or in the unicellular *Anacystis nidulans*. Such uniform cultures of vegetative cells consumed only small amounts of H_2 , often scarcely detectable, when grown aerobically (see Eisbrenner *et al.*, 1978). However, when grown anaerobically in the presence of NH_4^+ and H_2 , intact cells of all three cyanobacteria rapidly utilized H_2 in a strictly O_2 -dependent reaction in the dark (Fig. 1). Rates of the oxyhydrogen reaction in such cultures considerably exceeded those of endogenous respiration. It can be deduced that the uptake hydrogenase is also induced or activated in vegetative cells of N_2 -grown *Anabaena cylindrica* and *Nostoc muscorum* by incubating the cultures with H_2 . Under aerobic growth conditions, the activity of the oxyhydrogen reaction is probably low in vegetative cells but not in heterocysts, in agreement with Peterson & Wolk (1978).

The anaerobic, photosynthetic pathway of H_2 utilization. The preparation of particles from *Nostoc muscorum* performing light-dependent NADP⁺ reduction permitted the measurement of the rates of H_2 utilization by this pathway in both heterocysts and vegetative cells

Table 1. *Distribution of nitrogenase and hydrogenase in heterocysts and vegetative cells of Anabaena cylindrica*

Anabaena cylindrica was grown for 3 d anaerobically under H_2 before assay. The total activities were determined in an extract prepared from 11 ml of a centrifuged culture; they are given in $nmol\ h^{-1}$. Specific activities are expressed as $\mu mol\ h^{-1}\ (mg\ chlorophyll)^{-1}$.

Assay	Crude extract	Heterocysts	Vegetative cell extract
Nitrogenase			
Specific activity	0.86	5.0	0.04
Total activity per assay	779	625	23
Activity as % of total	100	80	3
H_2 evolution by hydrogenase ($Na_2S_2O_4$ - and MV-dependent)			
Specific activity	2.1	2.0	3.1
Total activity per assay	1935	245	1740
Activity as % of total	100	12	89
H_2 uptake by hydrogenase (PMS-dependent)			
Specific activity	24.6	53.8	25.2
Total activity per assay	22175	6720	13860
Activity as % of total	100	30	62

Fig. 1. The oxyhydrogen reaction in intact cells of *Anacystis nidulans* (Δ), *Anabaena cylindrica* ($\circ$) and *Nostoc muscorum* ($\bullet$) grown on NH_4^+ . The cyanobacteria were grown for 2 d under $H_2/N_2/CO_2$ (20:75:5, by vol.) before assay. The gas phase in the assay vessels consisted of Ar plus 10 mm- H_2 , and O_2 as indicated.

(Table 2). NADPH formation by illuminated particles from vegetative cells was achieved with H_2O as the electron donor, and the reaction was severely inhibited by DCMU, indicating the involvement of both photosystems. H_2 produced virtually no enhancement of $NADP^+$ reduction in either the absence or presence of DCMU, indicating that H_2 did not substitute for H_2O in vegetative cells of *Nostoc muscorum* grown in the absence of combined nitrogen or with NH_4^+ , either aerobically or anaerobically in the presence of H_2 . In contrast, with particles from heterocysts, $NADP^+$ reduction was obtained with DCPIP/ascorbate but not with H_2O as the electron donor, in agreement with the well-documented absence of photosystem II in the heterocysts. H_2 significantly supported $NADP^+$ reduction by particles from heterocysts, with rates being approximately 50% of those obtained with DCPIP/ascorbate. The addition of ferredoxin to all particles only slightly increased the overall activity.

