

## Regulation of Hydrogenase Activity in Vegetative Cells of *Anabaena variabilis*†

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Heterocyst-free ( $\text{NH}_4^+$ -grown) cultures of the cyanobacterium *Anabaena variabilis* produce a hydrogenase which is reversibly inhibited by light and  $\text{O}_2$ . White or red light at an intensity of 5,000 lx inhibited greater than 95% of the activity. Oxygen at concentrations as low as 0.5% inhibited more than 85% of the hydrogenase in the vegetative cells of  $\text{CO}_2$ - $\text{NH}_4^+$ -grown cultures. The vegetative cell hydrogenase is also sensitive to strong oxidants like ferricyanide. In the presence of strong reductants like  $\text{S}_2\text{O}_4^{2-}$ , hydrogenase activity was not inhibited by light. However, hydrogenase activity in the heterocysts was insensitive to both light (greater than 5,000 lx) and  $\text{O}_2$  (10%). Heterocysts and light-insensitive hydrogenase activity appear simultaneously during differentiation of the vegetative cells into heterocysts (an  $\text{NH}_4^+$ -grown culture transferred to  $\text{NH}_4^+$ -free,  $\text{CO}_2$ -containing medium). This light-insensitive hydrogenase activity was detected several hours before the induction of nitrogenase activity. These results suggest a mode of regulation of hydrogenase in the vegetative cells of *A. variabilis* that is similar to "redox control" of hydrogenase and other "anaerobic" proteins in enteric bacteria like *Escherichia coli*.

Several microorganisms are capable of both evolution and consumption of  $\text{H}_2$  under appropriate growth conditions. Cyanobacteria, capable of  $\text{N}_2$  fixation, evolve  $\text{H}_2$ , and it is generally accepted that this  $\text{H}_2$  evolution is catalyzed by the enzyme nitrogenase in the heterocysts (2). Heterocysts are also capable of  $\text{H}_2$  consumption catalyzed by an enzyme system containing hydrogenase. Several studies have indicated that vegetative cells from heterocystous cyanobacteria contain a reversible hydrogenase, but its physiological role in the cell is unknown. It has been postulated that the role of this hydrogenase in cellular metabolism is to remove excess reducing power generated by the cell as  $\text{H}_2$  (7, 12, 15). This process is similar to that found in fermentative bacteria growing under anaerobic conditions (11). However, under normal photosynthetic growth conditions, heterocyst-free cyanobacteria are not known to evolve  $\text{H}_2$  as a metabolic end product. This anomaly prompted us to study the regulation of hydrogenase activity by the vegetative cells of *Anabaena variabilis* as a function of different physiological conditions. The results presented in this communication show that the hydrogenase in the vegetative cells is present in an inactive state and that the enzyme is reversibly activated upon the removal of light and  $\text{O}_2$ .

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### MATERIALS AND METHODS

*A. variabilis* Kütz was grown in Allen and Arnon medium (A) at one-half strength (A/2; reference 10) in 125-ml Erlenmeyer flasks on a shaker at 30°C under continuous light (4,000 lx) as previously described (10). This medium was supplemented with 10 mM fructose to enhance the growth rate of the culture and 5 mM phosphate as a buffer. Cultures grown in the presence of  $\text{CO}_2$  as the sole carbon source were continuously bubbled with 0.8%  $\text{CO}_2$  in air at a flow rate of 30 ml/min. After about 4 to 6 days, filaments were harvested in mid-log phase of growth and washed once in minimal medium. This washed cell suspension was used for various assays. Growth of the culture was monitored as the increase in cell density measured as optical density at 750 nm (10). To determine the optical density, a sample (1.5 ml) of the culture was sonicated in a sonicator bath (Branson ultrasonic cleaner; model B-12) for 15 s to break up filament length. This step provides a uniform filament suspension and aids in the reproducibility of optical density measurements. The resulting values were converted to packed cell volume and dry weight by using a predetermined standard curve. A 1- $\mu\text{l}$  volume of *A. variabilis* cells is equivalent to 1  $\mu\text{l}$  of packed cell volume (see also reference 23). A volume of 12  $\mu\text{l}$  of packed cells corresponds to 1 mg of dry weight of cells and to an optical density at 750 nm of 4.8. Chlorophyll content of the cells was not used in our experiments because of the fluctuations in chlorophyll content with the age of the culture.

**Enzyme assays.** Nitrogenase and hydrogenase activities were assayed at room temperature (24°C) using 1.5 ml of algal suspension in 10-ml vials (Wheaton). To

assay for nitrogenase ( $C_2H_2$  reduction activity), the gas phase contained  $C_2H_2$  (10%) and Ar (90%). Gas samples of 50  $\mu$ l were withdrawn at appropriate time intervals, and the amount of  $C_2H_4$  present in the gas phase was determined with a Varian model 940 gas chromatograph equipped with a Porapak R column and flame ionization detector. Hydrogenase was assayed by the tritium exchange method (17). For these assays 100  $\mu$ l of  $^3H_2$  (11.2 mCi/mmol; New England Nuclear Corp.) was injected into reaction vessels as described above, but with He in the gas phase. These samples were incubated at room temperature in the dark or in the light. After appropriate time periods, 50- $\mu$ l samples of the liquid phase were withdrawn, diluted threefold with water, and mixed vigorously to remove any dissolved  $^3H_2$ .  $^3H_2O$  present in the samples was counted in a liquid scintillation counter, after the addition of 4.5 ml of Aquasol-2 (New England Nuclear Corp.). Formation of  $^3H_2O$  from  $^3H_2$  by nitrogenase is negligible, as shown by others (5).

