The Uptake and Production of Molecular Hydrogen by Unicellular Cyanobacteria

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Nine strains of unicellular non-nitrogen-fixing cyanobacteria had high rates of hydrogen evolution and uptake compared with filamentous nitrogen-fixing strains. Maximal hydrogen uptake activities were observed in assays including oxygen under both light and dark conditions suggesting the participation of the 'Knallgas' reaction. Nitrogenase activity, measured as acetylene reduction, was not found in aerobic cultures of any of the unicellular strains tested, and the capacity to synthesize nitrogenase under anaerobic conditions was absent in all five of the strains tested under these conditions. Thus the capacity to synthesize nitrogenase is not a pre-requisite for hydrogenase synthesis and hydrogen evolution by these unicellular cyanobacteria. Rates of hydrogen evolution and uptake by six of the unicellular strains were increased following adaptation with CO₂/H₂ gas mixtures.

INTRODUCTION

Hydrogenase, which reversibly activates molecular hydrogen, is believed to be present whenever hydrogen is evolved or consumed by micro-organisms, the notable exception being the ATP-dependent evolution of hydrogen observed under certain physiological conditions, which is catalysed by nitrogenase (Stewart, 1980; Bothe, 1982).

Gaffron & Rubin (1942) reported hydrogen formation by an anaerobically incubated culture of the green alga *Scenedesmus obliquus*, and though a reversible hydrogenase was demonstrated in a unicellular cyanobacterium, *Synechococcus elongatus*, following anaerobic pre-incubation (Frenkel *et al.*, 1950), green algae were long preferred to cyanobacteria for experimental work.

Hydrogen formation due to a cyanobacterial nitrogenase *in vitro* was first demonstrated in the nitrogen-fixing cyanobacterium, *Anabaena cylindrica* (Haystead *et al.*, 1970), and it is generally agreed that nitrogenase-mediated hydrogen evolution in cyanobacteria and other photosynthetic bacteria are similar processes which have all the characteristics of a nitrogenase system requiring light-generated ATP (light-dependence on photosystem I only, sensitivity to uncouplers of photophosphorylation and inhibition by nitrogen but not carbon monoxide). Hydrogen production by nitrogen-fixing cells and nitrogen fixation (acetylene reduction) decrease in parallel when fixed nitrogen is added to the culture medium (Stewart, 1980), and the use of carbon monoxide and acetylene at concentrations shown to inhibit uptake hydrogenase activities has provided evidence that a natural function of hydrogenase may be to recycle hydrogen lost by nitrogenase, thereby reducing the energy loss inherent in this process.

As hydrogen evolution due to nitrogenase action is irreversible, all known cyanobacterial hydrogen uptake reactions are presumed to be mediated by hydrogenase. Hydrogen uptake has been shown to occur filamentous cyanobacteria, irrespective of whether they fix nitrogen, and Tel-Or *et al.* (1977) demonstrated an increase in hydrogenase activity in *Anabaena cylindrica* and *Nostoc muscorum* cultures grown under hydrogen. Eisbrenner *et al.* (1978) reported similar

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Abbreviations: chl, chlorophyll *a*; DCMU, *N*-(3,4-dichlorophenyl)-*N,N*-dimethylurea.

results in a range of cyanobacteria including a unicellular non-nitrogen-fixer, *Anacystis nidulans* (*Synechococcus*). Evidence suggests that cyanobacterial hydrogenase is multifunctional as envisaged by Dixon (1972) for aerobic nitrogen-fixing organisms such as rhizobia. In cyanobacteria, the oxygen-dependent hydrogen uptake proceeds via the respiratory chain, providing additional ATP for cellular metabolism (Bothe *et al.*, 1977*b*; Peterson & Burris, 1978), an additional supply of reducing equivalents for nitrogen fixation and the removal of oxygen from the nitrogenase site, thereby protecting the enzyme from damage by this gas (Bothe *et al.*, 1977*b*; Gallon, 1981).

Peschek (1979*a,b,c*) has studied hydrogen metabolism in *Anacystis nidulans* 1402-1 (*Synechococcus* 6301) and his results indicate a membrane-bound hydrogenase involved in phosphorylative electron flow from hydrogen to oxygen via the respiratory chain, and a membrane-bound 'photoreduction' hydrogenase initiating a light-driven electron flow from hydrogen to various low potential acceptors, including endogenous ferredoxin, though as yet it is unclear whether two distinct functional enzymes are involved. Other work on hydrogen metabolism by unicellular cyanobacteria has involved the use of field isolates selected for hydrogen evolution due to nitrogenase, although pure cultures were not used (Berchtold & Bachofen, 1979). This communication details studies on hydrogen metabolism in nine pure cultures of unicellular cyanobacteria which do not exhibit nitrogenase activity.