Table 2. H_2 -dependent $NADP^+$ reduction by particles from heterocysts and vegetative cells of *Nostoc muscorum*

Potential electron donor	Inhibitor	Activity [$\mu\text{mol h}^{-1} (\text{mg chlorophyll})^{-1}$]		
		N_2 -grown cells		NH_4^+ -grown cells
		Vegetative cells	Heterocysts	
H_2O	—	40	1.6	22
H_2O	DCMU	1.4	0.5	2.4
H_2O, H_2	—	38	22	28
H_2O, H_2	DCMU	2.1	21	4.4
DCPIP/ascorbate	—	46	54	53
DCPIP/ascorbate	DCMU	43	56	48

Fig. 2. H_2 formation and H_2 uptake by *Anacystis nidulans* grown anaerobically in the presence of H_2 . The cultures were grown under $H_2/N_2/CO_2$ (20:75:5, by vol.). The oxyhydrogen reaction (○) was measured in intact cells. $Na_2S_2O_4$ - and MV-dependent H_2 evolution (●) and PMS-dependent H_2 uptake (△) were determined in crude extracts obtained by French press treatment ($25\,000\text{ lbf in}^{-2}$). For further details see Bothe *et al.* (1980). Note the difference in the scales for H_2 evolution and H_2 uptake.

The nature of the 'reversible' hydrogenase

Do heterocystous cyanobacteria contain a 'reversible' soluble hydrogenase in both cell types in addition to the membrane-bound uptake hydrogenase(s)? To avoid complications associated with enzyme distribution among different cell types this question was first explored in the unicellular, non-heterocystous *Anacystis nidulans*. Both the oxyhydrogen reaction and $Na_2S_2O_4$ - and MV-dependent H_2 formation were observed at low activity in aerobically grown cultures. Growth in the presence of H_2 induced both H_2 uptake activity, assayed either with PMS or O_2 , and H_2 evolution activity, essentially in a parallel fashion (Fig. 2). Such parallelism may be taken as an indication of the existence of only one enzyme catalysing both activities; on the other hand, a coincidental induction of both enzymes under H_2 cannot entirely be ruled out. Evolution and uptake activities were then compared in extracts of *Anacystis nidulans* obtained by various breakage procedures (Table 3). In unbroken cells, the $Na_2S_2O_4$ - and MV-dependent H_2 evolution was low, but clearly demonstrable (see also Daday *et al.*, 1979). This activity was strictly dependent on $Na_2S_2O_4$ and MV and increased more, the more drastic the method used to break the cells. Highest activities were obtained upon treatment of the cells with high pressure ($28\,000\text{ lbf in}^{-2}$) in the French press or by sonication at maximal power. In contrast, both PMS- and O_2 -dependent H_2 uptake activities decreased more, the more drastic the treatment to disrupt the membranes (Table 3). The oxyhydrogen

Table 3. *Effect of different breakage methods on hydrogenase activities in Anacystis nidulans*

Anacystis nidulans grown under H₂/N₂/CO₂ (20:75:5, by vol.) for 6 d was assayed in Fernbach flasks containing (final vol. 4 ml) cells or cell extracts (0.08 to 0.12 mg chlorophyll) and 100 µmol HEPES buffer pH 7.6. For H₂ evolution, the gas phase was Ar and 60 µmol Na₂S₂O₄ and 20 µmol MV were present. For H₂ uptake, the gas phase consisted of Ar plus 10 mM-H₂, and 10 mM-O₂ or 40 µmol PMS was present as indicated. The experiments were performed in the dark at 28 °C for 1 to 4 h.

Treatment*	Activity [µmol h ⁻¹ (mg chlorophyll) ⁻¹]		
	H ₂ evolution (Na ₂ S ₂ O ₄ - and MV-dependent)	H ₂ uptake (PMS-dependent)	H ₂ uptake (O ₂ -dependent)
None	4.7	238	148
Lysozyme	5.0	167	145
French press (1000 lbf in ⁻²)	4.7	215	110
French press (28 000 lbf in ⁻²)	13.4	181	65
Sonication (40 W)	5.8	155	123
Sonication (130 W)	11.1	114	78

* Lysozyme treatment was done with 1 mg lysozyme (ml cells)⁻¹, incubated anaerobically with shaking at 30 °C for 30 min. Sonication was done 6 times for 1 to 2 s at 40 W or 40 times for 1 to 2 s at 130 W.

Table 4. *Effect of treatment with Triton X-100 on hydrogenase activities in filaments and isolated heterocysts of Anabaena cylindrica*

Aerobically grown *Anabaena cylindrica* was treated, where indicated, by incubation for 15 min with 1% (v/v) Triton X-100. Heterocysts were prepared as described in Methods. Details of the assay conditions are given in the legend to Table 3.

