Arch. Microbiol. 116, 125–132 (1978)

Archives of Microbiology
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# Hydrogen Metabolism in Isolated Heterocysts of *Anabaena* 7120

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*Abstract.* Isolated heterocysts of *Anabaena* 7120 evolve $H_2$ in an ATP-dependent nitrogenase-catalyzed process that is inhibited by $N_2$ and $C_2H_2$. Heterocysts have an active uptake hydrogenase that only requires an electron acceptor of positive redox potential, e.g., methylene blue, dichlorophenolindophenol or potassium ferricyanide. $O_2$ supplied at low partial pressures is a very effective physiological oxidant for $H_2$ uptake. High concentrations of $O_2$ are inhibitory to $H_2$ uptake. The oxyhydrogen reaction in heterocysts appears to be mediated by a cytochrome-cytochrome oxidase system, and it supports ATP synthesis via oxidative phosphorylation. Attempts to demonstrate acetylene reduction in isolated heterocysts employing $H_2$ as an electron donor were unsuccessful. It is suggested that the uptake hydrogenase functions to conserve reductant that otherwise would be dissipated via nitrogenase-catalyzed $H_2$ evolution.

*Key words:* Heterocyst – Blue-green algae – *Anabaena* – Nitrogenase – Hydrogenase.

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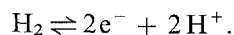
Hydrogenase from microorganisms catalyzes the reaction



$$H_2 \rightleftharpoons 2e^- + 2H^+.$$

Such classical hydrogenases typically are reversible and have no requirement for ATP. Most hydrogenases are easily inactivated by $O_2$, and in algae they may be formed during several hours of dark, anaerobic adaptation; they are deadapted readily by photosynthetically generated $O_2$ (Gaffron, 1942; Gaffron and Rubin, 1942; Ben-Amotz et al., 1975). $H_2$ may be evolved either by nitrogenase or hydrogenase in certain photosynthetic microorganisms, and this may confuse the source of the $H_2$ (Gest and Kamen, 1949; Bulen et al., 1965; Haystead et al., 1970).

Nitrogenase- and hydrogenase-catalyzed $H_2$ evolution can be distinguished. $H_2$ evolution from nitrogenase is ATP-dependent and is inhibited by $N_2$ and $C_2H_2$ but not by CO. No adaptation treatment is necessary under $N_2$-fixing conditions (Winter and Burris, 1968; Rivera-Ortiz and Burris, 1975). In contrast, classical hydrogenases are sensitive to CO, do not require ATP and are unaffected by $N_2$ (Hoberman and Rittenberg, 1943).

Hydrogenase and nitrogenase may have a functional association in vivo. Absence of measurable $H_2$ evolution from nitrogenase has been attributed to the presence of an "uptake hydrogenase" in *Azotobacter chroococcum* (Hyndman et al., 1953; Smith et al., 1976) and in certain leguminous nodules (Dixon, 1967, 1972). Bothe et al. (1977) have described a hydrogenase-nitrogenase system in *Anabaena cylindrica* which resembles the *A. chroococcum* system.

The potential significance of such hydrogenase-nitrogenase associations was pointed out by Schubert and Evans (1976) who conducted a survey of $H_2$ evolution by leguminous root nodules. $H_2$ evolution by nitrogenase dissipates ATP and reductant, and efficiency of $N_2$ fixation in root nodules could be increased by directing energy to $N_2$ reduction instead of $H_2$ evolution. As $H_2$ evolution is an inherent property of nitrogenase and is not completely suppressed even at infinite $N_2$ concentration (Rivera-Ortiz and Burris, 1975), we must consider recycling of $H_2$ via an uptake hydrogenase as a potential way for conserving energy that otherwise would be lost through $H_2$ evolution (Burris and Peterson, 1977).

Evidence is presented in this paper indicating that heterocysts not only evolve $H_2$ via nitrogenase, but also recapture $H_2$ with an uptake hydrogenase and utilize the energy of $H_2$ oxidation to produce ATP by oxidative phosphorylation.

0302-8933/78/0116/0125/$ 01.60

## MATERIALS AND METHODS

*Organism and Preparations. Anabaena* 7120 (ATCC designation 27893, *Nostoc muscorum*) was grown aerobically in the medium of Allen and Arnon (1955). The algae were harvested with a Sharples centrifuge and stored in liquid $N_2$ until used. For many of the experiments on $H_2$ evolution, filaments were disrupted by sonication, followed by sedimentation of the heterocysts into colloidal silica by gravity (scaled-up procedure, Peterson and Burris, 1976). For all of the experiments on $H_2$ uptake and some of the experiments on $H_2$ evolution, heterocysts were isolated by lysozyme treatment followed by mild sonication and washing without the colloidal silica treatment. In this procedure (modified from Tel-Or and Stewart, 1976) the algae were washed and resuspended in 30 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid), 30 mM PIPES (piperazine-N-N'-bis-2-ethanesulfonic acid), 1.0 mM $MgCl_2$, with 0.35 M mannitol and a pH of 7.2 After lysozyme was added to a final concentration of 1.0 mg/ml, the pH was adjusted to 7.5 with KOH; this retarded the decomposition of $Na_2S_2O_4$ during the subsequent 30 min incubation at 35° C. Lysozyme disrupted the filaments and produced detached heterocysts, vegetative cells, and filament fragments, and it made vegetative cells more susceptible to sonication. Sonication was at setting 6 or 7 (about 50 watts input) for 90 s on a Branson Sonifier (Heat Systems-Ultrasonics). The suspension was centrifuged at $500 \times g$ for 5 min; the resulting pellet of heterocysts was resuspended and washed 2 or 3 times with the mannitol-containing buffer by centrifugation at $500 \times g$ for 5 min. Microscopic examination showed such suspensions were composed almost entirely of heterocysts.