**Nitrogenase induction of an  $NH_4^+$ -grown culture.** All cultures grown on  $NH_4^+$ , whether  $NH_4^+-CO_2$  or  $NH_4^+$ -fructose, were serially transferred to a new medium three to four times until they were completely devoid of heterocysts, as checked in the microscope. All  $NH_4^+$  cultures were grown in A/2 medium buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 10 mM) and sodium-potassium phosphate (5 mM) at pH 7.3. For the induction of heterocysts and nitrogenase, filaments from 10 ml of an  $NH_4^+$ -grown culture were centrifuged at room temperature in a table-top centrifuge. The filaments were suspended in 10 ml of A/2 medium supplemented with 4 mM fructose and transferred to 70-ml bottles (Wheaton). These bottles were sealed with serum stoppers, and the gas phase was changed from air to  $N_2$  (98%) and  $O_2$  (2%). These cultures were incubated at room temperature at saturating light (5,000 lx). At appropriate times, 0.5-ml samples were withdrawn for the determination of heterocyst frequency, growth, and nitrogenase and hydrogenase activities.

**Effect of light intensity on hydrogenase activity.** Samples from cultures grown in the presence of  $NH_4^+$  (8 mM) and fructose (4 mM) or  $NH_4^+$  (8 mM) and  $CO_2$  (0.8%) were collected by centrifugation and resuspended in A/2 medium containing 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU; 6  $\mu$ M) and chloramphenicol (200  $\mu$ g/ml). This DCMU concentration inhibited photosynthetic  $O_2$  production by greater than 95%. These cultures were transferred to 10-ml vials and incubated at 24°C under He. Illumination by different light intensities was achieved with a set of 20 W daylight fluorescent tubes placed at various distances and using Corning neutral density filters (S-7-98) to cover a broad light intensity range. The effect of red light on hydrogenase activity was monitored with a Corning filter (CS2-64) with transmittance between 648 and 725 nm.  $^3H_2$  added was 100  $\mu$ l per flask, and 50- $\mu$ l samples were withdrawn after 2 and 4.5 h for  $^3H_2O$  determination.

## RESULTS

The enzyme hydrogenase, present in the vegetative cells of heterocystous blue-green algae, has been generally assayed by monitoring the amount of  $H_2$  produced either from endogenous

reserves or from  $S_2O_4^{2-}$  as electron donor (6, 8, 21). The low levels of hydrogenase in these cells would require large amounts of cells per assay. The presence of  $S_2O_4^{2-}$ , a strong reductant, would certainly alter the metabolic state of the cell. In the experiments designed to determine the physiological role of hydrogenase, however, it is essential to monitor the enzyme in whole cells with minimum alterations to the metabolic state. This was achieved by using the  $H_2-H^+$  exchange property of hydrogenase in the following experiments and monitoring the enzyme activity with  $^3H_2$  serving as the substrate. The product,  $^3H_2O$ , is easily detectable by sensitive methods, and the assay requires only small amounts of cells.

Hydrogenase activity was not detectable in an  $NH_4^+$ -grown, heterocyst-free culture in the light (first 24 h) (Fig. 1). After transfer of this culture to a medium free of combined N and containing only  $N_2$  as the nitrogen source, no growth was observed for about 24 h, the time required for the development of heterocysts. Fully developed heterocysts were observed only about 24 h after the transfer into N-free medium. The heterocyst frequency in the population then continued to increase for another 2 days. Growth resumed with the development of heterocysts and with the induction of nitrogenase activity, and the filaments continued to grow throughout the experimental period.

Nitrogenase activity was detectable after about 38 h, a lag of about 14 h after the initial observation of differentiated heterocysts. On the other hand, hydrogenase activity could be detected by the  $^3H_2$ -exchange assay as soon as heterocysts were observed. Nitrogenase activity was detected only when the frequency of heterocysts increased to a value of more than 3%. Between 5 and 10% heterocyst frequency, nitrogenase activity increased exponentially; this increase was 20.6 U (nanomoles of  $C_2H_4$  produced per milligram of dry weight per hour) for every 1% increase in heterocysts, indicating a net synthesis of the active protein. Under these experimental conditions, hydrogenase activity increased at a linear rate of 3.4 U (nanomoles of  $^3H_2O$  produced per milligram of dry weight per hour) per every 1% increase in heterocyst frequency, without any apparent lag. The differential rate of production of hydrogenase and nitrogenase activities, with respect to the production of heterocysts, suggests that the vegetative cells may contain a hydrogenase that is not detectable under photosynthetic conditions in the presence of light and  $O_2$ . During the differentiation of a cell into a heterocyst, this activity becomes apparent.

