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SOLAR ENERGY CONVERSION THROUGH BIOPHOTOLYSIS

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ABSTRACT

This report covers the progress during the third year of this project. The state-of-the-art of biophotolysis was reviewed and a bioengineering analysis carried out. The conclusions were that practical biophotolysis systems are feasible; however, they will require, in most cases, relatively long-term R & D. The biophotolysis system developed under this project, utilizing heterocystous blue-green algae, was demonstrated both indoors and outdoors with a model converter system using the heterocystous blue-green alga Anabaena cylindrica. Maximal light energy conversion efficiencies were 2.5% indoors and about 0.2% outdoors, averaged for periods of about two weeks. Achievement of such rates required optimization of N_2 supply and culture density. A small amount of N_2 in the argon gas phase used to sparge the cultures was benefitial to the stability of a long-term hydrogen-production activity.

A relatively small amount of the hydrogen produced by these cultures can be ascribed to the activity of the reversible hydrogenase which was studied by nitrogenase inactivation through poisoning with tungstate. The regulation of nitrogenase activity in these algae was studied through physiological and immunochemical methods. In particular, the oxygen protection mechanism was examined. Thermophilic blue-green algae have potential for biophotolysis; hydrogen production was studied in the laboratory. Preliminary experiments on the photofermetation of organic substrates to hydrogen was studied with photosynthetic bacteria.

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I. INTRODUCTION

Biophotolysis may be defined as the decomposition of water into hydrogen and oxygen by sunlight and a biological catalyst. Difficulties arise with the definition when indirect processes were considered, such as the production of hydrogen from organic substrates by photosynthetic bacteria. Thus, a broader term has been recently proposed--photobiological hydrogen production (1). This report details the research carried out during the third year of this project on photobiological hydrogen production (see 2, 3 for previous final reports). Basically, this project has for its goal the development of practical processes for hydrogen production using photosynthetic microorganisms as catalysts. This project originated with early studies on biophotolysis using both biochemical model systems (4) and cultures of blue-green algae (5). For more detailed reviews of photobiological hydrogen production, the reader is referred to Chapters II and XIV.

The main approach to biophotolysis being followed is the use of nitrogen-starved cultures of heterocystous nitrogen-fixing blue-green algae. These cultures exhibit simultaneous hydrogen and oxygen production (5, 6) which can be demonstrated with a simple outdoor rooftop converter consisting of vertical glass tubes (3,7). This project had as one of its main objectives the continued development of this outdoor biophotolysis system, as well as the elucidation of the basic biochemical and physiological mechanisms underlying the biophotolysis reaction of these algae. Only with such basic knowledge will it be possible to develop and perfect a practical system. In addition, this project was to continue exploratory research in the use of photosynthetic bacteria for hydrogen production.

Specific accomplishments during this year of the project were the demonstration that reversible hydrogenase can be demonstrated in vivo to produce hydrogen in a partially oxygen-resistant reaction (suggesting its presence in the heterocysts) (Chapter IV). Although the rates were low, this opens the possibility that this enzyme may be used in this process. The effects of small concentrations of nitrogen in the argon gas phase used to operate the biophotolysis system were studied in detail (Chapter III). The results demonstrate a significant improvement in the operation of such a system with an optimal concentration of N_2 (about 1%). This study was applied to an outdoor demonstration of this biophotolysis system (Chapter VI). With improved gas flow regulation, steady rates of hydrogen production were observed in short-term (one day) experiments. Hydrogen production in longer-term experiments (up to one month) varied as a function of solar insolation and, also, the state of the culture. Hydrogen production by thermophilic blue-green algae was also studied (Chapter V). The effects of N_2 gas, temperature, and inhibitors were measured. Uptake of hydrogen by these algae appears to be a generally more severe problem than in the mesophyllic Anabaena cylindrica strain used. A major part of this project concentrated on the study of the regulation of nitrogenase in the heterocysts (Chapters VII to XI). The role of oxygen and heavy metals in nitrogenase biosynthesis was studied (Chapters VII and X). The oxygen protection mechanisms were studied in detail (Chapter IX). Antibody probes for the nitrogenase (Chapter VIII) were used to study heterocyst regulation (Chapter X) and localization (Chapter XII). The mechanism of the nitrogenase enzyme was briefly explored by study of its reversibility (Chapter XIII). Only a very limited amount of work was carried out with photosynthetic bacteria (Chapter XII). Of major significance to biophotolysis is the bioengineering analysis presented in Chapter XIV which suggests the basic technical-economic feasibility of

photobiological hydrogen production and suggests new areas for future investigations.

