Hydrogen Production by Nitrogen-Starved Cultures of Anabaena cylindrica

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Nitrogen-starved cultures of the alga Anabaena cylindrica 629 produced hydrogen and oxygen continuously for 7 to 19 days. Hydrogen production attained a maximum level after 1 to 2 days of starvation and was followed by a slow decline. The maximum rates were 30 ml of H2 evolved per liter of culture per h or 32 μl of H2 per mg of dry weight per h. In 5 to 7 days the rate of H2 evolution by the more productive cultures fell to one-half its maximum value. The addition of 10⁻⁴ to 5 × 10⁻⁴ M ammonium increased the rate of oxygen evolution and the total hydrogen production of the cultures. H2-O2 ratios were 4:1 under conditions of complete nitrogen starvation and about 1.7:1 after the addition of ammonium. Thus, oxygen evolution was affected by the extent of the nitrogen starvation. Thermodynamic efficiencies of converting incident light energy to free energy of hydrogen via algal photosynthesis were 0.4%. Possible factors limiting hydrogen production were decline of reductive supply and filament breakage. Hydrogen production by filamentous, heterocystous blue-green algae could be used for development of a biophotolysis system.

In 1896 Jackson and Elms (13) found that an Anabaena sp. from a Massachusetts reservoir immediately began to form hydrogen when placed in a sealed bottle. It is uncertain whether the hydrogen was produced by this blue-green alga or by contaminating bacteria. However, the alga did not visibly decolorize or decompose until about 4 days later, after the gas evolution had ceased. In 1942 Gaffron and Rubin (10) reported that hydrogen was evolved by anaerobically incubated Scenedesmus obliquus, a green alga. Further work by Gaffron and other researchers has led to the following characterization of green algae hydrogen production (14). It requires a period of 2 to 30 h of anaerobic adaptation for the reaction to commence. The period of peak productivity (4 μl of H2 per mg of dry weight per h [12]) is only a few hours long, and the reaction completely ceases after approximately 1 day. Gas-phase oxygen concentration above about 0.2% is inhibitory. Moderate and high light intensities photosynthetically produce this concentration of oxygen unless O2 evolution by photosystem II is blocked or chemically absorbed. Hence, H2 evolution does not occur simultaneously with O2 evolution, except at low levels of the latter or as a transient phenomenon (6). Although hydrogen production from water by photosynthetic systems (biophotolysis) has recently been receiving attention as a method of solar energy conversion (2, 3, 5, 17, 18), the above mentioned properties of H2 production by green algae make them doubtful candidates for practical biophotolysis systems.

In 1974 Benemann and Weare (4) used actively growing cultures of the heterocystous blue-green alga Anabaena cylindrica 629 to demonstrate simultaneous hydrogen and oxygen production. The alga was grown in nitrate-free media under an atmosphere of air and CO2 and, when incubated in the light under an atmosphere of argon and CO2, immediately started producing hydrogen. The indication was that the nitrogen-fixing enzyme system was responsible for the hydrogen evolution. The production of hydrogen by this system was about two to three times as much as has been reported using green algae. The hydrogen-oxygen ratio was approximately 1:7. This system could be developed for biophotolysis.

Nitrogenase is a very oxygen-labile enzyme (8, 11). However, light-saturated, unstarved cultures of A. cylindrica exhibit no significant O2 inhibition at atmospheric oxygen tensions (23, 24). It is generally accepted that nitrogenase activity is localized in heterocysts that lack O2-evolving photosystem II (7, 21, 22), have high respiration rates (9), are surrounded by a thick cell wall (15), and have a reducing intracellular environment (19). In a proposed model of heterocyst function (23), reductant flows into

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the heterocysts from vegetative cells, energy is supplied by photo- and oxidative phosphorylation, and fixed nitrogen flows out of the heterocysts to the vegetative cells. The physiology of heterocystous blue-green algae as it relates to hydrogen production as a method of solar energy conversion is discussed.

Our prerequisites for considering any system for biophotolysis are: (i) sustained hydrogen and oxygen evolution for many weeks; (ii) a 2:1 ratio of hydrogen to oxygen; (iii) high specific rates of hydrogen production and photosynthetic efficiency; and (iv) no limitation to scaling up the system. In this paper nitrogen-starved cultures of A. cylindrica are used to approach these goals.