METHODS

Cyanobacteria and culture conditions. The following unicellular strains were obtained from the Institut Pasteur, Paris (earlier names are given in parentheses, see Rippka *et al.*, 1979): *Synechococcus* PCC 6307 (*Coccochloris peniocystis*), *Synechococcus* PCC 6301 (*Anacystis nidulans*), *Synechocystis* 6714 (*Aphanocapsa* sp.), *Synechocystis* 6308 (*Aphanocapsa* and *Gloeocapsa alpicola*), *Gloeobacter* 7421, *Microcystis* 7820. *Synechococcus* strains 6308 and 602 were a gift from Dr S. V. Shestakov, Moscow State University, USSR, and *Aphanocapsa montana* was provided by Dr R. A. Lewin, Scripps Institute of Oceanography, La Jolla, Calif., USA.

All strains were grown axenically and photoautotrophically in BG11 medium (Stanier *et al.*, 1971), which contained (g l⁻¹): NaNO₃ (1.5), K₂HPO₄ (0.04), MgSO₄·7H₂O (0.075), CaCl₂·2H₂O (0.036), citric acid (0.006), ferric ammonium citrate (0.006), EDTA (magnesium salt) (0.001), Na₂CO₃ (0.02), and a trace metals solution at a final concentration of 1 ml l⁻¹. The trace metals solution contained (g l⁻¹): H₃BO₃ (2.86), ZnSO₄·7H₂O (0.222), Na₂MoO₄·2H₂O (0.39), CuSO₄·5H₂O (0.79), and Co(NO₃)₂·6H₂O (0.049). Following autoclaving and cooling, the pH of the medium was 7.1. Cultures were grown in Erlenmeyer flasks at 25 to 30 °C and a photon fluence rate of 20 µmol m⁻² s⁻¹, which was provided at the surface of the flasks by white fluorescent tubes. All cultures were continuously agitated on an MSE orbital shaker. For CO₂/H₂ adaptation experiments, cells were grown in Quickfit flasks, equipped with two-way adaptors and air filters, in an identical manner to aerobically-grown cultures. Cultures were also maintained and checked for purity on agar plates (final concentration 0.8%, w/v) prepared by mixing autoclaved double strength Oxoid Technical Agar no. 3 with autoclaved double strength BG11 medium in equal proportions.

Measurement of hydrogen uptake by whole cells. The uptake of molecular hydrogen was measured using 5 ml cells in gas-tight 30 ml McCartney bottles. The bottles were flushed with argon for 20 min and hydrogen was syringed into the bottles to start the reaction. Hydrogen uptake rates were linear throughout the incubation period for up to 4 h, and were measured by injecting 0.25 ml gas samples into a Perkin-Elmer F-30 gas liquid chromatograph equipped with a molecular sieve column and a thermal conductivity detector with argon as the carrier gas. The bottles were shaken under light at a photon fluence rate of 20 to 30 µmol m⁻² s⁻¹.

Measurement of hydrogen evolution by whole cells. Hydrogen evolution was measured using 3 ml cell suspensions in gas-tight Bijou bottles of approximate internal volume 7.8 ml under an atmosphere of argon. The suspensions were flushed with argon for 20 min and the bottles shaken under light at a photon fluence rate of 20 to 30 µmol m⁻² s⁻¹. 1 ml gas samples were removed after a given time period and assayed using the Perkin-Elmer gas liquid chromatograph. Rates of hydrogen evolution were observed to be linear for at least 5 to 6 h.

Measurement of nitrogenase activity. The acetylene reduction method (Stewart *et al.*, 1967) was used. Experiments were done in Bijou bottles under argon. Cell suspensions (3 ml) were routinely flushed with argon for 20 min, acetylene of a given concentration was syringed into the bottle, and the bottles were continuously agitated under a photon fluence rate of 20 to 30 µmol m⁻² s⁻¹ for light assays. Gas samples of known volume were assayed using a Varian gas liquid chromatograph, model 940 (Varian Associates, Walton-on-Thames, UK), equipped with a flame ionization detector and a Porapak R column. Results were calculated using both the acetylene and ethylene peaks obtained from the chromatograph. Cells were grown in BG11 medium containing nitrate and resuspended in either fresh BG11 containing nitrate or BG11 medium without nitrate for the determination of acetylene reduction activity. Thus nitrogenase activity was tested both in the presence and absence of combined nitrogen.

Attempts were made to induce nitrogenase synthesis and activity in the unicellular cyanobacteria by imposing anaerobiosis as described by Rippka & Waterbury (1977).

Lysozyme treatment of cells. In certain experiments, cells were treated with lysozyme in an attempt to permit the entry of inhibitors. Cell suspensions were incubated with lysozyme (Grade 1, Sigma), at a final concentration of 1 mg per ml cyanobacterial culture, for 45 min at 25 to 30 °C with constant shaking.