No qualitative differences were apparent in the $H_2$ metabolism of heterocysts isolated by either method. 1.0 mM $Na_2S_2O_4$ was present during isolation of heterocysts when $H_2$ evolution was to be observed, but $Na_2S_2O_4$ was omitted from suspensions prepared for $H_2$ uptake. All operations were conducted under an $O_2$-free gas phase of either $H_2$ or $N_2$.

*Amperometric Measurement of $H_2$.* The system was essentially that of Wang et al. (1971). The electrode had a platinum anode and a silver-silver chloride cathode (No. 5331, Yellow Springs Instrument Co., Yellow Springs, Ohio). The electrode surface was covered with a film of half-saturated KCl and 25 μ thick Teflon membrane before use. During operation, the polarizing potential across the electrode was set at + 0.60 v versus the Ag-AgCl cathode. The output current from the electrode was amplified and fed into a 1.0 mv (full span) recorder. The electrode was centered in a glass water-jacketed reaction chamber (30° C) whose total volume was 1.9 ml. The reaction chamber was sealed during use with a ground glass ball mounted on a stem; the ball seated firmly into the constricted neck of the reaction chamber whose contents were mixed with a magnetic stirring bar.

The electrode response was specific for $H_2$. No interference by $N_2$, $O_2$, or argon was observed. Likewise, no reaction mixture components interfered, except that CO caused a transient response that was followed by a drastic decrease in electrode sensitivity.

Without preconditioning, the electrode was relatively insensitive, nonlinear, and unstable. Preconditioning involved application of timed alterations in polarizing potential between + 0.2 and + 0.8 v at a rate of 50 cycles/min from a battery-operated electronic oscillator for a total of 10 min. For best performance, a fresh Teflon membrane was applied, and the electrode was preconditioned and calibrated before each series of measurements.

The electrode was calibrated by injection of μl portions of $H_2$-saturated distilled $H_2O$ or buffer. The final concentration of $H_2$ was calculated from the $\alpha$ value for $H_2$ at 25° C of 0.0175 (Umbreit et al., 1972, p. 62). The response to $H_2$ was linear over all ranges of $H_2$ concentration employed, and the response time was nearly instantaneous. The noise level was always less than 5% of the signal response. Prolonged incubation in the presence of a fixed concentration of $H_2$ revealed a slow decay in the signal (about 17%/h under favorable conditions), that introduced only a minor error. This slow decay may arise from leakage of $H_2$ from the reaction chamber. $H_2$ concentrations down to less than 1.0 nmole/ml could be measured routinely and reliably.

*Manometric Determination of $H_2$ Uptake.* Gas uptake by heterocysts was determined using submarine volumometers (Umbreit et al., 1972, p. 104). Nine ml Warburg flasks with one side-arm were used routinely with a total reaction mixture volume of 1.0 ml and with 0.2 ml of 20% KOH and a fluted filter paper wick in the center well to absorb $CO_2$. Reaction mixture components less heterocysts were placed in the main chamber of the flask before it was placed on the manometer. The top of the side-arm ground-glass capillary plug was sealed with a rubber stopper. The system was evacuated and flushed through the side-arm three times with $H_2$ or argon, and the chosen gas phase then was admitted. A long hyperdermic needle was used to inject the heterocyst suspension into the side-arm. Excess pressure was relieved, and the side-arm plug was rotated 90°.

Before placing vessels in the 30° C water bath for 10 min thermo-equilibration, the side-arm contents were dumped into the main chamber. Gas uptake was corrected to standard temperature and pressure (STP) (Umbreit et al., 1972, p. 105).

*Phosphorylation in Heterocysts.* Oxidative phosphorylation was determined by incorporation of $^{32}PO_4^{-3}$ into organic phosphate in the presence of ADP. Heterocysts from freshly harvested *Anabaena* 7120 were used. Esterfied phosphate (as $AT^{32}P$) was trapped as glucose-6-phosphate generated by hexokinase from D-glucose, or it was trapped with 2-deoxy-D-glucose. 2-deoxy-D-glucose was the preferred acceptor for phosphate, as its phosphorylated derivative probably is not metabolized by heterocysts. Reactions always were run in the dark in Warburg vessels as described or in 21 ml stoppered vaccine bottles. Reactions were terminated with 0.5 ml of 20% trichloroacetic acid.