MATERIALS AND METHODS

Organism and culture methods. A. cylindrica 629 was grown as described previously (23) in modified Allen and Arnon (1) medium (modifications: 2 μg of Fe per ml as ethylenediaminetetraacetic acid complex and 5.0 x 10⁻³ M NaHCO₃ at 25 to 28°C and gassed with approximately 0.3% CO₂ in air to keep the pH between 7.5 and 8.2. When the desired cell density was reached, cultures were switched to argon-CO₂ (day 0 of experiments described in text).

Growth was measured routinely with a model 800-3 Klett-Summerson photoelectric colorimeter with a no. 66 red filter. Dry-weight determinations were made by filtering portions of culture on preweighed filters, washing with 4 volumes of distilled water, and drying to constant weight at 80°C. The conversion factor between dry weight and Klett reading was 0.0035 mg of dry weight per ml equals 1 Klett unit. This correlation showed a 15% variability between different samples. All of these measurements were based on suspended material only, since settled algae did not exhibit any H₂ production. Occasionally, spurious cell density values were obtained when cultures were inadvertently shaken or disturbed, causing settled material to become temporarily suspended (see day 3, Fig. 2b). Contamination was checked by streaking petri dishes containing 20 g of agar per liter, 1 g of tryptone per liter, 1 g of glucose per liter, and 1 ml of 2 M K₂HPO₄ per liter and incubating at 27 to 28°C for at least 10 days. In addition, spot checks were done by examining samples under the microscope.

Illumination. Two basic lighting arrangements were used. The first consisted of a two-sided light box with four Sylvania F72T12 cool white fluorescent bulbs on each side. One-liter capacity Roux bottles filled with water lined each side and served as heat filters. The light intensity, measured with a YSI-Kettering model 65 radiometer, at all heights on the sides of a 2-liter capacity culture vessel (outer diameter, 12.5 cm) was 2.0 x 10⁶ ergs/cm² per s and at all heights on the sides of 250-ml capacity culture vessel (outer diameter, 4.5 cm) was 1.8 x 10⁶ ergs/cm² per s. Reproducibility of light measurements was within about 10%.

The second lighting arrangement consisted of two General Electric 150-W reflector spotlights placed on opposite sides of 250-ml capacity culture vessels. A 5-liter capacity flask filled with 1.2 g of CuSO₄·5H₂O solution per liter was placed between each spotlight and the growth vessel to absorb heat. Light intensity was measured at 12 heights on each side of the container; the results were added and then averaged, giving the values quoted in the text. Spotlights were arranged so that maximum intensity coincided with the center of the width of the container. Although intensities fell off somewhat as the probe was moved away from this point, only the maximum intensities were recorded. The total intensity for the region at the bottom of the cylinder (equal to about 25 ml) was approximately 10% of the average intensity. The spotlights were far enough from the container that the culture did not receive any light that went undetected by the light probe. Using these procedures ensured that the photosynthetic efficiencies calculated are low estimates.

Acetylene reduction assays. Fernbach flasks (5.7 ml, serum stopped) were prepared by flushing for 10 min with argon and then injected with predetermined amounts of other gases or solutions or both. One milliliter of acetylene was added last after the flasks were vented to atmospheric pressure, rendering 15% acetylene by volume. Samples were withdrawn from culture vessels with sterile 25-ml pipettes and transferred with argon-CO₂ flowing above the surface of the flask when necessary, and a 2-ml portion was slowly injected into each assay flask with a 20-gauge needle and 10-ml syringe. With this technique, contamination from the O₂ in the air was kept to a minimum. Flasks were incubated for 20 min on a shaker at 27°C under a light intensity of 6.0 x 10⁵ ergs/cm² per s. Reactions were terminated by injecting 0.25 ml of approximately 25% (wt/vol) trichloroacetic acid in water. Controls showed no ethylene production. Ethylene production was measured using a series 200 Varian Aero- graph gas chromatograph equipped with a column packed with Poropak R and a flame ionization detector. The carrier gas was N₂. Overall precision for the acetylene reduction assay was about 8%.