Adaptation of cultures with molecular hydrogen. Cultures were grown under a gas mixture of 5% (v/v) carbon dioxide in hydrogen, before being grown aerobically for 4 d and then transferred back to growth under 5% carbon dioxide in hydrogen.

Other methods. The growth of cultures was monitored by measuring their optical density using an EEL colorimeter with a red OR1 filter. Chlorophyll *a* concentrations were measured by methanol extraction (MacKinney, 1941). Dry weight determinations were made by filtering 5 ml cell suspensions onto a pre-dried, pre-weighed Whatman GF/C filter disc and drying to a constant weight at 60 °C.

Chemicals and gases. Lysozyme, antimycin A and rotenone were obtained from Sigma and all other chemicals were from BDH. All gases were from Air Products, Glasgow, UK, except acetylene and ethylene which were obtained from the British Oxygen Company.

RESULTS

Uptake of molecular hydrogen

Experiments were done with *Synechococcus* strains 6803 and 6301 to elucidate the mechanism of hydrogen uptake by these organisms. Hydrogen was consumed in an oxygen-requiring 'Knallgas' type of reaction with an optimal oxygen concentration between 1.0 and 1.5 mmol l⁻¹. Additionally, experiments in which the hydrogen concentration was varied showed the optimum to be about 1.0 mmol l⁻¹. Hence for the measurement of hydrogen uptake by whole cells 1.05 mmol l⁻¹ hydrogen and 1.05 mmol l⁻¹ oxygen were used throughout. Table 1 shows the extent of hydrogen uptake by the nine strains grown photoautotrophically in pure culture. All the strains consumed molecular hydrogen, though variation occurred between strains. Although the rates observed with certain strains were low, these rates were reproducible and were found to be linear over 5 h. Certain strains (*Microcystis* 7820, *Synechococcus* strains 6307 and 6301) had low rates of hydrogen uptake, whereas others (e.g. *Synechococcus* strains 6803 and 602, *Synechocystis* 6308 and *Gloeobacter* 7421) had higher rates of hydrogen uptake. All the strains were cultured on BG11 medium containing nitrate. They have been shown by Rippka and co-workers (see Rippka *et al.*, 1979), and in this paper, not to possess detectable nitrogenase activity. Maximal hydrogen uptake activities were usually observed in the presence of oxygen, either under light or dark conditions, suggesting the participation of the 'Knallgas' reaction. Little or no hydrogen uptake was observed in assays where the concentration of oxygen was low, i.e. in the presence of DCMU in the light and in dark assays without oxygen addition. Rates of hydrogen consumption by *Synechococcus* 6301 were higher than the value of 6 µmol H₂ h⁻¹ (mg chl)⁻¹ reported earlier for light plus added oxygen-dependent uptake by this strain (Eisbrenner *et al.*, 1981), though rates of hydrogen uptake and evolution by cyanobacteria reported by different laboratories often vary and explanations have been proposed to account for this (Bothe *et al.*, 1978).

Effect of selected electron transport inhibitors on hydrogen uptake

As the results in Table 1 suggested the participation of a 'Knallgas' type reaction, presumably involving a membrane-bound respiratory electron transport chain, the action of the electron transport chain inhibitors antimycin A, rotenone and KCN was tested on hydrogen uptake by *Synechococcus* strains 6803 and 6301. Experiments were done with lysozyme-treated cells (see Methods) to facilitate the entry of inhibitors into the cells, and with control cells (not lysozyme-treated). Rates of hydrogen uptake by both strains were higher in lysozyme-treated cells than in untreated cells (Table 2), and the treated cells were used to determine the extent of inhibition of hydrogen uptake. Respiratory chains have to date been little characterized in cyanobacteria, although it is safe to assume that they possess segments of the respiratory chain in common with mitochondria and other bacteria (Gel'man *et al.*, 1967; Lemberg & Barrett, 1973). KCN, an inhibitor of terminal respiratory cytochrome oxidase, completely inhibited hydrogen uptake by both *Synechococcus* strains. Rotenone, a flavoprotein inhibitor (Chance & Hollunger, 1963),

Table 1. *Uptake of molecular hydrogen by nine strains of unicellular cyanobacteria*

All assays were done in duplicate or quadruplicate. Hydrogen uptake was monitored as the disappearance of hydrogen from the gas phase. Concentrations of both hydrogen and oxygen in the assay were both initially 1.05 mmol l^{-1} . DCMU was added to a final concentration of $5.4 \times 10^{-6} \text{ mol l}^{-1}$. Boiled cell controls were done for each of the nine strains and the values obtained (approximately 5% of the rates found with living cells) were subtracted from the values obtained in each of the nine assays. Chlorophyll *a* concentrations in all experiments were between 3 and $5 \mu\text{g ml}^{-1}$. The photon fluence rate was $30 \mu\text{mol m}^{-2} \text{ s}^{-1}$. ND, Not determined.