When the filaments were assayed for hydrogenase activity in the dark instead of in the light

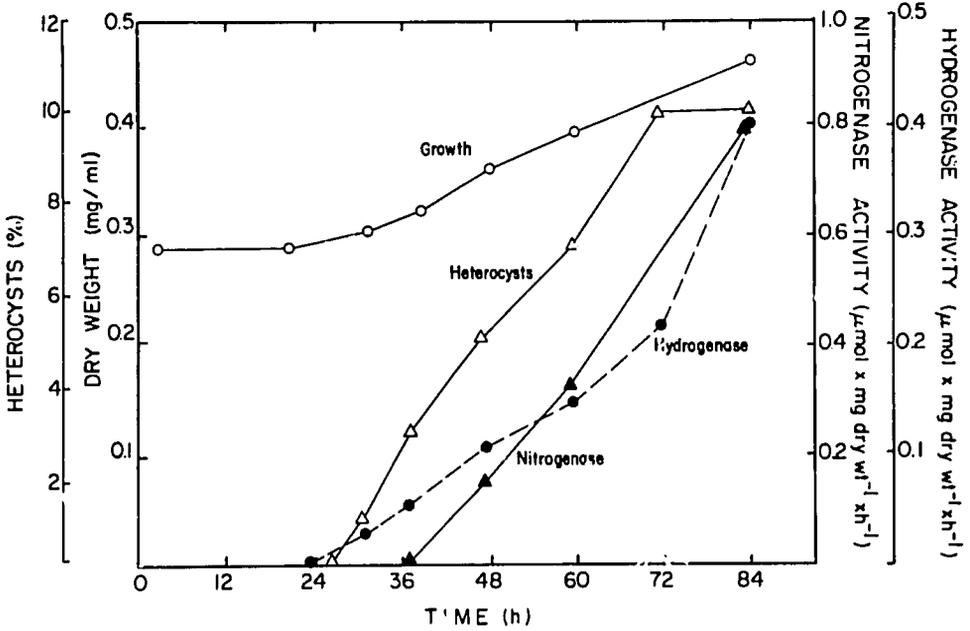


FIG. 1. Induction of hydrogenase and nitrogenase in vegetative cells of *A. variabilis*. An  $\text{NH}_4^+$ -fructose-grown culture was washed and transferred to A/2 medium supplemented with 4 mM fructose. At the times indicated, samples were taken for measurement of growth, heterocysts, nitrogenase activity, and hydrogenase activity.

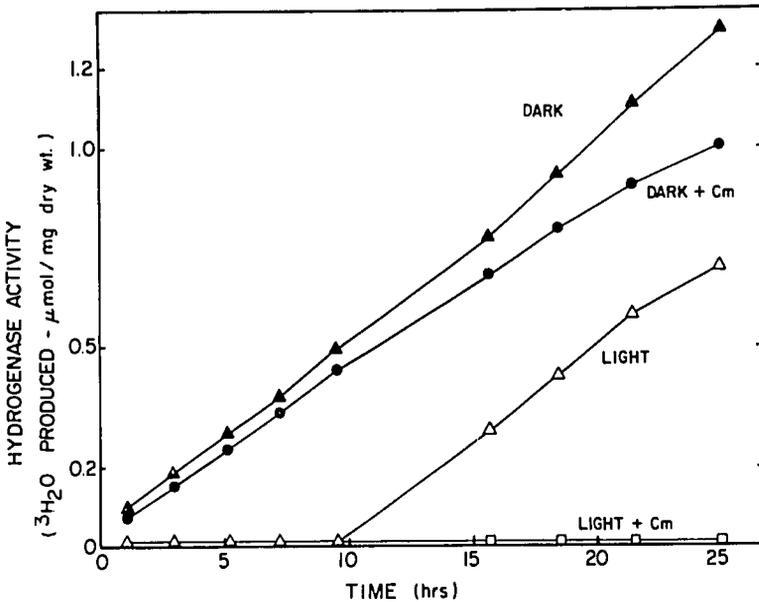


FIG. 2. Light inhibition of vegetative cell hydrogenase and the onset of light-insensitive hydrogenase activity in differentiating cells of *A. variabilis*. Cultures previously grown in  $\text{NH}_4^+$  (8 mM) medium supplemented with 4mM fructose were washed free of  $\text{NH}_4^+$  and suspended in A/2 medium with fructose (4 mM). Hydrogenase activity in these cells was assayed under four different conditions: dark, dark with chloramphenicol (Cm; 200  $\mu\text{g/ml}$ ), light (5,000 lx), and light plus chloramphenicol. Tritium gas (100  $\mu\text{l}$ ) was added at the beginning of the experiment, and the amount of  $^3\text{H}_2\text{O}$  present in the culture supernatant was determined at the time periods indicated.