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II. RECENT DEVELOPMENTS IN HYDROGEN PRODUCTION BY MICROALGAE

ABSTRACT

Biophotolysis is the biological decomposition of water into hydrogen and oxygen. A number of different possible biological catalysts can be considered in biophotolysis. The ones based on cultures of algae and photosynthetic bacteria appear to be capable of being developed into practical applications. The most near-term applications would be the development of a process for the "photofermentation" of wastes involving mixed heterotrophic and photosynthetic bacterial cultures. Such a system may be used with low nitrogen wastes and could be considered as an alternative to methane fermentations in some cases. Microalgae may be used in a more direct process of photosynthesis in which water is decomposed in single or two-stage systems. Two-stage systems would separate the hydrogen and oxygen production reactions and have the advantage of requiring only a relatively small area for collecting the hydrogen. Examples of two-stage systems involve the pumping and recycling of an algal culture from a shallow open pond to a deeper glass-covered pond. Alternatively, the hydrogen and oxygen reactions can alternate between night and day, respectively. The latter approach requires large glass-covered surfaces for collecting the hydrogen. The practical photosynthetic efficiency that may be achieved with well-controlled biophotolysis systems, of about 3%, limit the allowable capital investment, requiring very low-cost materials and designs. One design may involve long tubular, thin glass of 4-8 cm in diameter, as this material is very inexpensive. In one proposal, which has already been demonstrated under outdoor conditions, nitrogen-fixing blue-green algae are used to simultaneously produce hydrogen and oxygen. Many difficult biological and engineering problems remain and exploration of different approaches to biophotolysis will require extensive basic and applied research.

RECENT DEVELOPMENTS IN HYDROGEN PRODUCTION BY MICROALGAE

INTRODUCTION

The production of hydrogen from water and sunlight by photosynthetic catalysts is known as biophotolysis. The concept of photosynthetic solar energy conversion through water splitting is not new; it can be found in one of the pioneering books of solar energy (1). However, it only received attention by the research community recently when the newly created Research Applied to National Needs, Division of the National Science Foundation (NSF-RANN) initiated a program on biological solar energy conversion which supported a number of projects on biophotolysis. The attractiveness of biophotolysis is the potential of hydrogen as a "fuel of the future," the simplicity of the biophotolysis concept, and the appeal of photosynthetic solar energy conversion. However, the technical difficulties of developing a practical biophotolysis system have resulted in a waning of interest in, and support for, this research.

In this presentation, we discuss the various proposed photosynthetic systems that are, or may be, capable of biophotolysis; review the scientific basis of biophotolysis; present recent experimental results from this and other laboratories; analyze the technical problems of developing practical and economic biophotolysis systems; and speculate on the future of this approach to biological solar energy conversion.

BIOPHOTOLYSIS CONCEPTS

There are two basic types of biophotolysis systems that can be envisioned: single-stage systems in which hydrogen and oxygen are produced simultaneously and two-stage systems in which hydrogen and oxygen are produced in separate chambers or at different times. The latter systems have obvious advantages--they avoid an explosive mixture of hydrogen and oxygen, or the considerable expense of separating these gases, and allow the hydrogen producing reaction to take place in the absence of oxygen. Since it appears very difficult, if not impossible, to devise a catalyst capable of evolving hydrogen in the presence of free oxygen, any proposed single-stage biophotolysis system must cope with this key problem. In addition, two-stage systems do not need to cover the larger oxygen-evolving stage, lowering the overall material inputs. These conceptual advantages of two-stage systems were pointed out by Professor Krampitz in his address last year to this symposium (2). It is, therefore, appropriate to review two-stage systems first.