Strain	Hydrogen uptake [$\mu\text{mol H}_2 \text{ h}^{-1} (\text{mg chl})^{-1}$]				
	Light only	Light + O ₂	Light + DCMU	Dark only	Dark + O ₂
<i>Synechococcus</i> 6803	42.22	33.38	10.76	12.76	14.73
<i>Synechococcus</i> 602	0	32.40	ND	0	29.46
<i>Synechococcus</i> 6301	0.98	12.76	3.92	13.74	14.73
<i>Synechococcus</i> 6307	13.74	16.69	ND	0	2.94
<i>Microcystis</i> 7820	8.83	5.89	0	0	0.98
<i>Gloeobacter</i> 7421	5.89	98.20	8.83	33.38	55.97
<i>Synechocystis</i> 6308	2.94	54.01	7.85	0	56.95
<i>Synechocystis</i> 6714	11.78	20.62	ND	0	14.73
<i>Aphanocapsa montana</i>	15.71	29.46	0	ND	27.49

Table 2. *Uptake of hydrogen by Synechococcus strains 6803 and 6301: the effect of electron transport chain inhibitors*

Oxygen and hydrogen were initially present in all assays at 1.5 mmol l^{-1} . Duplicate assays were done under a microaerobic atmosphere of argon after an argon flushing period of 20 min. The photon fluence rate was $30 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Hydrogen uptake rates were measured over 4 h following the commencement of the assays. Potassium cyanide was added dissolved in water. Antimycin A and rotenone were added dissolved in ethanol; ethanol-only controls were run and the hydrogen uptake rates were the same as those measured in light + oxygen assays in both lysozyme-treated and untreated cells. The final concentrations of the inhibitors were: antimycin A, $3 \times 10^{-4} \text{ mol l}^{-1}$; rotenone, $10^{-5} \text{ mol l}^{-1}$ and KCN, $10^{-3} \text{ mol l}^{-1}$. The chlorophyll *a* concentrations were: *Synechococcus* 6803, $2.57 \mu\text{g ml}^{-1}$ and *Synechococcus* 6301, $2.60 \mu\text{g ml}^{-1}$.

Inhibitor	Lysozyme	Hydrogen uptake [$\mu\text{mol H}_2 \text{ h}^{-1} (\text{mg chl})^{-1}$]	
		<i>Synechococcus</i> 6803	<i>Synechococcus</i> 6301
—	+	25.92	21.70
—	—	23.56	11.39
Potassium cyanide	+	0	0
Potassium cyanide	—	0	0
Rotenone	+	9.42	0
Rotenone	—	14.14	9.72
Antimycin A	+	1.17	6.48
Antimycin A	—	29.46	0

completely inhibited hydrogen uptake by lysozyme-treated *Synechococcus* 6301 and caused a 64% inhibition of hydrogen-uptake in the light control by *Synechococcus* 6803. Antimycin A, which inhibits electron transfer between cytochromes *b* and *c* in mitochondria (Rieske & Zaugg, 1962), caused a 70% inhibition and a 95% inhibition of the lysozyme-treated light control rates in strains 6301 and 6803 respectively. A lack of inhibitory effects on respiration by antimycin A has previously been reported in cyanobacteria (Biggins, 1969; Bothe *et al.*, 1977*b*; Peschek, 1980), although Houchins & Burris (1981) reported an inhibition of light and dark hydrogen uptake reactions in membrane particles from *Anaebaena* 7120 by $50 \mu\text{M}$ -antimycin A. The results in Table 2 therefore suggest that such hydrogen uptake, functioning maximally in the presence of oxygen, may involve a typical respiratory electron transport chain with oxygen as the terminal acceptor.

Fig. 1. Effect of carbon monoxide on hydrogen evolution by *Synechococcus* 6803. Assay mixtures were incubated for 3 h under an atmosphere of argon, following a period of argon flushing of 20 min. Points are the mean of duplicate assay determinations; variation is denoted by the vertical bars. The photon fluence rate was $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ and the chlorophyll *a* concentration was $4.295 \mu\text{g ml}^{-1}$.