	Activity [µmol h ⁻¹ (mg chlorophyll) ⁻¹]		
	H ₂ evolution (Na ₂ S ₂ O ₄ - and MV-dependent)	H ₂ uptake (no addition)	H ₂ uptake (PMS-dependent)
Whole filaments, untreated	5.8	1.0	5.2
Filaments, treated	8.1	0.9	2.3
Isolated heterocysts, untreated	3.2	2.4	14.3
Isolated heterocysts, treated	7.4	0.6	3.8

reaction was particularly sensitive to the method of cell breakage, presumably due to its dependence on the respiratory chain.

Similar results were obtained with *Anabaena cylindrica* (Table 4). When intact filaments or isolated heterocysts were treated with the detergent Triton X-100, H₂ uptake activity with PMS greatly decreased, whereas the Na₂S₂O₄- and MV-dependent H₂ evolution activity increased. In all cases the highest H₂ formation rates were obtained when the most drastic methods were applied.

DISCUSSION

A 'reversible' soluble hydrogenase, catalysing H₂ evolution in vivo, has repeatedly been claimed to occur in cyanobacteria (Tel-Or *et al.*, 1978; Hallenbeck & Benemann, 1978, 1979; Daday *et al.*, 1979). The results presented here indicate that the specific activities of this 'reversible' hydrogenase in preparations are largely dependent on the isolation procedures. The 'reversible' hydrogenase appears to be an integral membrane protein participating in one or both pathways of H₂ utilization. When bound to membranes, it may scarcely be accessible to Na₂S₂O₄ and MV, presumably due to the 'sidedness' of the membranes. When the hydrogenase is solubilized, H₂ utilization can proceed only in the presence of a suitable electron acceptor (PMS), but no longer via the photosynthetic and respiratory pathways, since the electron transport chains are disrupted. Conversely, a physiological, unidirectional,

membrane-bound uptake hydrogenase should catalyse the reverse reaction when solubilized, provided the assays are supplemented with the suitable concentrations of reductants. An increase in H_2 formation activities upon solubilization of hydrogenase has also been observed for other organisms (e.g. Sim & Vignais, 1979) and for *Anacystis* by Peschek (1979 *b*).

It is difficult to find a physiological role for a H_2 -evolving hydrogenase in *Anabaena cylindrica*, *Nostoc muscorum* or *Anacystis nidulans* normally grown under photoautotrophic conditions. These cyanobacteria respire and do not need to remove excess reductant by forming H_2 . Formation of H_2 by intact photoautotrophic cyanobacteria is catalysed by nitrogenase and not (or at best to a minuscule extent) by hydrogenase. Among aerobic, N_2 -fixing micro-organisms a soluble hydrogenase, catalysing H_2 evolution, would be a unique enzyme of cyanobacteria which is not observed in *Azotobacter*, *Rhizobium* and others. H_2 formation by such an enzyme would require an electron donor system with a low potential redox couple (at least -400 mV); all hydrogenases isolated from cyanobacteria fail to couple to ferredoxin, flavodoxin or other natural low potential electron carriers. All these arguments rule out the existence of a soluble hydrogenase in *Anacystis nidulans*, *Anabaena cylindrica* or *Nostoc muscorum* capable of acting in vivo at significant rates. It is possible, however, that part of the enzyme is more tightly bound to membranes and part is more readily washed off. In addition, the structural integrity of the membranes which bind hydrogenase may vary from organism to organism.

All the aforementioned evidence indicates that the H_2 -evolving hydrogenase is an artefact of disruption procedures. Similarly, the relatively high H_2 evolution activity found in extracts from vegetative cells is likely to be artificial. Vegetative cells more readily break than heterocysts, and this fragility may release the hydrogenase of vegetative cells into the solubilized state where it is more accessible to $Na_2S_2O_4$ and MV. Moreover, with all the isolation procedures for vegetative cells, some heterocysts break and may leak out hydrogenase. Under aerobic growth conditions, the oxyhydrogen reaction (in agreement with Peterson & Wolk, 1978) and the light-dependent, anaerobic pathway of H_2 uptake (as determined by H_2 -dependent NADPH formation in this communication) is readily demonstrable in heterocysts and only to a limited extent in vegetative cells of *Anabaena cylindrica* and *Nostoc muscorum* and in *Anacystis nidulans*.