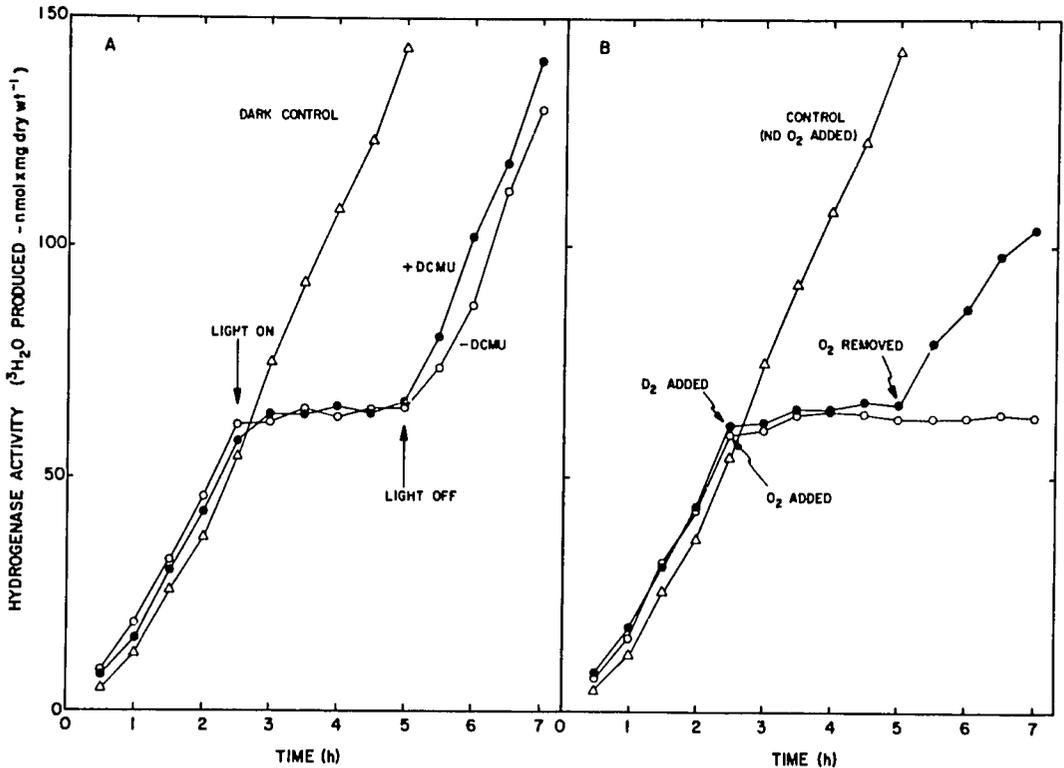


FIG. 3. Reversibility of inhibition of hydrogenase activity by light (A) or  $O_2$  in *A. variabilis*. A fructose- $NH_4^+$ -grown culture was washed, suspended in A/2 medium, and assayed for hydrogenase activity. After the gas phase of the reaction vessels was replaced with He,  $^3H_2$  (25  $\mu$ l) was added. Tritiated water in the assay supernatant was determined after certain time intervals. Chloramphenicol (250  $\mu$ g/ml) was present to prevent further protein synthesis. (A) After 2.5 h, two of the dark, anaerobic samples (one containing 6  $\mu$ M DCMU and another without DCMU) were transferred to light (5,000 lx). After 2.5 h of illumination, both samples were removed from light and incubated further in the dark. (B) After 2.5 h,  $O_2$  was added to two of the anaerobic samples at a final concentration of 10% in the gas phase. After 5 h  $O_2$  was removed and replaced by He in one of the  $O_2$ -containing samples.

(5,000 lx), the  $NH_4^+$ -grown culture produced detectable hydrogenase activity even before the appearance of mature heterocysts, and this activity was not affected by the protein synthesis inhibitor chloramphenicol (Fig. 2). In this experiment also, the hydrogenase activity in an illuminated sample could be observed only after the heterocysts were detectable (10 h). The differentiation of vegetative cells into heterocysts, accompanied by the production of light-insensitive hydrogenase activity, could be abolished by chloramphenicol. This set of experiments indicates that an  $NH_4^+$ -grown culture contains a hydrogenase activity detectable only under dark anaerobic conditions, and only the heterocysts contain a hydrogenase activity that can be measured in the presence of light.

It is known that the enzyme hydrogenase is sensitive to oxygen. *A. variabilis* is capable of producing oxygen in the presence of light by utilizing photosystem II. This raises the possibility that the lack of hydrogenase activity in the

vegetative cells under light might be a consequence of the effect of  $O_2$  on hydrogenase. To test this possibility, the effect of light on hydrogenase activity was determined both in the presence and absence of DCMU, an inhibitor of photosynthetic  $O_2$  evolution, and of chloramphenicol, an inhibitor of new protein synthesis. Light inhibited hydrogenase activity immediately in media both with and without DCMU (Fig. 3A). DCMU had no effect on hydrogenase activity in the dark. When the light was turned off, hydrogenase activity resumed immediately; this rate was comparable to that in a culture that had been kept in the dark throughout the experiment (5.64 U for the DCMU culture, 6.00 U for the culture without DCMU, and 5.40 U for the dark culture). Recovery of hydrogenase activity from light inhibition, in the culture without DCMU, exhibited a lag of about 25 min before the maximum rate was achieved. This could be due to the  $O_2$  produced by photosystem II. These experimental results show that the hydrogenase