Table 3. Evolution of molecular hydrogen by unicellular cyanobacteria

All values are the means of triplicate assays. Carbon monoxide and acetylene were present in the assays at final concentrations of $13.4 \mu\text{mol l}^{-1}$ and 1.34 mmol l^{-1} respectively. All assays were done under a microaerobic atmosphere of argon, following an argon flushing period of 20 min, and were incubated for 3 h. Chlorophyll *a* concentrations were between 3 and $5 \mu\text{g ml}^{-1}$. The photon fluence rate was $30 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Strain	Hydrogen evolution [$\mu\text{mol H}_2 \text{ h}^{-1} (\text{mg chl})^{-1}$]				
	Light only	Light CO + C ₂ H ₂	Light CO only	Light C ₂ H ₂ only	Dark CO + C ₂ H ₂
<i>Synechococcus</i> 6803	0.11	0.17	0.12	0.20	0.26
<i>Synechococcus</i> 602	0.48	0.61	0.66	0.31	0.66
<i>Synechococcus</i> 6301	0.08	0.08	0.02	0.09	0.08
<i>Synechococcus</i> 6307	0.02	0.01	0	0	0
<i>Microcystis</i> 7820	0.11	0.16	0.11	0.15	0.02
<i>Gloeobacter</i> 7421	0.60	1.38	0.36	0.69	0.59
<i>Synechocystis</i> 6308	0.06	0.13	0.07	0.03	0.07
<i>Synechocystis</i> 6714	0.04	0.05	0.07	0.03	0.07
<i>Aphanocapsa montana</i>	0.40	0.23	0.19	0.28	0.06

Hydrogen evolution

Preliminary time course experiments showed that *Synechococcus* strains 6803 and 6301 had linear rates of hydrogen evolution over 5 h. The effect of carbon monoxide on hydrogen evolution by *Synechococcus* 6803 was determined (Fig. 1). A slight increase in the rate of hydrogen evolution occurred up to about $13 \mu\text{M}$ -carbon monoxide, although rates decreased at higher concentrations. This slight initial stimulation and subsequent inhibition of hydrogen production at increasing carbon monoxide concentrations found in a unicellular strain where nitrogenase activity was not detectable requires an explanation for carbon monoxide effects on hydrogenase rather than nitrogenase. Observations of hydrogen evolution by *Synechococcus* strains 6803 and 6301 in the light were extended to the remaining seven strains. The effects of carbon monoxide and acetylene on hydrogen evolution rates were assessed; in particular it was determined whether the use of those gases together would stimulate hydrogen evolution, as shown for nitrogen-fixing *Anabaena cylindrica* (Bothe *et al.*, 1977a). All strains evolved hydrogen (Table 3), although the rates of evolution by *Synechococcus* 6301, *Synechocystis* 6714 and *Microcystis* 7820 were very low, and *Synechococcus* 6307 had virtually no capacity to evolve hydrogen. In contrast, *Synechococcus* strains 6803 and 602, *Gloeobacter* 7421 and *Aphanocapsa*

montana had relatively high rates of hydrogen evolution, which varied according to the assay conditions and are of interest considering that nitrogenase, which is believed to be primarily responsible for hydrogen evolution in filamentous cyanobacteria (Bothe *et al.*, 1978), is undetectable in all of these unicells by the acetylene reduction method (see below). The low rates of hydrogen evolution by *Synechocystis* strains 6308 and 6714, *Mycrocystis* 7820 and *Synechococcus* 6301 were virtually unaffected by changes in the assay conditions (Table 3), suggesting the presence of a low basal capacity for hydrogen evolution in these strains, which may be due to the uptake hydrogenase acting in a reversible manner. The four strains showing the greatest rates of hydrogen evolution (*Synechococcus* 6803 and 602, *Gloeobacter* 7421 and *Aphanocapsa montana*) exhibited variation depending on the assay conditions with rates of hydrogen evolution in the dark being comparable to those obtained in the light, with the exception of *Aphanocapsa montana*. Carbon monoxide and acetylene, alone and/or together, either did not affect hydrogen evolution (*Synechococcus* 6301) or caused only low increases (*Synechococcus* 6803 and *Gloeobacter* 7421). Increases in hydrogen evolution rates by filamentous nitrogen-fixing strains due to carbon monoxide and acetylene addition can be partly explained by the action of these gases on various sites, binding and/or catalytic, that are present on the nitrogenase complex, in addition to their inhibitor effects on the uptake hydrogenase-mediated reaction (Bothe *et al.*, 1978; Eisebrenner *et al.*, 1978), although nitrogenase cannot be invoked in the present studies.

Attempts to induce nitrogenase synthesis and activity

Rippka & Waterbury (1977) developed a technique to induce the synthesis and function of nitrogenase in non-heterocystous cyanobacteria in anaerobiosis. As found earlier for *Synechococcus* strains 6301, 6307 and 6803, *Gloeobacter* 7421 and *Synechocystis* 6714 (Rippka *et al.*, 1974, 1979; Rippka & Waterbury, 1977), no nitrogenase activity was detected in acetylene reduction assays of these cultures after transfer of late exponential/early stationary phase cells to anaerobic conditions. We obtained similar results with *Aphanocapsa montana*. We therefore conclude that a capacity to synthesize nitrogenase is not a pre-requisite for hydrogen evolution in the unicellular strains tested, and hence the hydrogen evolution recorded (Table 3) must be hydrogenase-mediated, via either an uptake hydrogenase acting in a reversible manner, or possibly a distinct functional hydrogenase acting in an evolution mode.