Basically, a two-stage biophotolysis system would involve the photosynthetic production of a reduced, oxygen-stable, reductant(s) capable of being used in the second stage for production of hydrogen. Because the first oxygen-stable reductant produced by photosynthesis is the two electron carrier "TPNH" (reduced triphospho pyridine nucleotide), it has received considerable attention as a possible intermediate in biophotolysis (2). However, it is a large, high-molecular weight metabolite, and any number of reduced organic compounds derived from carbon dioxide fixation by the Calvin

Cycle may be more appropriate possibilities for storing photosynthetic reductant for conversion to hydrogen. A large variety of possibilities exist for the particular first and second stages used. In one proposed system, microalgae would be maintained in a glass-enclosed vessel and produce oxygen and store reductant (either extra- or intracellularly) during the daytime and release it as hydrogen during the night and following dawn (3). Such a two-stage, single-chamber biophotolysis concept is based on the well-known ability of microalgae to store reductant and to produce hydrogen in the absence of oxygen, in the dark or at low light levels. (The hydrogen metabolism of algae has been recently reviewed (4, 5) and is discussed in the next section.) Such a "diurnal" system would require careful control over, for example, algal density, metabolism, gas phase, etc. The main difficulties are the storage of reductant and rate of hydrogenase induction and metabolism. An experimental demonstration of such a biophotolysis system should be possible.

One concept, which stretches the definition of biophotolysis, uses organic substrates generated by microalgae, or even various types of organic wastes, as a source of reductant in a bacterial hydrogen fermentation. This concept suffers from the relatively low efficiencies of hydrogen fermentations as compared to anaerobic digestion to methane (6). However, a similar concept which is more closely related to biophotolysis has recently been proposed (7) and is being actively investigated (8). It involves a "photohydrogen fermentation" of organic compounds using photosynthetic bacteria. The ability of photosynthetic bacteria to evolve hydrogen has been known for almost three decades (9) and has been demonstrated to be catalyzed by nitrogenase (see next section). The advantage of photosynthetic bacteria is that they can efficiently use light energy to generate hydrogen quantitatively from a wide variety of organic compounds. Thus, photosynthetic bacteria could overcome the thermodynamic limitations of dark hydrogen fermentations. At present, a limitation of such a system is that it could only be carried out in the absence of a nitrogen source, since either fixed or molecular nitrogen inhibits the hydrogen reaction. However, use of mutants of photosynthetic bacteria (10) could overcome this problem. The potential ability of photosynthetic bacteria to efficiently decompose organic wastes into hydrogen and carbon dioxide would make such a system an alternative to methane digestion for the treatment of wastes, particularly low N wastes. The high rates of hydrogen produced per area, possible waste treatment credits, and the simplicity of the general process make this the most immediate possibility for near-term practical applications of biophotolysis.

Other concepts for two-stage systems have been proposed (2, 3, 7), basically involving the use of separate oxygen and hydrogen-producing vessels between which reductant is transferred through some sort of pumping system. Among the possible alternatives are the simple cycling of an algal culture between the two stages. In the first, possibly open air stage, microalgae would accumulate reductant and produce oxygen. They would then be pumped to a small, deeper glass-covered, mixed vessel where anaerobic conditions would result and hydrogen would be evolved by the activated hydrogenase enzyme of the algae. The low light intensity in the vessel would stimulate the reaction. As the algae are depleted of reductant, they would be pumped back to the first stage. To prevent growth of the algae, nitrogen starvation would be used. A variation of the concept is the use of immobilized or encapsulated microalgae and photosynthetic bacteria in the two-stages with soluble organic compounds transferred between the stages. Heterotrophic

bacterial metabolism of these reductants would have to be avoided. A possible drawback of such two-stage systems are the energy requirements for pumping between them; however, this does not appear as restrictive a limitation as had been stated earlier (11).

Finally, single-stage systems may be considered. The above-mentioned problem of oxygen inhibition of hydrogen evolution must be overcome in such systems. At present, the only demonstrated single-stage biophotolysis system is that involving heterocystous blue-green algae (12,13,14). The oxygen producing vegetative cells are accompanied, under conditions of nitrogen fixation, by specialized heterocyst cells capable of both nitrogen fixation and hydrogen evolution. In these algae, then, a microscopic separation of the oxygen and hydrogen production reactions exists, with reductant transported through the cytoplasm from the vegetative cells to the heterocysts. This system is, experimentally, the best developed approach to biophotolysis.