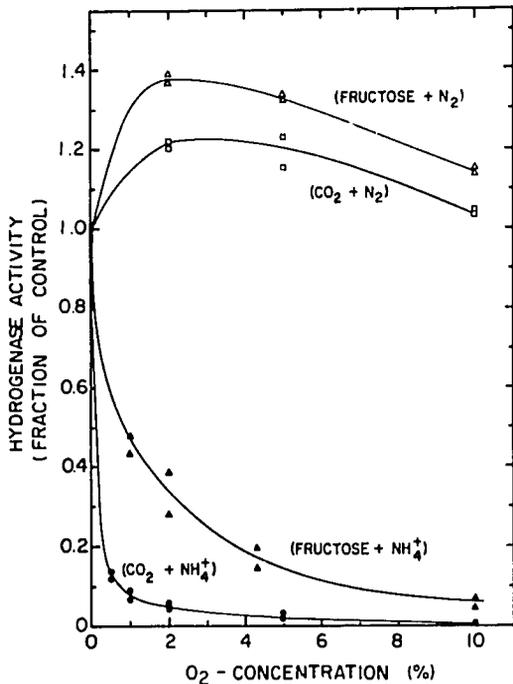


FIG. 4. Effect of  $O_2$  concentration on hydrogenase activity in  $NH_4^+$ -grown (heterocyst-free) and air-grown filaments (with heterocysts) of *A. variabilis*. The respective cultures were washed twice and suspended in A/2 medium containing chloramphenicol (250  $\mu$ g/ml). All assays were carried out in the dark. The specific activity of hydrogenase (units = nanomoles per milligram of dry weight per hour) in the dark for these cultures was as follows: fructose- $N_2$ , 33.6 U;  $CO_2$ - $N_2$ , 26.4 U; fructose- $NH_4^+$ , 60.0 U;  $CO_2$ - $NH_4^+$ , 6.0 U.

activity in the vegetative cells is reversibly inhibited by light and by  $O_2$ .

The lag period observed in the dark recovery of hydrogenase after illumination in the absence of DCMU (Fig. 3A) suggests that hydrogenase is also sensitive to  $O_2$  and that at the end of the lag period the  $O_2$  produced by photosynthesis was removed by respiration. Addition of  $O_2$  (10%) to a culture also inhibited the hydrogenase activity reversibly (Fig. 3B). However, upon complete removal of  $O_2$ , the hydrogenase activity only reached a maximum of about 55% (3.12 U) of the anaerobic control (5.64 U), indicating that some of the hydrogenase was irreversibly inactivated by 10%  $O_2$  in the gas phase.

We have further compared the sensitivity of hydrogenase to  $O_2$  in cultures previously grown under various conditions, with either  $CO_2$  or fructose as the carbon source and  $NH_4^+$  or  $N_2$  as the nitrogen source (Fig. 4). Hydrogenase activity in cells grown in a medium containing fructose and  $NH_4^+$  was less than 50% of the control in the presence of 1%  $O_2$  in the gas phase. This

inhibition between 1 and 10%  $O_2$  was exponential with increasing  $O_2$  concentration. However, the hydrogenase activity in cells grown in the presence of  $NH_4^+$ - $CO_2$  (0.8%) was even more sensitive to  $O_2$ . Greater than 85% of the activity was inhibited by 0.5%  $O_2$  in these cultures. In contrast, heterocyst-containing cultures ( $N_2$  as nitrogen source) cultivated in the presence of either  $CO_2$  or fructose as a carbon source showed a stimulation of hydrogenase activity at about 2%  $O_2$ . Obviously the hydrogenase activity in the heterocysts is not inactivated by  $O_2$ . The slower rate of decline in the hydrogenase activity in fructose- $NH_4^+$ -grown cultures (containing only vegetative cells) with increasing  $O_2$  concentration (compared to a  $CO_2$ / $NH_4^+$ -grown culture) suggests that cellular dark respiration utilizing endogenous reserves plays an important role in these experiments.

The inhibitory effect of light on hydrogenase activity may be achieved by a catalytic reaction, triggering a series of regulatory processes culminating in the inhibition of hydrogenase. Such a process would require only low light intensities. On the other hand, light may act as a substrate and normal photosynthetic reactions would be involved in the regulation of hydrogenase activity. To discriminate between these possibilities, the effect of light intensity on the activity of hydrogenase was determined, using white light (Fig. 5). Both  $CO_2$ - $NH_4^+$ - and fructose- $NH_4^+$ -grown cultures had similar kinetic responses to light intensity (Fig. 5, inset). Hydrogenase activity decreased with increasing light intensity in an exponential manner, reaching greater than 95% inhibition at 1,000 lx. Hydrogenase activity, although much lower in  $CO_2$ -grown cultures than in fructose-grown cultures, was sensitive to very low light intensities. Twenty-five percent of the activity was lost at a light intensity of 4 lx. In these experiments, the effect of red light on hydrogenase activity was similar to that of white light (data not presented).