Extraction of unreacted $^{32}PO_4^{-3}$ was based on a procedure described by Lindberg and Ernster (1956, p. 16). 0.5 ml of the precipitated sample was added to 4.3 ml of silicotungstic acid and 5.0 ml of a 1:1 (v/v) solution of isobutanol: benzene in an 18 × 150 mm test tube. 0.5 ml of 10% (w/v) ammonium molybate was added and the contents were vigorously mixed for 15–30 s. The organic phase was removed by aspiration. 5.0 ml of fresh isobutanol-benzene was added to the test tube, and 1.0 ml of 1.0 mM $KH_2PO_4$ was added together with another 0.5 ml of the ammonium molybdate. The tube was mixed and the organic phase was removed as before. Subsequent extractions with water-saturated isobutanol (Conover et al., 1963) and ether gave more complete extraction of unreacted $^{32}PO_4^{-3}$. 1.0 ml aliquots of the extracted sample were dissolved in Aquasol® and counted for a min on a Packard Tri-Carb scintillation counter. Counting efficiency was 75–80%, and duplicate derminations usually agreed to within 10% and often to within 5%.

## RESULTS

$H_2$ evolution by isolated heterocysts begins only after ATP is added, and thereafter the $H_2$ evolution proceeds linearly until the reductant is depleted. The effects of $N_2$ and $C_2H_2$ on $H_2$ evolution by isolated heterocysts are shown in Figure 1. $N_2$ inhibited 68%, whereas $C_2H_2$ completely suppressed $H_2$ evolution. No lags or transients were noted in the presence of argon or $N_2$. A small amount of $H_2$ was produced initially in

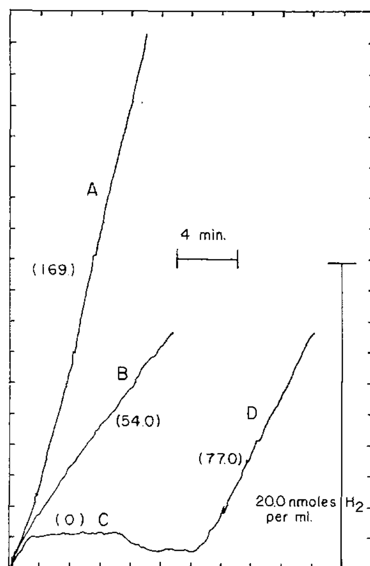



Fig. 1. Effects of $N_2$ and $C_2H_2$ on $H_2$ evolution by isolated heterocysts. Portions of an isolated heterocyst preparation were equilibrated with the following gas phases: *A* 100% argon; *B* 100% $N_2$; *C* 17% $C_2H_2$, 83% argon; *D* suspension C was bubbled with argon for 5 min. 1.0 mM $Na_2S_2O_4$ was present as reductant as was 5.0 mM ATP plus 7.0 mM $MgCl_2$, 20 mM creatine phosphate and 0.05 mg creatine phosphokinase as an ATP-generating system. Values in parentheses denote specific activities as nmoles $H_2$/(mg chl. $a \times$ min). 26.0 µg chl. *a* was present in heterocysts per ml of suspension

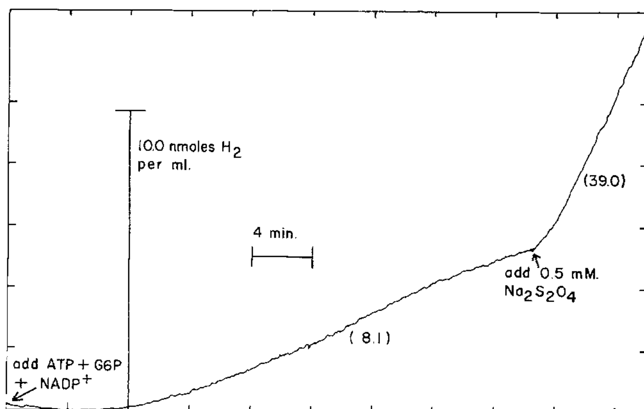



Fig. 2. $H_2$ evolution by isolated heterocysts employing 10.0 mM glucose-6-phosphate and 0.3 mM $NADP^+$ as an electron donor system. ATP was present as in Figure 1. Heterocysts were isolated in the presence of $Na_2S_2O_4$ as described in "Materials and Methods". Prior to assay, heterocysts were resuspended in buffer containing a limiting amount of $Na_2S_2O_4$. Such preparations exhibited nitrogenase activity only when glucose-6-phosphate (G6P) and $NADP^+$ were present (data not shown). (↑) denotes addition of 0.5 mM $Na_2S_2O_4$. Specific activities are in parentheses as in Figure 1

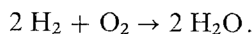
the presence of $C_2H_2$, but this ceased within the first min. Inhibition by $C_2H_2$ was partially reversed by sparging the suspension with argon for 5 min.