Hydrogen production assays. Samples were collected by withdrawing 1 ml of gas from the effluent tube of the experimental vessel. Duplicate or triplicate samples were always taken. These 1-ml samples were injected into a model A-90-P3 Aerograph gas chromatograph equipped with a column packed with molecular sieve 5A and a thermal conductivity detector. Argon was used as the carrier gas. When correlated with standard curves, hydrogen concentrations were obtained. Volume specific rates were calculated using data on culture volume and gassing rates. The latter were measured using a soap bubble flowmeter. Overall precision was 10%. Mass specific rates were calculated from volume rates, Klett measurements, and the Klett-dry weight conversion factor. All additions to cultures, culture sampling, and flow rate changes were made either immediately after the withdrawal of gas samples or many hours before. This was necessary to allow the system to be at steady state for hydrogen production determinations.
Oxygen evolution assays. Assays to determine oxygen evolution were performed using procedures identical to those used to determine hydrogen production, except that the gas chromatograph column temperature was lowered and injections were made using a 1-ml Precision Pressure-Lok sampling syringe to keep air contamination to a minimum. However, precision was only 15 to 20% due to the impossibility of totally eliminating air contamination and the reduced sensitivity of the detector to oxygen when using argon as the carrier gas.

Photosynthetic efficiencies. The efficiency of photosynthetic conversion of light to H₂ is the free energy of the total amount of H₂ produced divided by the total energy of the light incident on the culture vessels, times 100. Free energy was used since it is the proper measure of maximum work. The value of 51.6 kcal/mol of H₂ was calculated from the reaction:

\[ \text{H}_2 (g, 1 \text{ atm}) + \frac{1}{2} \text{O}_2 (g, 1 \text{ atm}) = \text{H}_2\text{O} (g, 1 \text{ atm}) \]

298°K. Each daily measurement of hydrogen per unit volume of culture per unit time was taken as the average volume-specific rate for the 12 preceding and 12 subsequent h. The rate of the first 12 h after switching from air to argon was taken to be equal to the unstarved culture activities reported by Bene mann and Weare (4). For the 1 to 2 days (prior to day 0) during which a culture was illuminated and growing, H₂ production was nil and averaged as such. Incident light energy was calculated by multiplying light intensity by area of illumination. This area was the diameter of the container times the height of the liquid. Photosynthetic efficiencies were not calculated for experiments run in the low-intensity light box, because the vessels were illuminated from all angles and the light probe only detected light incident within a 65° light cone.

Chlorophyll \( a \) determinations. Samples were collected on filters wetted with 2 drops of a saturated MgCO₃ solution, extracted by boiling for 45 s in 90% (vol/vol) methanol-water, left in the dark for 15 to 30 min, and centrifuged at 5,000 \( \times g \) for 15 min, and the absorbance was measured in a Beckman DB spectrophotometer at 665 and 750 nm (for background). Chlorophyll \( a \) content was calculated from the following formula: chlorophyll \( a \) (\( \mu \)g/sample) = 13.9 \( \left( A_{665} - A_{750} \right) \) (8 ml/cm), where 8 ml was the volume of aqueous methanol used for extraction and 1 cm was the length of the spectrophotometer cell. Duplicate samples showed a 2% variation at most. Reextraction with hot methanol yielded only 4% of the first results. Values for unstarved cultures varied from 4.4 to 5.6 \( \mu \)g of chlorophyll \( a \) per mg of dry weight, depending on light intensity and cell density. Extraction with acetone, aided by sonic treatment was only 75% as effective as the above method.

RESULTS

Hydrogen production. Four quantities that are important for designing a system that makes efficient use of space and incident light energy were measured; (i) rates of hydrogen production per unit volume of culture, (ii) rates per unit mass or chlorophyll \( a \) content, (iii) photosynthetic efficiency, and (iv) percentage of hydrogen in the gas phase.