It is possible that the inhibitory effect of  $O_2$  and light is mediated by their ability to serve as oxidants in the cell. To test this possibility, the effect of several artificial electron carriers (acceptors) on hydrogenase activity was tested both in fructose-grown and  $CO_2$ -grown cells (Table I). Dichlorophenolindophenol (DPIP;  $E_0' = +217$  mV), methylene blue ( $E_0' = +11$  mV), and ferricyanide ( $E_0' = +360$  mV) inhibited the hydrogenase activity in the vegetative cells of  $CO_2$ -grown cultures. Low potential electron carriers, like benzyl viologen ( $E_0' = -360$  mV) and methyl viologen ( $E_0' = -440$  mV), had little or no effect. The pattern of inhibition was different with a fructose- $NH_4^+$ -grown culture, which had a much higher specific activity of hydrogenase than a  $CO_2$ - $NH_4^+$ -grown culture. In a fructose-

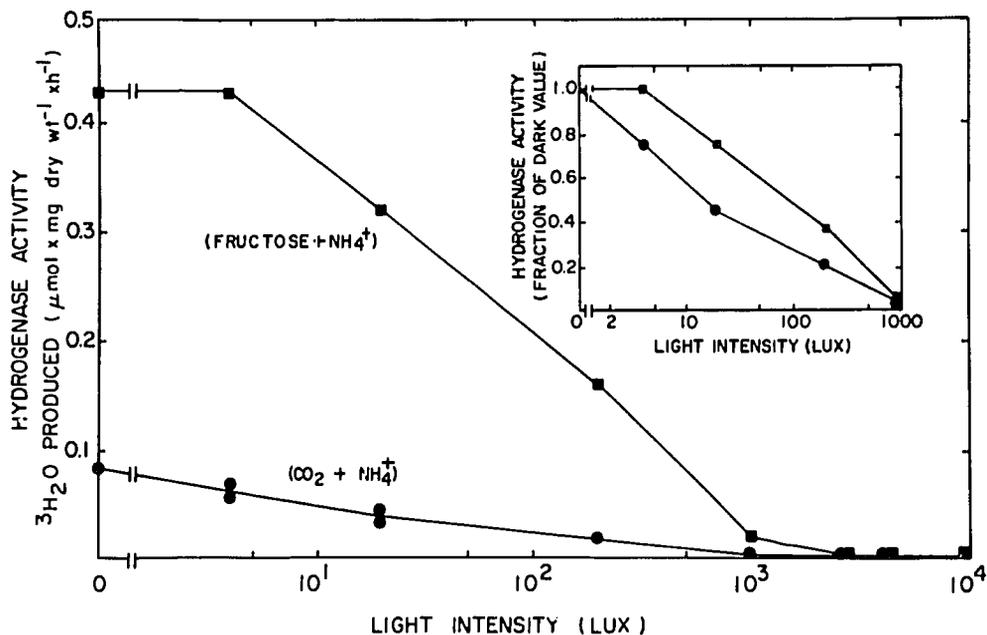


FIG. 5. Effect of light intensity on vegetative cell hydrogenase in *A. variabilis*. Cultures were washed once in A/2 medium and assayed in A/2 medium in the presence of DCMU (6  $\mu$ M) and chloramphenicol (200  $\mu$ g/ml). Other assay conditions were as described in the text. Symbols: ■, fructose- $\text{NH}_4^+$ ; ●,  $\text{CO}_2$ - $\text{NH}_4^+$ .

grown culture, DPIP had only a minimal inhibitory effect and benzyl viologen and methyl viologen had no effect. With methylene blue and ferricyanide, on the other hand, immediate inhibition of hydrogenase was observed. Except for DPIP, in which case the low concentration may have prevented a prolonged inhibition (higher concentrations totally inactivate hydrogenase), there was a good correlation between the redox potential of electron carriers and inhibition.

TABLE 1. Effect of artificial electron carriers on hydrogenase activity in *A. variabilis*<sup>a</sup>

Electron carrier	$E_0'$ (mV)	Hydrogenase activity (nmol of $^3\text{H}_2\text{O}$ produced mg dry wt $^{-1}$ h $^{-1}$ )	
		$\text{CO}_2/\text{NH}_4^+$ -grown filaments	Fructose/ $\text{NH}_4^+$ -grown filaments
None		17.3	130
Methyl viologen	-440	17.3	117
Benzyl viologen	-360	12.5	117
Methylene Blue	+11	3.1	37
DPIP	+217	5.7	110
Ferricyanide	+360	0.0	44

<sup>a</sup> Electron carrier at a concentration of 2 mM (except for DPIP at 0.2 mM) was added to a culture in the dark, 6 h after the addition of  $^3\text{H}_2$ , DCMU (6  $\mu$ M), and chloramphenicol (200  $\mu$ g/ml), to initiate the reaction. Hydrogenase activities were monitored for another 9 h. See the text for other experimental details.