BIOCHEMISTRY AND METABOLISM OF BIOPHOTOLYSIS

Any biophotolysis system must be based on an intimate understanding of the underlying biochemistry and metabolism of the microalga or bacteria used. Thus, knowledge about the hydrogenase enzymes and their rates of reaction, induction and activation, electron transport pathways, interaction with photosynthesis, and metabolism generally must all be investigated during biophotolysis research and development. An impressive amount of information already exists about these subjects; however, further basic research is required in many specific areas to allow development of the biphotolysis concepts detailed in the preceding section. Only then will it be possible to optimize the reactions and maximize solar conversion efficiencies, the main requirement if biophotolysis is to succeed practically. Therefore, biophotolysis research and development must entail a heavy dose of basic biochemistry and metabolic biology research. Unfortunately, sometimes basic and applied research have been confused or basic research has been neglected in favor of prematurely attempted practical applications. This has resulted in a lack of progress which led to the waning of support by federal agencies mentioned in the introduction. In this section only the fundamental aspects of hydrogenase biochemistry and relevant algal-bacterial metabolism are discussed; recent reviews contain further details (4,5,15).

Three basic types of enzymes are known to metabolize hydrogen--a reversible hydrogenase that can either evolve or take up hydrogen gas, an irreversible uptake hydrogenase, and an irreversible hydrogen evolution enzyme that has been shown to be identical to the nitrogenase enzyme. The exact mechanism of these enzymes is still being studied. They all involve iron-sulphur proteins. The reversible hydrogenase is coupled to an electron carrier of low redox potential (similar to that of the $H_2/2H^+$ couple at pH 7.0 of -420 mv). The normal carrier is usually one of the electron carriers ferredoxin or flavodoxin; however, in some cases, other electron carriers are involved, particularly with the reversible hydrogenase of heterocystous blue-green algae recently isolated (16), for which the physiological electron carrier still remains to be identified. The basic feature of this hydrogenase is its equilibrium reaction with hydrogen; thus, it does not function effectively in evolving hydrogen at high partial pressures of hydrogen gas. Although some reversible hydrogenases are irreversibly inactivated by the presence of oxygen, in many cases, particularly the hydrogenase of micro-

algae, oxygen inactivation is slow and reversible. Indeed, in these algae, the hydrogenase can be activated *in vivo* or *in vitro* by short-term exposure to anaerobic conditions. This has direct application to the development of biophotolysis systems. Whether the reversible hydrogenase reaction itself (as distinct from the enzyme) can be protected against molecular oxygen must still be determined. The energy of activation of molecular oxygen reacting with nascent hydrogen is very low and the reaction is probably diffusion controlled. By contrast, the uptake hydrogenase can apparently function in the presence of oxygen, as it is found in some aerobic bacteria and blue-green algae. However, its exact oxygen tolerance is not known and, anyway, the uptake of hydrogen is only a detraction from any biophotolysis system, as it lowers the amount or rate of hydrogen produced. Thus, it must be eliminated, possibly by mutation, from any blue-green algae or photosynthetic bacteria used in hydrogen production. It should be noted that very low concentrations (down to 1%) of hydrogen can be effectively assimilated by this irreversible uptake hydrogenase (17).

The use of the irreversible hydrogen evolution reaction of nitrogenase in biophotolysis has been the focus of considerable research. Basically, this reaction is a laboratory artifact, reflecting the lack of control over nitrogenase activity in the absence of molecular nitrogen. When no nitrogen is available, this enzyme continues to be reduced and hydrolyze ATP, and it spontaneously reduced protons from water to hydrogen. As in nature, conditions of nitrogen limitation would seldom, if ever, be found; there was no need for the nitrogenase enzyme to evolve a regulatory mechanism to cope with those situations. This laboratory curiosity can be used to "trick" the blue-green algae or photosynthetic bacteria which possess the nitrogase enzyme to evolve hydrogen. Green algae are more highly evolved and have lost the nitrogen-fixing enzyme system; thus, they do not exhibit nitrogenase mediated hydrogen evolution. The involvement of ATP in the nitrogenase reaction allows hydrogen evolutions to proceed essentially unidirectionally even in the presence of high partial pressures of hydrogen. This is, of course, an advantage of this enzyme in biophotolysis; the relatively low turnover number, inhibition by nitrogen gas, instability, and use of energy in the form of ATP cancel this advantage.