The two pathways of H_2 utilization are apparently affected in a different way by incubating the cells with H_2 . The oxyhydrogen reaction is then readily demonstrable in *Anacystis nidulans* and in NH_4^+ -grown *Anabaena cylindrica* and *Nostoc muscorum* (Fig. 1) and thus independently of nitrogen fixation. The rate of the oxyhydrogen reaction exceeds that of endogenous respiration; this cannot adequately be explained at present (see also Peschek, 1979 *b*). In contrast, incubation of the cells under H_2 does not significantly enhance H_2 consumption by the light-dependent, anaerobic pathway. H_2 only poorly supports $NADP^+$ reduction by particles from vegetative cells of either N_2 - or NH_4^+ -grown *Nostoc muscorum* (this communication). Consequently, H_2 virtually does not enhance CO_2 fixation in photoreduction experiments (Bothe & Eisbrenner, 1978), in agreement with recent observations (but not interpretations) of Peschek (1979 *a*). H_2 -dependent CO_2 fixation by the above mentioned cyanobacteria never exceeds 5% of the H_2O -dependent activity, and the organisms are unable to grow photosynthetically with H_2 as the electron donor (in DCMU-treated cultures) or in the dark. In heterocyst preparations, H_2 is the best electron donor for C_2H_2 reduction (Eisbrenner *et al.*, 1978; Peterson & Wolk, 1978) and significantly supports $NADP^+$ reduction (this communication). Since heterocysts lack ribulose-1,5-bisphosphate carboxylase, they cannot perform H_2 -dependent CO_2 fixation.

Are the two pathways of H_2 utilization catalysed by functionally distinct uptake hydrogenases? The observation that incubation under H_2 alters the pathways differentially may be taken as positive evidence, in agreement with recent findings by Peschek (1979 *a, b*). On the other hand, Eisbrenner & Bothe (1979) suggested from inhibitor studies that plastoquinone is a common intermediate of both pathways located on the thylakoid

membranes. Such a concept of electron carriers common to respiration and photosynthesis has recently been substantiated by the observation that plastocyanin and cytochrome c_{553} participate in both photosynthetic and respiratory activities of *Anabaena variabilis* (Lockau, 1981). If such a concept is correct, H_2 consumption by both pathways would necessitate only a single hydrogenase but would be subject to different, still unknown, control mechanisms. At present it is safer to speak about two pathways of H_2 utilization until the occurrence of only one or of two uptake hydrogenases has unambiguously been demonstrated.

Note added in proof. Recently, evidence has been presented for the so-called reversible hydrogenase (Hallenbeck *et al.*, 1981). The reported activity is, however, less than $1 \mu\text{mol } H_2 \text{ evolved h}^{-1} (\text{g dry wt})^{-1}$. Any membrane-bound uptake hydrogenase should be able to catalyse such extremely poor H_2 -evolving rates.

This work was kindly supported by grants from the Deutsche Forschungsgemeinschaft. The authors are indebted to Miss B. Klein for skilful assistance.