Figure 1 shows hydrogen evolution sustained for 1 to 2 weeks with 2-liter cultures under a light intensity of 2.0 \( \times 10^4 \) erg/cm² per s (see Materials and Methods). In most cases, measurements were terminated before activities ceased. Two of the cultures (Fig. 1A; no. 1 in Fig. 1B) shown in the figure showed light bacterial contamination after 7 days (less than 500 bacteria/ml). Bacterial contamination did not have a noticeable effect on hydrogen production in these or any other experiments. The percentage of hydrogen in the gas phase was typically

![](image)

**Fig. 1.** Hydrogen production by 2-liter cultures of \( A. \) cylindrica under low light intensity \( \left( 2.0 \times 10^4 \right) \) ergs/cm² per s). (A) Culture density was initially 60 Klett units, decreasing to 50 during experiment. Chlorophyll \( a \) content was 5.6 \( \mu \)g per mg of dry weight on day 0 and 6.7 \( \mu \)g per mg of dry weight on day 1. Small arrows indicate addition of \( 10^{-4} \) M \( \text{NH}_4\text{Cl} \). The large arrow (day 19) indicates addition of \( 2.1 \times 10^{-3} \) M DCMU. (B) Three cultures assayed for 8 days. Symbols: Cell density initially was 70 Klett units, increasing to 115 (○); initially 75 Klett units, increasing to 100 (○); initially 50 Klett units, increasing to 75 (+). Nitrogenous additions were made along schedules similar to experiment (A), but in larger amounts.
about 0.2% at a gassing rate of 1.9 liters/h.

Another experimental arrangement using 250-ml capacity culture vessels was designed to allow the use of denser cultures, higher light intensities, and lower gassing rates. Flow rates of about 600 ml/h provided mixing in these narrow vessels. Figure 2 shows rates of hydrogen production by such cultures. Under a light intensity of $4.0 \times 10^4$ erg/cm² per s culture A (with a cell density equal to 250 Klett units) produced 31.8 $\mu$l of H₂ per mg of dry weight per h (peak value), which is equivalent to 7.2 $\mu$l of H₂ per $\mu$g of chlorophyll a per h and is 2.5 to 3 times the rates previously reported for unstarved cultures (4). Culture B was half as dense and under lights half as intense; it produced a maximum of 29.7 $\mu$l of H₂ per mg of dry weight per h. The photosynthetic efficiencies (averaged over the duration of the experiments) were 0.4 and 0.3%, respectively. The denser culture produced 0.8% H₂ in the gas phase at a flow rate of 630 ml/h (day 3). The other culture produced 0.3% H₂ at a flow rate of 800 ml/h (day 3).

**Effects of light levels and DCMU.** Experiments using 250-ml capacity culture vessels in the low intensity light bank ($1.8 \times 10^4$ erg/cm² per s) were also performed. A culture with a cell density of 100 Klett units produced 20.0 $\mu$l of H₂ per mg of dry weight per h, whereas one of 50 Klett units produced 34.3 $\mu$l of H₂ per mg of dry weight per h (both are peak values). This demonstrates that self shading by the cultures results in light limitations under these conditions. The question of whether the effect from this limitation was due to submaximal photosystem I activity in the heterocysts or to reduced photosystem II activity in the vegetative cells, which would lead to lowered reductant supplies, arose. DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] was used to distinguish the roles of the two photosystems. A culture was grown to a cell density of 70 Klett units in a 250-ml capacity culture vessel under the low intensity lights. It was growing exponentially at this point. The culture was switched from air-CO₂ to argon-CO₂. Within 1 day growth ceased, and it had turned from green to yellow. These are among the most apparent effects of nitrogen starvation. The yellowing is due to loss of phycoerythrin (16). Chlorophyll a determinations showed that the content of this pigment did not decrease when the culture yellowed. In fact, a slight increase was observed. After 2 days of starvation, the culture was assayed and found to be producing 19.4 $\mu$l of H₂ per mg of dry weight per h. Immediately after this assay, 2.1 $\times 10^{-5}$ M DCMU was added, and the culture
was switched to a high light intensity (3.9 \times 10^6 \text{ erg/cm}^2 \text{ per s}). Three and one-half hours later, production was found to be 30.8 \mu l \text{ of H}_2 \text{ per mg of dry weight per h, which is considered maximal. The initial effect of increasing the light intensity must have been to stimulate photosystem I activity in the heterocysts. (The DCMU precluded any stimulation of photosystem II activity.) This showed that the increased capacity for nitrogen-fixing activity attained in these experiments did not saturate at the low light intensity. The culture was kept under high light, and after another 17 h was producing only 2.1 \mu l \text{ of H}_2 \text{ per mg of dry weight per h. Other experiments confirmed these results. When DCMU was added after the first or second day of starvation, activity declined by the end of the third day to, at the most, 2\% of its value at the time DCMU was added. When DCMU was added at varying points after the third day, rates declined faster (see Fig. 1A). A previous investigation (5) has shown that adding DCMU to green cultures at the same time as switching them to argon (corresponding to day 0 in this report) prevents the two- to threefold increase in nitrogenase activity and heterocyst formation. In summary, the experiments with DCMU indicate that low light limits photosystem I in the heterocysts of nitrogen-starved cultures and that stored reductant can sustain H_2 production for 1 or 2 days only.}