Adaptation of cyanobacterial cultures with molecular hydrogen

The growth of filamentous cyanobacteria with gas mixtures containing molecular hydrogen has been reported to increase hydrogenase levels when compared with aerobically grown cultures, suggesting that hydrogenase can be induced in cyanobacteria. Fujita *et al.* (1964) reported an activation of hydrogen consumption and production when filaments of aerobically-grown *Anabaena cylindrica* were incubated for a few hours under hydrogen. Tel-Or *et al.* (1977) reported a 5- to 20-fold stimulation of hydrogenase activity in *A. cylindrica* and *Nostoc muscorum* following growth under an atmosphere containing 20% hydrogen, compared to aerobically-grown cultures, and similar observations were reported (Eisebrenner *et al.*, 1978) with six strains of cyanobacteria including *Synechococcus* 6301. Six unicellular strains were gassed with molecular hydrogen in an attempt to influence rates of hydrogen uptake and evolution (Table 4). Cultures were first grown aerobically at 25 °C for 4 d and the resulting exponential phase cells were then transferred to growth under an atmosphere of 5% (v/v) carbon dioxide in hydrogen for a further 4 d, after which hydrogenase activities were assayed. Between 2- and 15-fold increases in hydrogen uptake and between 2- and 8-fold increases in hydrogen evolution occurred as a result of gassing cultures with molecular hydrogen. Molecular hydrogen has been reported to inhibit photosynthesis in *Anabaena* sp. TA1 by inhibiting electron flow between photosystem II and photosystem I (Antarikanonda *et al.*, 1980) whereas Peschek (1979a) has routinely grown *Synechococcus* under molecular hydrogen.

Table 4. Increase in rates of hydrogen uptake and hydrogen evolution by six unicellular strains of cyanobacteria following adaptation with 5% carbon dioxide in hydrogen

Exponential phase cultures were gassed with 5% (v/v) carbon dioxide in hydrogen using Quick-fit flasks and attachments (Methods) for 4 d before use. Percentage increases are the means of increases found in four or five assays using an identical rationale of treatments as used for aerobic control cultures (see legends to Tables 1 and 3). Each assay was done in duplicate. The percentage increases were calculated from the rates of hydrogen exchange by aerobic cultures. Absolute values are given in Tables 1 and 3. ND, Not determined.

	Percentage increase	
	Hydrogen uptake	Hydrogen evolution
<i>Synechococcus</i> 6803	548	192
<i>Synechococcus</i> 6301	580	432
<i>Synechococcus</i> 6307	1500	ND
<i>Synechocystis</i> 6714	279	ND
<i>Aphanocapsa montana</i>	203	824
<i>Gloeobacter</i> 7421	300	270

DISCUSSION

The results reported here have confirmed and extended earlier findings on hydrogen exchange by *Synechococcus* 6301 (Peschek, 1979*a,b,c*, 1980; Bothe *et al.*, 1980), and have characterized hydrogenase activity in a further eight strains of unicellular cyanobacteria from various groups. The absence of nitrogenase synthesis in anaerobiosis by all strains tested indicates that hydrogen exchange can occur in pure strains of unicellular cyanobacteria in the absence of nitrogenase activity and synthesis (Howarth *et al.*, 1981).

Rates of hydrogen uptake and production vary from strain to strain, and the mechanism of hydrogen uptake in the unicellular strains appears to be similar to hydrogen uptake in the filamentous cyanobacteria as shown by assays in which oxygen was used, in conjunction with both light and dark assays. Prior addition of oxygen to the assays for hydrogen uptake in the light caused increases in the rate of hydrogen uptake by *Synechococcus* strains 602 and 6301, *Gloeobacter* 7421, *Synechocystis* strains 6308 and 6714 and by *Aphanocapsa montana*, but significant levels of anaerobic light-dependent hydrogen uptake occurred with *Synechococcus* strains 6803 and 6307, *Synechocystis* 6714 and *A. montana*, in agreement with one of the proposed modes of hydrogen uptake, a light anaerobic reaction (Peschek, 1979*b*). Evidence for a second mode of hydrogen uptake, via an oxygen-dependent 'Knallgas' reaction, as demonstrated for *Synechococcus* 6301 (Peschek, 1979*c*), was found for all the strains used in the present study.