REFERENCES

- BENEMANN, J. R. & WEARE, N. M. (1974). Nitrogen fixation by *Anabaena cylindrica*. III. Hydrogen supported nitrogenase activity. *Archives of Microbiology* **101**, 401–408.
- BOTHE, H. & EISBRENNER, G. (1978). Aspects of hydrogen metabolism in blue-green algae. In *Hydrogenases, their Catalytic Activity, Structure and Function*, pp. 353–369. Edited by H. G. Schlegel & K. Schneider. Göttingen: Verlag Erich Goltze.
- BOTHE, H. & EISBRENNER, G. (1981). The hydrogenase–nitrogenase relationship in nitrogen fixing organisms. In *Biological Nitrogen and Sulfur Metabolism*, pp. 141–150. Edited by H. Bothe & A. Trebst. Berlin: Springer.
- BOTHE, H., TENNIGKEIT, J. & EISBRENNER, G. (1977a). The utilization of molecular hydrogen by the blue-green alga *Anabaena cylindrica*. *Archives of Microbiology* **114**, 43–49.
- BOTHE, H., TENNIGKEIT, J., EISBRENNER, G. & YATES, M. G. (1977b). The hydrogenase–nitrogenase relationship in the blue-green alga *Anabaena cylindrica*. *Planta* **133**, 237–242.
- BOTHE, H., DISTLER, E. & EISBRENNER, G. (1978). Hydrogen metabolism in blue-green algae. *Biochimie* **60**, 277–289.
- DADAY, A., LAMBERT, G. R. & SMITH, G. D. (1979). Measurement in vivo of hydrogenase-catalysed hydrogen evolution in the presence of nitrogenase enzyme in cyanobacteria. *Biochemical Journal* **177**, 139–144.
- EISBRENNER, G. & BOTHE, H. (1979). Modes of electron transfer from molecular hydrogen in *Anabaena cylindrica*. *Archives of Microbiology* **123**, 37–45.
- EISBRENNER, G., DISTLER, E., FLOENER, L. & BOTHE, H. (1978). The occurrence of the hydrogenase in cyanobacteria. *Archives of Microbiology* **118**, 177–184.
- HALLENBECK, P. C. & BENEMANN, J. R. (1978). Characterization and partial purification of the reversible hydrogenase of *Anabaena cylindrica*. *FEBS Letters* **94**, 261–264.
- HALLENBECK, P. C. & BENEMANN, J. R. (1979). Hydrogen from algae. In *Topics in Photosynthesis*, vol. 3, pp. 333–364. Edited by J. Barber. Amsterdam: Elsevier/North Holland Biomedical Press.
- HALLENBECK, P. C., KOCHIAN, L. V. & BENEMANN, J. R. (1981). Hydrogen evolution catalysed by hydrogenase in cultures of cyanobacteria. *Zeitschrift für Naturforschung* **36c**, 87–92.
- KLINGENBERG, M. (1970). Nicotinamid-adenin-dinucleotide (NAD, NADP, NADH, NADPH). Spektrophotometrische und fluorimetrische Verfahren. In *Methoden der Enzymatischen Analyse*, 2nd edn, vol. 2, pp. 1975–2004. Edited by H. U. Bergmeyer. Weinheim: Verlag Chemie.
- LOCKAU, W. (1981). Evidence for a dual role of cytochrome c_{553} and plastocyanin in photosynthesis and respiration of the cyanobacterium *Anabaena variabilis*. *Archives of Microbiology* **128**, 336–340.
- PESCHEK, G. A. (1979a). Anaerobic hydrogenase activity in *Anacystis nidulans*: H_2 -dependent photo-reduction and related reactions. *Biochimica et biophysica acta* **548**, 187–202.
- PESCHEK, G. A. (1979b). Aerobic hydrogenase activity in *Anacystis nidulans*: the oxyhydrogen reaction. *Biochimica et biophysica acta* **548**, 203–215.
- PESCHEK, G. A. (1980). Restoration of respiratory electron transport reactions in quinone-depleted particle preparations from *Anacystis nidulans*. *Biochemical Journal* **186**, 515–523.
- PETERSON, R. B. & BURRIS, R. H. (1978). Hydrogen metabolism in isolated heterocysts of *Anabaena* 7120. *Archives of Microbiology* **116**, 125–132.
- PETERSON, R. B. & WOLK, C. P. (1978). Localization of an uptake hydrogenase in *Anabaena*. *Plant Physiology* **61**, 688–691.
- SIM, E. & VIGNAIS, P. M. (1979). Comparison of the membrane-bound and detergent-solubilized hydrogenase from *Paracoccus denitrificans*. *Biochimica et biophysica acta* **570**, 43–55.
- TEL-OR, E., LUIK, L. W. & PACKER, L. (1978). Hydrogenase in N_2 -fixing cyanobacteria. *Archives of Biochemistry and Biophysics* **185**, 185–194.
- TETLEY, R. M. & BISHOP, N. I. (1979). The differential action of metronidazole on nitrogen fixation, hydrogen metabolism, photosynthesis and respiration in *Anabaena* and *Scenedesmus*. *Biochimica et biophysica acta* **546**, 43–53.