Low, but detectable rates of $H_2$ evolution occurred when glucose-6-phosphate plus $NADP^+$ served as the electron donor (Fig. 2). A pronounced lag in $H_2$ evolution was evident, and addition of 1.0 µmole of $Na_2S_2O_4$ to the reaction mixture increased the rate nearly five-fold. The apparently low production of $H_2$ supported by the physiological electron donor system (glucose-6-phosphate plus $NADP^+$) may be explained partly by the reoxidation of evolved $H_2$ by an uptake hydrogenase in these heterocysts.

Isolated heterocysts took up $H_2$ vigorously when a suitable electron acceptor was added. Responses to several acceptor systems are compared in Figure 3 which indicates rates of $H_2$ uptake in the presence of 2,6-dichlorophenol indophenol (DCPIP) or methylene blue (MB). In most cases, rates of $H_2$ uptake were linear or slightly hyperbolic. When no acceptor was added, uptake of $H_2$ was not detectable. Figure 4 illustrates $H_2$ uptake in the presence of $K_3Fe(CN)_6$, $NADP^+$, and $NAD^+$, and with MB for comparison with other experiments. Ferricyanide was a very effective electron acceptor, whereas the pyridine nucleotides functional poorly. Although methyl viologen (MV) is an effective electron acceptor in many hydrogenase systems, it was ineffective in the heterocyst system (data given by Peterson, 1976). Addition of a crude preparation of *Anabaena* 7120 ferredoxin in the presence of $NADP^+$ gave little increase in $H_2$ uptake (data given by Peterson, 1976). Illumination of heterocysts in 600 ft-c of incandescent light in the presence or absence of $NADP^+$ did not enhance $H_2$ uptake relative to uptake in the dark.

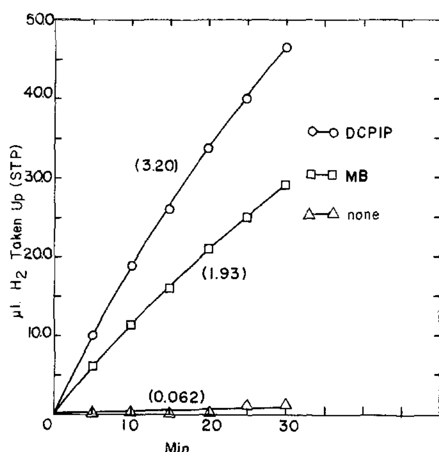
There was no effect of 5% CO on $H_2$ uptake (Peterson, 1976). $O_2$ is a potent inhibitor of hydrogenases in cell-free preparations, but the hydrogenase in the intact heterocyst system is relatively insensitive to $O_2$ at the $pO_2$ of air. Heterocyst suspensions exposed to air for 40 min prior to assay under 100% $H_2$ retained 70% of their activity.

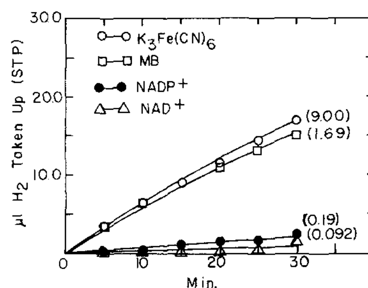
The heterocyst-hydrogenase system not only exhibits stability to $O_2$, but also is capable of using $O_2$ as an effective oxidant. The anaerobic rate (in argon) of endogenous $H_2$ uptake by heterocysts measured amperometrically was 0.035 nmoles $H_2$ taken up/(µg Chl $a \times$ min) compared to 1.10 in the presence of air. Thus, air supported a 30-fold enhancement in rate. Exposure to high $pO_2$ causes inhibition of $H_2$ uptake activity relative to 1−2% $O_2$. The stoichiometry of $H_2$ and $O_2$ uptake was investigated by monitoring the change in $H_2$ concentration amperometrically in the presence of limiting levels of $O_2$ (Fig. 5). The average experimental ratio of $H_2:O_2$ was 2.4 in six determinations, suggesting that the overall reaction can be written



$$2\,H_2 + O_2 \rightarrow 2\,H_2O\,.$$

High levels of $O_2$ inhibited the oxyhydrogen reaction (Fig. 6). The optimum $pO_2$ was not determined



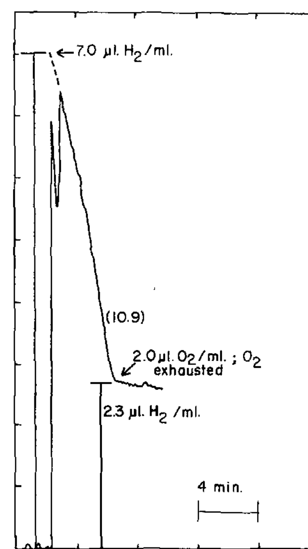




Fig. 3. Manometric determination of $H_2$ uptake by isolated heterocysts in the presence of 10.0 mM 2,6-dichlorophenolidophenol (DCPIP), 10.0 mM methylene blue (MB),.and without added electron acceptor. The gas phase was 100% $H_2$ and the temperature was 30° C. The heterocysts present in a fluid volume of 1.0 ml contained 26.4 µg of chlorophyll *a*. The numbers in parentheses refer to rates of $H_2$ uptake as nmoles $H_2$/(µg chl. $a \times$ min). Each point represents a mean of duplicates. See that "Materials and Methods" section for details