If electron acceptors with higher redox potential can act as inhibitors of hydrogenase activity, then it is possible that low potential electron donors like  $\text{S}_2\text{O}_4^{2-}$  ( $E_0' = -527$  mV) can reverse the light-mediated inhibition. Thus, an experiment was devised to test the effect of different concentrations of reductant (dithionite and cysteine) on the reversal of light-mediated inhibition of hydrogenase (Fig. 6). DCMU (6  $\mu$ M) was included in these experiments to prevent  $\text{O}_2$  evolution and to create microaerobic conditions. The inhibition by light was relieved by 2 mM  $\text{S}_2\text{O}_4^{2-}$  (Fig. 6). Cysteine ( $E_0' = -340$  mV), a sulfhydryl reagent, reversed the light inhibition of hydrogenase only partially, even at a concentration of 10 mM. Both cysteine (2 mM) and  $\text{S}_2\text{O}_4^{2-}$  (2 mM) also enhanced the hydrogenase activity in the dark (25 and 80%, respectively). Other compounds that were either redox compounds or both redox compound and sulfhydryl reagent were also tested for their effect on hydrogenase activity in the light. Ascorbate at 5 mM ( $E_0' = +70$  mV) had no significant effect, similar to reduced glutathione ( $E_0' = -340$  mV). Dithiothreitol ( $E_0' = -332$  mV) at 5 mM reversed the light inhibition by one-third; calcium-(poly) sulfide and  $\text{H}_2\text{S}$  reversed the inhibition completely. These differences in the reversal of light inactivation by sulfhydryl reagents with similar redox values may be due to differences in the ability of these compounds to traverse the permeability barrier of the cell.

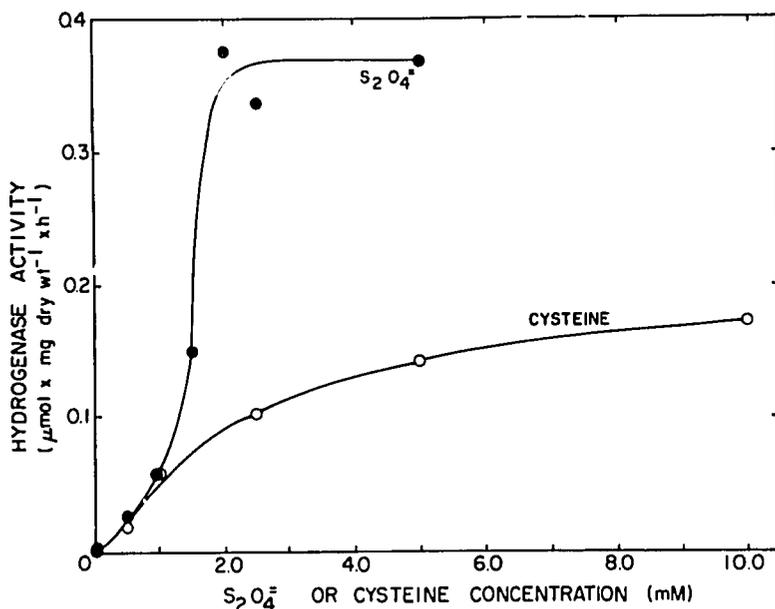


FIG. 6. Effect of dithionite and cysteine on vegetative cell hydrogenase in *A. variabilis*. A culture grown in the presence of  $\text{NH}_4^+$ -fructose was washed once, suspended in A/2 medium in the presence of DCMU ( $6 \mu\text{M}$ ) and chloramphenicol ( $200 \mu\text{g/ml}$ ), and assayed for  $^3\text{H}_2\text{O}$  production. Incubation was carried out in the light for 7 h.

## DISCUSSION

Hydrogenase is a protein capable of catalyzing  $\text{H}_2$  production from endogenous electron donors or utilizing  $\text{H}_2$  as a source of reductant (1). This enzyme is detectable in several bacteria and green algae (24). Among cyanobacteria, hydrogenase can be observed in unicellular and filamentous as well as in  $\text{N}_2$ -fixing organisms (16, 18). It is generally assumed that the role of hydrogenase in the heterocysts of nitrogen-fixing, filamentous cyanobacteria is to recycle the  $\text{H}_2$  produced by the enzyme nitrogenase (3, 21). Hydrogenase is also present in the vegetative cells of  $\text{N}_2$ -fixing, filamentous cyanobacteria, which are not active sites of  $\text{N}_2$  fixation (8, 22). The physiological role, as well as the control, of this vegetative cell hydrogenase and the hydrogenase in non-nitrogen-fixing cyanobacteria is largely unknown. The vegetative cell hydrogenase is readily reversible and presumably present in the cytoplasm (8, 12). The hydrogenase activity in the vegetative cells is very low compared to that in the heterocysts in the same filament. This very low activity led Bothe and his co-workers to suggest that reversible hydrogenase is usually not present in vegetative cells of *Nostoc* and that the observed activity may represent a heterocyst contamination of the vegetative cell preparations or an artifact of solubilized hydrogenase released from membranes (6). One of the major problems contributing to the confusion over the

existence of hydrogenase in the vegetative cells of  $\text{N}_2$ -fixing cyanobacteria is the enzyme's low activity, coupled with the low sensitivity of the assay procedures measuring the appearance or disappearance of  $\text{H}_2$ .

The data presented in this paper clearly show that the vegetative cells of *A. variabilis* produce detectable levels of hydrogenase activity. In these experiments the  $\text{H}_2$ - $\text{H}^+$  exchange capability of the hydrogenase was used to monitor the presence and activity of the enzyme. This assay system employs  $^3\text{H}_2$  and is extremely sensitive and independent of electron donors and acceptors. Hydrogenase activity was also monitored by determining  $\text{H}_2$  evolution from dithionite-methyl viologen, and found to be rather low (data not presented). Since both methods were used with intact filaments in the complete absence of heterocysts in the growth medium, the occurrence of reversible hydrogenase in vegetative cells is thus unequivocally confirmed. This hydrogenase can also be released by osmotic shock (data not presented).