**Duration of hydrogen production.** Hydrogen production lasted from 7 days to 2 weeks. In most cases, dense cultures were no longer assayed after activities had dropped to unstarved culture rates (about 12 \mu l \text{ of H}_2 \text{ per mg of dry weight per h}). These cultures typically produced H_2 above this level for 6 to 8 days with three additions of ammonium. Some 2-liter cultures had lost only 50\% of their maximal activity after approximately 2 weeks (see Fig. 1A). Additions of NH_4Cl equivalent to \text{ 10}^{-4} \text{ M were made eight times during this period. The effect of adding ammonium is not apparent. It did not inhibit hydrogen evolution. In many cases, if the additions were made early, the rates stayed near maximum for several days. Dense cultures that had the first addition of ammonium after 1 or 2 days of starvation did not decline significantly in mass- or volume-specific rates until the fourth or fifth day. Cultures that received no nitrogenous additions lost mass-specific activity steadily after 1 day of starvation. This leveled off at unstarved culture rates by the fourth day. However, volume-specific rates of these completely starved cultures dropped steadily to very low values. The cell density decreased markedly, and dead material began to accumulate.}

**Oxygen production.** Simultaneous oxygen and hydrogen evolution rates from a bacteria-free culture (light intensity, 4.0 \times 10^6 \text{ erg/cm}^2 \text{ per s}) of cell density equal to 250 Klett units are shown in Table 1. The hydrogen-oxygen ratio was 4:0:1 near the beginning when levels of stored reductant were still high. Addition of ammonium decreased this ratio. Initially, it was due to an increase in oxygen evolution. This is supported by the observation that the cultures turned from yellow to yellow-green after ammonium was added. It is assumed that the light-harvesting pigments were being re-synthesized, leading to increased photosystem II activity. Thus, addition of a nitrogen source can be a tool for manipulating the hydrogen-oxygen ratio. At later stages in the experiment, hydrogen evolution decreased, thereby further decreasing the hydrogen-oxygen ratio. The data on NH_4Cl additions indicate that these additions help sustain maximal activity if made early, lengthen hydrogen production lifetimes, and promote stoichiometric ratios of hydrogen to oxygen. This may be due to increased photosystem II activity and to use of the nitrogen in cell maintenance and growth.

**Nature of hydrogenase system.** To see whether the evolution of hydrogen was catalyzed by nitrogenase alone or nitrogenase and other hydrogenases, hydrogen production assays were done in the presence of acetylene. The assays were done by the same method as the C_2H_2 reduction assays, except that the sam-

<table>
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<th>ml of H_2/liter per h</th>
<th>ml of O_2/liter per h</th>
<th>% H_2 in gas phase</th>
<th>% O_2 in gas phase</th>
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* Day 0 coincides with switching from air-CO_2 to argon-CO_2.