Fig. 4. $H_2$ uptake by isolated heterocysts in the presence of $K_3Fe(CN)_6$, methylene blue (MB), $NADP^+$ and $NAD^+$ (concentrations of each, 10.0 mM). 16.1 µg chlorophyll *a* present in heterocysts in all flasks except $K_3Fe(CN)_6$ replicates which contained 3.22 µg chlorophyll *a*. See Figure 3 for conditions; rates in parentheses as Figure 3

Fig. 5. Amperometric determinations of $H_2$ uptake by isolated heterocysts in the presence of a limiting quantity of $O_2$. Initial composition of the gas phase in equilibrium with the sample was 7.2% $O_2$, 40% $H_2$, 52.8% Ar. 6.3 µg chlorophyll *a* per ml was present in heterocysts. The $H_2:O_2$ uptake ratio was calculated from the change in $H_2$ concentration and initial $O_2$ concentration in the sample (in this sample $H_2:O_2 = 2.4$). Note the increase in rate of $H_2$ uptake as $O_2$ becomes depleted. A period of about 30 s elapses between addition of the heterocyst suspension to the electrode chamber and the activation of the stirring mechanism to give a stable response to $H_2$ concentration. This interval is noted by a transient. Subsequent $H_2$ uptake is extrapolated back to time zero (*dashed line*) indicating an initial $H_2$ concentration of 7.0 µl $H_2$/ml. Value in parentheses is $H_2$ uptake rate as nmoles $H_2$/(µg chl. $a \times$ min). See "Materials and Methods" and Figure 1 for other details

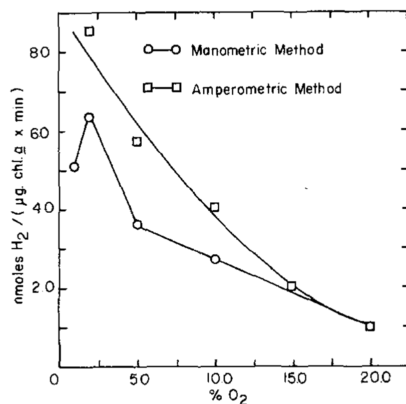



Fig. 6. Effect of the $pO_2$ on the oxyhydrogen reaction in isolated heterocysts. Manometric determinations were made under 40% $H_2$, $O_2$ as indicated, and the balance Ar. See Figure 3 for other conditions. The amperometric determinations were conducted by equilibrating samples of heterocysts under 40% $H_2$, $O_2$ as indicated, and the balance Ar. Samples were placed in the $H_2$ electrode chamber, and the initial rate of $H_2$ uptake was recorded

exactly, but it appears to be 1–2% $O_2$. Representative rates of $H_2$ uptake in the presence of various oxidants, including $O_2$, appear in Table 1. Only acceptors with positive $E^{0\prime}$ values function effectively in this system.

Respiratory inhibitors were tested for their effects on the oxyhydrogen reaction in heterocysts. HCN caused 88% inhibition at a concentration of 0.1 mM, but this concentration of HCN inhibited only 29% when MB was the oxidant and $O_2$ was absent.

The oxyhydrogen reaction was inhibited by 74% in the dark by 85% CO. Exposure of the heterocysts to 4500 ft-c intensity white light effected a dramatic reversal in CO inhibition to only 36%, compared to the control without CO (Fig. 7). When MB was the oxidant, CO inhibition was much reduced and was not light reversible, and in agreement with results discussed earlier, 5% CO exerted little or no inhibition of the oxyhydrogen reaction (Peterson, 1976). The effect of 17% $C_2H_2$ on $H_2$ uptake with potassium ferri-

Table 1. Summary of effectiveness of various electron acceptors in mediating $H_2$ uptake by isolated heterocysts

| Acceptor system | nmoles $H_2$ / µg chl. $a \times$ min | $E^{0\prime}$ of oxidant $(v)\,y$ [a] |
|---|---|---|
| None | 0.06 | – |
| 10 mM MV | 0.21 | – 0.44 |
| 10 mM $NAD^+$ | 0.09 | – 0.320 |
| 10 mM $NADP^+$ | 0.19 | – 0.324 |
| 10 mM $NADP^+$ + 0.3 mg Fd/ml [b] | 0.53 | ca. – 0.400 |
| 10 mM MB | 1.93 | + 0.011 |
| 10 mM DCPIP | 3.20 | + 0.217 |
| 10 mM $K_3Fe(CN)_6$ | 11.1 | + 0.36 |
| $O_2$ (< 2%) | 10.9 | + 0.816 |

[a] $E^{0\prime}$ (pH 7) values from: CRC Handbook of Biochemistry (H. A. Sober, ed.), pp. J – 27. Cleveland, Ohio: Chemical Rubber 1968