Increases in hydrogen uptake and production rates following exposure of the cultures to hydrogen may be explained by *de novo* protein synthesis, possibly involving the hydrogenase itself, or stimulation of pre-existing enzyme(s). However, this cannot be inferred directly from the results because the whole cell hydrogen uptake and production processes involve several components besides hydrogenase, including electron transport chain compounds, and it is not known how these additional components may be limiting, and how their levels may be changed by exposure to hydrogen.

The biological role of hydrogenase(s) in unicellular non-nitrogen-fixing cyanobacteria is little understood. A function in the recovery of hydrogen, obligatorily produced by nitrogenase, in the provision of reductant to nitrogenase and in the consumption of oxygen to protect nitrogenase (Dixon, 1972; Stewart, 1980; Stewart *et al.*, 1982; Bothe, 1982) clearly cannot be invoked in this case. Hydrogen sulphide is the best documented source of reductant in cyanobacterial anoxygenic photosynthesis (Padan & Cohen, 1982), but the use of hydrogen as an electron source for the photoreduction of nitrite, nitrate and carbon dioxide has been demonstrated with *Synechococcus* 6301 (Peschek, 1978, 1979*b*; Bothe *et al.*, 1980). The production of hydrogen by 71 field isolates of cyanobacteria, although at low rates, is presumably largely via nitrogenase, since the organisms were selected for their ability to fix nitrogen (Berchtold & Bachofen, 1979).

The present results from nine pure cultures of unicellular strains suggests that the capacity to metabolize hydrogen via hydrogenase may be widespread among non-nitrogen-fixing cyanobacteria.