* NH_4Cl was added 2 to 4 h after the assays.
ples were flushed with argon to remove hydrogen already produced. The samples were taken from a culture that produced 23.4 μl of H₂ per mg of dry weight per h when assayed from the effluent, i.e., the normal in situ H₂ assay. When assayed in Fernbach flasks without acetylene, it produced 17.8 μl of H₂ per mg of dry weight per h and with acetylene 1.1 μl of H₂ per mg of dry weight per h. The first two results were expected to be similar. Most likely the 25% difference was due to hydrogen uptake (5), which would be much more effective in a small, stoppered flask than in a large, sparged container. A pH change due to flushing could also account for the 25% difference. In any case, it is clear that most, and probably all, of the hydrogen production was due to nitrogenase, since acetylene inhibited most of the activity.

Acetylene reduction. To learn about the effects of nitrogen starvation over a period of days, cultures were assayed for acetylene reduction under various conditions. Two-liter cultures were grown under normal nitrogen-fixing conditions to a cell density of 50 to 70 Klett units (approximately 0.17 to 0.25 mg of dry weight per ml).

Figure 3 shows some typical time courses of acetylene reduction activity in the light under argon. These are the same cultures as those shown in Fig. 1B. The rates of hydrogen production were much less than the rates of acetylene reduction, mainly as a result of differences in the assay procedures. Hydrogen evolution was assayed in situ under limiting light (see the previous section for a discussion of light limitation), whereas acetylene reduction was assayed in small flasks on a shaker bath under saturating light intensities. In addition, hydrogen evolution can be diminished by hydrogen uptake. Despite these differences, the principal features of Fig. 1B and 2 indicate a good correlation between acetylene reduction and hydrogen production. Acetylene reduction activities were consistently between 12 and 16 μl of C₂H₂ produced per mg of dry weight/h for unstarved (green) cultures. During all our experimentation, cultures turned yellow and reached maximum rates of both acetylene production and hydrogen production within 1 to 2 days of the onset of starvation (switching from air-CO₂ to argon-CO₂).

Maximum activities for starved cultures were 2.5 to 3.5 times as great as those for unstarved cultures. A substantial decrease (approximately 90%) in O₂ evolution was found in this and previous studies (4). In all cases, concomitant with the rise in nitrogenase activity was a rise in the frequency of intercalary heterocysts from 5% to 12 to 15%. This is in agreement with previous findings (16). The period of maximum activity lasted 1 or 2 days. In most cultures the ensuing decline in activity was accompanied by a dramatic breakage of filaments. Unstarved cultures and those that had been starved only 1 to 2 days consisted mostly of filaments with 200 to 400 cells. After another day or two of starvation, this number was reduced to 10 to 30. Terminal and unattached heterocysts became common. This suggests that filament breakage was the major limitation of the system. However, in experiments run on cultures that exhibited high resistance to oxygen (see below), the initial decline of activity preceded any significant filament breakage by a full day. Thus, filament breakage was not the only factor leading to the decline of nitrogenase activity. The addition of a source of nitrogen (in our experiments, NH₄Cl or nitrogen gas or both) slowed down this decline and even led to increases in activities as can be seen in Fig. 3. The lifetimes of the acetylene reduction activities showed the same trends as the H₂ production lifetimes of the 2-liter cultures.

Oxygen inhibition. Reported effects (20, 24) of oxygen on acetylene reduction by heterocystous algae are quite variable, ranging from 25% stimulation to 50% inhibition at atmospheric oxygen tensions. In these experiments, no oxygen stimulation was found and resistance was variable. Figure 4 shows oxygen inhibition curves for two cultures. Culture A showed high resistance to oxygen; culture B was less resistant. Before starvation, culture A was inhibited by oxygen in the light only when the level of oxygen in the gas phase became greater than 40%. Culture B showed inhibition above 20% oxygen. Dark assays exhibited stimulation by
Fig. 4. Oxygen inhibition of acetylene reduction activities by 2-ml samples from 2-liter cultures. Open symbols represent assays performed under a light intensity of $6.0 \times 10^4$ ergs/cm$^2$ per s; closed symbols represent dark activities. Numbers in parentheses correspond to the number of days after switching from air-CO$_2$ to argon-CO$_2$. One hundred percent activities correspond to assays performed in the light under argon-acetylene. (A) One hundred percent activity equals 13.3, 40.7, 20.8, and 13.4 $\mu$L of C$_2$H$_4$ per mg of dry weight per h for days 0, 1, 2, and 3, respectively. $10^{-5}$ M NH$_4$Cl was added after assay on days 1 and 2. (B) One hundred percent activity equals 14.8, 39.1, 30.1, 16.8, and 15.3 $\mu$L of C$_2$H$_4$ per mg of dry weight per h for days 0, 1, 2, 3, and 4, respectively. $10^{-5}$ M NH$_4$Cl was added after assay on days 2 and 3.