[b] A partially purified preparation of ferredoxin from *Anabaena* 7120 was obtained as follows: 25 g of cell paste was suspended in 100 ml of 20 mM $K_2HPO_4$, 10 mM $MgCl_2$ at pH 7.5. The suspension was passed through a French press at 20000 psi then centrifuged at $27000 \times g$ for 30 min. The supernatant was applied to a column of Whatman DE 52 and washed with 0.2 M NaCl in buffer. Ferredoxin was eluted with 0.8 M NaCl in buffer. The ion exchange column step was repeated once. The ferredoxin preparation was desalted and concentrated by filtration through a UM-2 membrane (Amicon Diaflo) under 50 psi argon

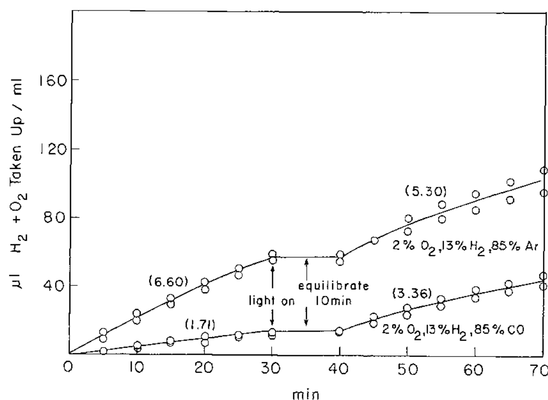



Fig. 7. Inhibition by 85% CO of the oxyhydrogen reaction in isolated heterocysts and its reversal by white light (4500 ft-c). Gas exchange was not monitored during the 10 min thermoequilibration period following turning on the light. 8.6 µg chlorophyll *a* was present in heterocysts. See Figure 3 for conditions; rates in parentheses are expressed as in Figure 3

cyanide as oxidant was tested, and it did not inhibit the reaction.

Heterocysts phosphorylated ADP in the dark in the presence of $O_2$, and the phosphorylation was linear with time. ATP formed in the reaction mixture was converted to ADP in situ via the hexokinase-glucose (or 2-deoxy-D-glucose) reaction. Table 2 shows that addition of ADP is obligatory for phosphorylation to occur. The uncoupler of phosphorylation, m-Cl-CCP (carbonyl cyanide, m-chlorophenyl hydrazone; Heytler and Prichard, 1962) inhibited phosphorylation in heterocysts by 35% and 47% at concentrations of 2.0 and 4.0 µM, respectively.

Table 2. Effect of omission of ADP and presence of (m-Cl-CCP) on $^{32}PO_4^{-3}$ uptake in isolated heterocysts

| Conditions | nmoles $PO_4^{-3}$ esterified/assay | Average |
|---|---|---|
| Control (TCA killed) | 0 | 0 |
| | 0 | |
| Complete, uninhibited | 84.3 | 88.1 |
| | 91.9 | |
| – ADP | 2.8 | 3.2 |
| | 3.6 | |
| + 2.0 µM m-Cl-CCP [a] | 54.2 | |
| | 60.4 | |
| + 4.0 µM m-Cl-CCP | 48.6 | 46.9 |
| | 45.3 | |

*Note.* Assay system consisted of 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid], 4.0 mM $MgCl_2$, 0.35 M mannitol, 2.0 mM ATP, 10.0 mM $K_2HPO_4$, 15.0 mM 2-deoxy-D-glucose (a gift from Dr. Laurens Anderson), 10 units of yeast hexokinase (Sigma), 5.34 µg · chl. *a* as heterocysts, and 5.7 µ-curies $^{32}PO_4^{-3}$ at pH 8.0 in a total fluid volume of 1.0 ml in 21 ml stoppered vaccine bottles. The gas phase in this experiment was 2% $O_2$, 98% $H_2$ and the incubation time was 50 min at 30° C. See "Materials and Methods" for further details

[a] Carbonyl cyanide m-chlorophenyl hydrazone

Table 3. Effect of $H_2$ on the rate phosphorylation by isolated heterocysts

| Conditions | nmoles $PO_4^{-3}$ esterified/assay | |
|---|---|---|
| | Exp. 1 | Exp. 2 |
| 2% $O_2$, 98% Ar | 34.5 (0.019) | 52.5 (0.038) |
| 2% $O_2$, 40% $H_2$, 58% Ar | 91.0 (0.026) | 98.0 (0.029) |

*Note.* Values in parentheses are calculated P : O ratios. Assays were conducted in Warburg flasks with the reaction medium described in the note attached to Table 2. There were 8.15 and 5.34 µg · chl. *a* per flasks in Exps. 1 and 2, respectively. Each value is a mean of duplicates. See "Materials and Methods" for further details

Table 3 shows the results of two experiments designed to determine whether $H_2$-mediated $O_2$ uptake is coupled to ATP synthesis. When $H_2$ was present together with 2% $O_2$, phosphorylation was 2 – 3-fold greater than when $H_2$ was absent. The relative amounts of $H_2$ and $O_2$ taken up when $H_2$ was added were calculated by assuming a $H_2 : O_2$ uptake ratio of 2 : 1.