The hydrogenase in the vegetative cells of *A. variabilis* was found to be sensitive to light when the  $^3\text{H}_2$ -exchange method was used to monitor the enzyme. An analogous decrease in hydrogenase activity with increasing light intensity was reported in green algae (14, 16). In these organisms, this light effect was interpreted to be due to the inactivation of hydrogenase by  $\text{O}_2$  generated by photosystem II. In *A. variabilis*,

however, the light-dependent inactivation is readily reversible, and the inhibition occurs even in the presence of DCMU, an inhibitor of photosynthetic  $O_2$  evolution (Fig. 3A). The immediate recovery of hydrogenase activity, in the absence of any new protein synthesis, would also argue against photosynthetic  $O_2$  production as a mechanism of light inactivation (see Fig. 3B).

Oxygen by itself, or derivatives of it such as superoxide, may also act as an inhibitor of hydrogenase activity. The amount of oxygen required for complete inactivation, however, far exceeds the minuscule amounts of photosynthetic  $O_2$  evolved in the presence of DCMU. Furthermore, the inclusion of DCMU resulted in  $O_2$  consumption in the light which approached dark respiration values, and net  $O_2$  evolution was not observed in the presence of DCMU. The data presented in Fig. 3 suggest that the effect of light is independent of  $O_2$  inactivation of hydrogenase.

It is interesting that as the vegetative cells mature into heterocysts, inhibition of hydrogenase activity by light or oxygen is totally relieved (Fig. 1 and 4), suggesting that either the controlling elements or the metabolic events leading to the control of hydrogenase in the vegetative cells are altered in the heterocysts. Heterocysts are known to be defective in system II photosynthesis and  $O_2$  evolution and lack  $CO_2$  fixation (9).

The level of hydrogenase activity in the cells is influenced by the carbon source in the growth medium ( $CO_2$  or fructose; Fig. 5, Table 1). In the presence of fructose, the hydrogenase activity was as high as 10 times the values obtained with  $CO_2$ -grown cultures (Fig. 4). Katoh and Ohki (13) demonstrated that the photosystem II activity in *A. variabilis* can be decreased by addition of high levels of citrate and Casamino acids to the growth medium. We have also observed that the net rate of  $O_2$  evolution by filaments can be reduced by increasing the fructose concentration in the medium (10). However, under the experimental conditions used in Fig. 4 and 5 and Table 1, both  $CO_2$ - and fructose-grown cultures produced about the same levels of chlorophyll (32.4 g/mg of dry weight). This indicates that the high levels of hydrogenase activity in the fructose culture are not a result of photosynthetic pigment level, but rather a consequence of photoheterotrophic growth conditions.

Hydrogenase activity is also subject to major changes depending on the type of redox compound present in the medium. Addition of strong reductants such as dithionite leads to reductant excess conditions, besides removing any oxidant present in the cell, even in the presence of light, and thus activation of hydrogenase oc-

curred (Fig. 6). On the other hand, artificial electron acceptors like ferricyanide, methylene blue, or DPIP with positive  $E_0'$  would decrease the reductant pool sizes, even in the dark, and thus inactivate hydrogenase (Table 1). These results suggest that under conditions of reductant excess hydrogenase is active, whereas at low levels of reductant the enzyme is inactive. One could perceive that hydrogenase activity in the vegetative cells could be modulated by the ratio of a redox couple such as  $NAD^+$ -NADH as a physiological electron carrier.

As a working hypothesis, we propose that hydrogenase synthesis and activity in the vegetative cells of *A. variabilis* are regulated by the redox state of the cell (presence or absence of light, reductant, or oxidant). Such a control system, termed redox control, has been postulated to regulate the synthesis of anaerobic proteins, including hydrogenase, in enteric bacteria (19), and the role of redox control in nitrogen fixation has been reviewed recently (20). Although the control of hydrogenase activity in the vegetative cells is modulated by the availability of reductant or oxidant, we do not know the actual biochemical mechanism or the intermediate steps involved in this control. Additional experiments are necessary to determine the molecular mechanisms of this control and to show how this hydrogenase activity differs from the one in the heterocyst.

Buchanan observed that thioredoxin acts as an intermediate in the modulation of several  $CO_2$ -fixation reactions in photo-synthetic organisms, including plants (4). In these systems thioredoxin is reduced by the photosystem, which in turn reduces certain enzymes, e.g., fructose-1,6-diphosphate phosphatase, into an active form. Thioredoxin, thioredoxin reductase, and photosynthetic particles can be replaced by dithiothreitol. A working scheme involving thioredoxin in the regulation of hydrogenase would not be inconsistent with the experimental results described in this paper.

A better understanding of the molecular mechanism of the regulation of hydrogenase by photosynthetic cultures could lead to development of new methods for achieving high rates of photosynthetic  $H_2$  production by using water as an electron donor and light as an energy source. A knowledge of the regulation of hydrogenase synthesis and activity will permit researchers to perform genetic alterations of cyanobacteria for maximum photohydrogen production.

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