oxygen through the 30% level for culture A. In all cases, the dark activities were lower than the light activities, with peak dark activities 40 to 60% of control. After 1 day of starvation, with the activities in the light under argon 2.5 to 3 times higher than the previous day, oxygen inhibited at lower concentrations both in the light and the dark. This trend continued through the next day. Typically, dark activities fell to zero at lower oxygen concentrations than did light activities. Addition of ammonium to culture A after day 1 did not lead to any effect on the inhibition by oxygen. After day 2, more ammonium was added, and the oxygen inhibition was relieved somewhat by day 3. Cell density increased by 25% from day 2 to day 3. Whether the increase in oxygen resistance was due to growth of new cells or changes in old ones is not clear. Culture B exhibited the same effects from ammonium addition.

DISCUSSION

The localization of nitrogen fixation in the heterocysts of A. cylindrica requires exchanges of metabolites between the different cells. The interrelationship between photosynthesis and nitrogen fixation is of importance to any study of hydrogen production. Normally growing, light saturated, nitrogen-fixing cultures have a plentiful supply of reductant which is actively maintained. Nitrogen fixation and cell mass
production are optimized under these conditions. A steady state at which about 5% of the cells are heterocysts is attained. The initial response to nitrogen starvation is to increase nitrogenase activity and to reduce photosynthetic capacity. Oxygen evolution decreases, the phycocyanins are broken down, causing yellowing of the cultures, and growth ceases. Differentiation of vegetative cells into heterocysts increases until the heterocyst frequency has nearly tripled. Hydrogen production and acetylene reduction reach their maximum rates. DCMU has little effect, but oxygen becomes a stronger inhibitor of nitrogenase activity. This indicates that reductant pools are still high, but not sufficient to support the greatly increased nitrogenase activity and at the same time help to fully maintain the mechanisms of oxygen protection. Since oxidative respiration consumes O₂ it must be considered as a protective mechanism. Yet, the dark activities fall to zero at lower O₂ tensions compared to activities in the light. Thus, there is extra protection against oxygen when the lights are on. This extra protection we attribute to photorespiration in heterocysts. As starvation continues, activities start to decline. Sensitivity to DCMU and oxygen increases, as do their synergistic effects. Therefore, reductant is in shorter supply, since nitrogenase activity is more immediately dependent on the much diminished photosystem II activity.

In the introduction the necessary prerequisites for biophotolysis were defined. Using the simple expedient of nitrogen starvation, cultures of A. cylindrica produced hydrogen and oxygen for over a week at a photosynthetic efficiency of about 0.4%. The basic limitation of the duration of production is heterocyst inactivation caused primarily by filament breakage. As shown previously, filament breakage at heterocysts correlates with loss of nitrogenase activity (2). Strategies will have to be developed to minimize mechanical breakage of filaments. If the maximum rates of hydrogen evolution that were observed could be sustained throughout the experiment, photosynthetic efficiencies would be at least doubled. As discussed above, the decline in H₂ evolution after the initial maximum is attributed to limitations in the supply of reductant to the heterocyst. Control of the regulation of reductant synthesis and flow will be required to maintain high rates. Since the photosynthetic efficiencies were low estimates (see Materials and Methods), improved experimental arrangements should increase efficiencies. Although there do not seem to be any limitations on the size of the system, problems with mixing and gas exchange will have to be solved when designing flat-plate systems more suitable for solar converters. Finally, stoichiometric oxygen production, an essential requirement of any biophotolysis system, appears to be attainable through manipulation of the nitrogen level in the system. The ratio of H₂ to O₂ reported in this paper approaches the desired ratio of 2:1. In conclusion, this paper shows that cultures of nitrogen-starved, heterocystous blue-green algae have significant promise in the further development of a biophotolysis system.

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