Table 4 records results of attempts to demonstrate support of acetylene reduction by $H_2$. Generally acetylene reduction under $H_2$ was little greater than under argon. Exp. 1, Part B, showed a significant response to $H_2$ in the presence of light, but this response was considerably less in Exp. 2. Furthermore, no dark

Table 4. Effect of $H_2$ and argon on acetylene reduction by isolated heterocysts

| Conditions | nmoles $C_2H_4$/(μg · chl. $a$ × 20 min | |
|---|---|---|
| | Exp. 1 | Exp. 2 |
| A. No exogenous electron donor was present | | |
| $H_2$ + light | 3.74 | 2.24 |
| Ar + light | 3.41 | 1.99 |
| $H_2$ + dark | 0 | 0 |
| Ar + dark | 0 | 0 |
| B. 10.0 mM glucose-6-phosphate and 0.3 mM $NADP^+$ present | | |
| $H_2$ + light | 3.02 | 5.00 |
| Ar + light | 2.09 | 4.65 |
| $H_2$ + dark | 1.23 | 3.11 |
| Ar + dark | 1.36 | 3.77 |

*Note.* The heterocysts were isolated after disruption of vegetative cells by lysozyme treatment followed by sonication (see "Materials and Methods"). The heterocysts were resuspended in buffer containing a limiting concentration of $Na_2S_2O_4$ to provide protection from $O_2$ (see legend to Fig. 2). Assays were conducted under 83% $H_2$ or Ar and 17% $C_2H_2$ at 30° C and under 700 ft-c light where indicated. ATP and an ATP-generating system were present; other details of the assay procedure are described in Peterson and Burris (1976)

acetylene reduction occurred in the presence of 0.3 mM $NADP^+$ alone under an atmosphere of 83% $H_2$, 17% $C_2H_2$ (Peterson, 1976).

## DISCUSSION

Our observations on the characteristics of $H_2$ evolution in heterocysts are consistent with published reports of $H_2$ evolution in intact filamentous $N_2$-fixing blue-green algae (Benemann and Weare, 1974b; Jones and Bishop, 1976; Bothe et al., 1977; Peters et al., 1976). This ATP-dependent $H_2$ evolution is strongly inhibited by $N_2$ and completely suppressed by $C_2H_2$. $N_2$ inhibits $H^+$ reduction by competing for electrons from nitrogenase. Nitrogenase-catalyzed $H_2$ evolution in cell-free preparations of *Azotobacter vinelandii* is inhibited 65% by 1.0 atm $N_2$, but extrapolation of the data to infinite $pN_2$ indicates that $H_2$ evolution is never completely blocked by $N_2$. In contrast, $C_2H_2$ is capable of blocking $H_2$ evolution completely.

Evidence for hydrogenase activity in aerobically grown blue-green algae has been reported (Packer et al., 1977; Bothe et al., 1977). Rates of $H_2$ uptake by heterocysts described here are about 20-fold higher than the highest rates we have observed for their acetylene reduction. The only requirement for vigorous $H_2$ uptake by heterocysts appears to be the presence of an appropriate oxidant; no anaerobic adaptation treatment is necessary.

Isolated heterocysts from *Anabaena* 7120 were capable of removing $H_2$ from the suspension to a level not detected with the $H_2$ electrode (less than 1.0 nmole/ml). No $H_2$ evolution by intact $N_2$-fixing *Anabaena* 7120 was observed. As $O_2$ acts very effectively as an electron acceptor during $H_2$ oxidation, it is understandable that it would block accumulation of $H_2$ in the medium. Nearly complete suppression or $H_2$ evolution by 21% $O_2$ was observed by Jones and Bishop (1976) in several strains of intact *Anabaena*. Our attempts to demonstrate $H_2$ evolution by *Anabaena* 7120 in the absence of $O_2$ were unsuccessful, indicating that this organisms may have alternative electron sinks.

The oxyhydrogen reaction in heterocysts appears to be mediated by a cytochrome-based respiratory chain that terminates with a CO-sensitive oxidase. Cytochrome oxidase is inhibited by CO and intense light reverses such CO-inhibition; this response is diagnostic of cytochrome-oxidase-mediated respiration (Keilin, 1966, p. 252). The oxyhydrogen reaction in heterocysts is strongly inhibited by 85% CO, and this inhibition is reversed partially by intense light. When $O_2$ is absent and methylene blue serves as the oxidant, the inhibition by CO is much reduced and unaffected by light. The oxyhydrogen reaction also is sensitive to low concentrations of HCN. The *Anabaena* 7120 oxyhydrogen reaction bears superficial resemblance to the oxyhydrogen reaction in *Scenedesmus* (Gaffron, 1942; Horwitz, 1957).