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REFERENCES

- ANTARIKANONDA, P., BERNDT, H., MAYER, F. & LORENZEN, H. (1980). Molecular hydrogen: a new inhibitor of photosynthesis in the blue-green alga (cyanobacterium), *Anabaena* sp. TA1. *Archives of Microbiology* **126**, 1-10.
- BERCHTOLD, M. & BACHOFEN, R. (1979). Hydrogen formation by cyanobacteria cultures selected for nitrogen fixation. *Archives of Microbiology* **123**, 227-232.
- BIGGINS, J. (1969). Respiration in blue-green algae. *Journal of Bacteriology* **99**, 570-575.
- BOTHE, H. (1982). Nitrogen fixation. In *Botanical Monographs*, vol. 19, *The Biology of Cyanobacteria*, pp. 87-104. Edited by N. G. Carr & B. A. Whitton. Oxford: Blackwell Scientific Publications.
- BOTHE, H., TENNIGKEIT, J., EISBRENNER, G. & YATES, M. G. (1977a). The hydrogenase-nitrogenase relationship in the blue-green alga *Anabaena cylindrica*. *Planta* **133**, 237-242.
- BOTHE, H., TENNIGKEIT, J. & EISBRENNER, G. (1977b). The utilization of molecular hydrogen by the blue-green alga *Anabaena cylindrica*. *Archives of Microbiology* **114**, 43-49.
- BOTHE, H., DISTLER, E. & EISBRENNER, G. (1978). Hydrogen metabolism in the blue-green algae. *Biochimie* **60**, 277-289.
- BOTHE, H., NEUER, G., KALBE, I. & EISBRENNER, G. (1980). Electron donors and nitrogenase in nitrogen-fixing microorganisms. In *Proceedings of an International Symposium on Nitrogen Fixation*, pp. 83-112.
- CHANCE, B. & HOLLUNGER, G. (1963). Inhibition of electron and energy transfer in mitochondria. I. Effects of amytal, thiopental, rotenone, progesterone and methylene glycol. *Journal of Biological Chemistry* **278**, 418-431.
- DIXON, R. O. D. (1972). Hydrogenase in legume root nodule bacteroids. Occurrence and properties. *Archives of Microbiology* **85**, 193-201.
- EISBRENNER, G., DISTLER, E., FLOENER, L. & BOTHE, H. (1978). The occurrence of the hydrogenase in some blue-green algae. *Archives of Microbiology* **118**, 177-184.
- FRENKEL, A. H., GAFFRON, H. & BATTLE, E. H. (1950). Photosynthesis and photoreduction in the blue-green alga, *Synechococcus elongatus* Näg. *Biology Bulletin* **99**, 157-162.
- FUJITA, Y., OHAMA, H. & HATTORI, A. (1964). Hydrogenase activity of a cell free preparation obtained from the blue-green alga *Anabaena cylindrica*. *Plant and Cell Physiology* **5**, 305-314.
- GAFFRON, H. & RUBIN, J. (1942). Fermentative and photochemical production of hydrogen in algae. *Journal of General Physiology* **26**, 219-240.
- GALLON, J. R. (1981). The oxygen sensitivity of nitrogenase - a problem for biochemists and microorganisms. *Trends in Biochemical Sciences* **6**, 19-23.
- GEL'MAN, N. S., LUKOYANOVA, M. A. & OSTROVSKI, D. N. (1967). *Respiration and Phosphorylation of Bacteria*. New York: Plenum Press.
- HAYSTEAD, A., ROBINSON, R. & STEWART, W. D. P. (1970). Nitrogenase activity in extracts of heterocystous blue-green algae. *Archives of Microbiology* **75**, 235-243.
- HOUCHINS, J. P. & BURRIS, R. H. (1981). Light and dark reactions of the uptake hydrogenase in *Anabaena* 7120. *Plant Physiology* **68**, 712-716.
- HOWARTH, D. C., CODD, G. A. & STEWART, W. D. P. (1981). The uptake and evolution of molecular hydrogen by unicellular cyanobacteria. *Society for General Microbiology Quarterly* **8**, 153.
- LEMBERG, R. & BARRETT, J. (1973). *Cytochromes*. London & New York: Academic Press.
- MACKINNEY, G. (1941). Absorption of light by chlorophyll solutions. *Journal of Biological Chemistry* **140**, 315-322.
- PADAN, E. & COHEN, Y. (1982). Anoxygenic photosynthesis. In *Botanical Monographs*, vol. 19, *The Biology of Cyanobacteria*, pp. 215-235. Edited by N. G. Carr & B. A. Whitton. Oxford: Blackwell Scientific Publications.
- PESCHEK, G. A. (1978). Reduced sulfur and nitrogen compounds and molecular hydrogen as electron donors for anaerobic CO₂ photoreduction in *Anacystis nidulans*. *Archives of Microbiology* **119**, 313-322.
- PESCHEK, G. A. (1979a). Evidence for two functionally distinct hydrogenases in *Anacystis nidulans*. *Archives of Microbiology* **123**, 81-92.
- PESCHEK, G. A. (1979b). Anaerobic hydrogenase activity in *Anacystis nidulans*. H₂-dependent photoreduction and related reactions. *Biochimica et biophysica acta* **548**, 187-202.
- PESCHEK, G. A. (1979c). Aerobic hydrogenase activity in *Anacystis nidulans*. The oxyhydrogen reaction. *Biochimica et biophysica acta* **548**, 203-215.
- PESCHEK, G. A. (1980). Electron transport reactions in respiratory particles of hydrogenase-induced *Anacystis nidulans*. *Archives of Microbiology* **125**, 123-141.
- PETERSON, R. B. & BURRIS, R. H. (1978). Hydrogen metabolism in isolated heterocysts of *Anabaena* 7120. *Archives of Microbiology* **116**, 125-132.
- RIESKE, J. S. & ZAUGG, W. S. (1962). The inhibition by antimycin A of the cleavage of one of the complexes of the respiratory chain. *Biochemical and Biophysical Research Communications* **8**, 421-426.
- RIPPKA, R. & WATERBURY, J. B. (1977). The synthesis of nitrogenase by non-heterocystous cyanobacteria. *FEMS Microbiology Letters* **2**, 83-86.
- RIPPKA, R., WATERBURY, J. B. & COHEN-BAZIRE, G. (1974). A cyanobacterium which lacks thylakoids. *Archives of Microbiology* **100**, 419-436.
- RIPPKA, R., DERUELLES, J., WATERBURY, J. B., HERDMAN, M. & STANIER, R. Y. (1979). Generic

- assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology* **111**, 1–61.
- STANIER, R. Y., KUNISAWA, R., MANDEL, M. & COHEN-BAZIRE, G. (1971). Purification and properties of unicellular blue-green algae. (Order Chroococcales). *Bacteriological Reviews* **35**, 171–205.
- STEWART, W. D. P. (1980). Some aspects of structure and function in N_2 -fixing cyanobacteria. *Annual Review of Microbiology* **34**, 497–536.
- STEWART, W. D. P., FITZGERALD, G. P. & BURRIS, R. H. (1967). *In situ* studies on N_2 fixation using the acetylene reduction technique. *Proceedings of the national Academy of Sciences of the United States of America* **58**, 2071–2078.
- STEWART, W. D. P., HAWKESFORD, M., ROWELL, P. & CODD, G. A. (1982). H_2 -production from sunlight and H_2O – the role of the uptake hydrogenase in N_2 -fixing cyanobacteria. In *Solar Energy R & D in the European Community*, series D, vol. 1, *Photochemical, Photoelectrochemical and Photobiological Processes*, pp. 165–169. Edited by D. O. Hall & W. Palz. Dordrecht: D. Reidel.
- TEL-OR, E., LUIJK, L. W. & PACKER, L., (1977). An inducible hydrogenase in cyanobacteria enhances N_2 -fixation. *FEBS Letters* **78**, 49–52.