Respiration catalyzed by cytochrome oxidase is saturated with $O_2$ at a low $pO_2$. The $Q_{O_2}(N)$ for heterocysts carrying out the oxyhydrogen reaction ranges from 500 – 650 μl $O_2$/(mg N × h) at a $pO_2$ of 0.02 atm or less. Inhibition by $O_2$ at progressively higher $pO_2$'s most likely reflects direct inhibition of the hydrogenase. The apparent reversibility of this $O_2$ inhibition (see Fig. 5) resembles the pattern of $O_2$ inhibition observed in *Proteus vulgaris* (Hoberman and Rittenberg, 1943; Fisher et al., 1954). Note that these observed rates of $O_2$ uptake by heterocysts are twice the $O_2$ uptake rates observed with intact *Anabaena* 7120 (Peterson and Burris, 1976). Fay and Walsby (1966) also observed higher respiratory rates in isolated heterocysts than in intact filaments of *A. cylindrica*.

Heterocysts are capable of oxidative phosphorylation. Tel-Or and Stewart (1976) have observed oxidative phosphorylation in heterocysts; their data, however, imply that photophosphorylation may be a more important source of ATP (see also Scott and Fay, 1972). Our data indicate that dark phosphorylation by heterocysts in the presence of $O_2$ is dependent upon the presence of ADP and is inhibited by the uncoupler carbonyl cyanide m-chlorophenyl hydrazone. A significant finding was that $H_2$ greatly in-

creased the rate of oxidative phosphorylation. Hence, $H_2$ evolved by nitrogenase in vivo may be recaptured by hydrogenase in heterocysts and the energy released through the oxyhydrogen reaction may be coupled to produce ATP. The low P:O ratios in heterocysts (Table 3) may reflect damage to them by the sonication employed in their isolation.

There is much interest currently in the hydrogen metabolism of $N_2$-fixing organisms. Hoch et al. (1960) observed that soybean nodules evolved $H_2$, and Dixon (1967) found that pea root nodules also took up $H_2$. Schubert and Evans (1976) have demonstrated a marked variation in the net $H_2$ production from the root nodules of various leguminous plants. The implication is that nodules that release little net $H_2$ actually metabolize the $H_2$ they produce and recapture energy that otherwise would be dissipated. Our observations of ATP formation coupled to the oxyhydrogen reaction in heterocysts furnish additional experimental evidence that $H_2$ metabolism in $N_2$-fixing organisms actually can be coupled to ATP formation.

Hydrogenase in isolated heterocysts resembles that in the particulate preparation of Fujita and Myers (1965), as no ATP-independent $H_2$ evolution could be shown. We have not demonstrated $NADP^+$ reduction by hydrogenase in heterocysts.

The heterocyst hydrogenase appears to reduce only acceptors that have positive potentials; Table 1 indicates that for acceptors with potentials up to 0.36 v, the more positive the potential the more rapid the reduction. The potential of the hydrogen electrode is below $-0.4$ v, but it appears that reduction of the acceptors tested is coupled into the respiratory electron transfer chain at much more positive potentials.

Although experiments reported here failed to demonstrate that the hydrogenase in heterocysts can generate reductant for nitrogenase, this possibility should not be ruled out. Wolk and Wojciuch (1971) observed strong enhancement by $H_2$ of acetylene reduction in intact filaments and detached heterocysts of *A. cylindrica*. Benemann and Weare (1974a) demonstrated $H_2$-dependent acetylene reduction in anaerobic, illuminated cultures of *Anabaena cylindrica* depleted of endogenous reductant. Ooyama (1976) has reported the isolation of $N_2$-fixing bacteria that oxidize $H_2$ as their source of energy for growth, $CO_2$ reduction and $N_2$ fixation. Fujita and Myers (1965) indicated that $H_2$-dependent reduction of $NADP^+$ occurs in a particulate preparation of hydrogenase from *A. cylindrica*. Perhaps $H_2$ recaptured from nitrogenase may be recycled as reductant for nitrogenase in vivo. We have been unable to establish which sink, $N_2$ fixation or respiration, competes most effectively for this reductant in heterocysts.

It could be argued on the basis of evidence presented by Smith et al. (1976) that $C_2H_2$ used in our nitrogenase assay could suppress hydrogenase activity, and hence, any $H_2$-supported acetylene reduction by isolated heterocysts. The results of Bothe et al. (1977) indicate that hydrogenase from *Clostridium pasteurianum* and *A. cylindrica* are much less sensitive to $C_2H_2$ than the *A. chroococcum* and *Klebsiella pneumoniae* enzymes. We found that $H_2$ uptake by isolated heterocysts with ferricyanide as the electron acceptor was unaffected by 17% acetylene, so the lack of $H_2$-supported nitrogenase activity in our isolated heterocysts was not attributable to inhibition of hydrogenase by $C_2H_2$.

*Acknowledgements*. This investigation was supported by the College or Agricultural and Life Sciences, University of Wisconsin-Madison, by National Science Foundation Grant GB-21422, by Public Health Service Grant AI-00848 from the National Institute of Allergy and Infectious Diseases and by Public Health Service Training Grant GM00236 BCH from the National Institute of General Medical Sciences.

We wish to thank David W. Emerich, Stephen L. Albrecht, and Bruce R. Selman for helpful discussions. We are also in debt to Ronald Zweifel and Paul Rosen for design and construction of apparatus.

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*Received November 14, 1977*