The mechanism of nitrogenase is complex. It involves molybdenum at its active site as is indicated by the loss of activity when this metal was replaced by tungsten (18). There are two proteins that make up the enzyme. They have recently been purified from a blue-green algae (19). One other noteworthy property of nitrogenase is its ability to reduce acetylene to ethylene, a reaction used as a common test for its activity. For biophotolysis applications, it may be desirable to develop a modified enzyme more suited to hydrogen production than nitrogen fixation. Thus, study of its mechanism, such as the reversal of the nitrogenase reaction (20), is of interest.

The metabolic interrelationships between the hydrogenase enzymes and the photosynthetic electron transport and reductant generating pathways are of prime interest in any biophotolysis system. Considerable work has been done in the case of the green algae since 1942 when Gaffron and Rubin (21) discovered their ability to evolve hydrogen. Unfortunately, the pathways are complex, variable between various algae, and not yet completely understood. The general pattern is that a period (minutes to hours) of anaerobic conditions activates a reversible hydrogenase in green algae following

which a period of dark hydrogen evolution ensues, whose rates and extents depend on the algal species and previous history of the cultures. Low light stimulates the reaction while oxygen or high light (which results in oxygen production) inhibit it. Simultaneous oxygen and hydrogen production by adapted green algal cultures exposed to carefully timed light flashes has been observed (22). Whether oxygen and hydrogen evolution can take place simultaneously in the same cells is still uncertain. At any rate, no simultaneous oxygen and hydrogen evolution can be sustained at significant rates. Thus, this system appears best suited to the two-stage biophotolysis systems discussed above. The activated reversible hydrogenase of green algae has the specific metabolic function of allowing photosynthetic electron transport to recover from anaerobic conditions (23).

In the case of heterocystous blue-green algae, the two-cell types, vegetative cells and heterocysts and three different hydrogenases combine into a complex metabolic system. A model of our current knowledge of heterocystic blue-green algal hydrogen metabolism is shown in Figure 1. All three hydrogenase enzymes are present in the heterocysts; however, the reversible hydrogenase is usually not present in high concentrations (24). The absence of nitrogenase from vegetative cells was a much debated issue in blue-green algal research, and complete proof is still lacking. With the purification of the blue-green algal nitrogenase (19) and the production of a specific antibody (25), immunological staining techniques can now be used to solve this problem. The regulation of blue-green algal nitrogenase is also of interest. It appears to be closely linked to the differentiation of heterocysts and not directly controlled by ammonia (26). The hydrogen metabolism of heterocystous blue-green algae are the subject of considerable recent research (23-34 and see below) and the study of the metabolism of heterocysts is of general interest as it has applications in, for example, the development of nitrogen-fixing blue-green algal biomass production for fuel and fertilizer (35).

The source of reductant for hydrogen production by a biophotolysis system is immediately the carbon dioxide-fixing Calvin Cycle and associated metabolic reactions and ultimately the photosynthetic solar energy conversion process itself. Thus, the fundamental consideration is the theoretical efficiency of any biophotolysis system. The maximum efficiency of photosynthesis has been recently calculated for the visible solar energy spectrum (400-700 mm at air mass 1.2) and found to be almost exactly 20% for the primary reactions of algae or bacteria which lead to stored chemical energy (36). This is close to the theoretical limit for any photochemical reaction. Considering that visible sunlight makes up only about 45% of total solar energy, that some inactive absorption is inevitable, that metabolic inefficiencies in carbon dioxide fixation, reductant transport and respiration reduce overall efficiency significantly, and that the diurnal variation in light intensity does not allow complete light absorption or utilization, the practical limits for conversion of (total) solar energy to hydrogen in biophotolysis is about 3%, the same estimated for conventional plant photosynthesis systems (37). This raises the question of what are the special advantages of biophotolysis which require it to be developed in a long-term research and development program when other biomass energy concepts appear nearly ready for commercialization. This issue is addressed below. First, recent experimental progress in biophotolysis is presented.

RECENT EXPERIMENTAL PROGRESS

Two areas of biophotolysis research are being actively pursued at present--experiments with in vitro systems and development of whole-cell microalgal systems. The in vitro studies use hydrogenase enzymes, photosynthetic particles, and other purified or crude cell-free preparations. They are an evolution of the initial work carried out in biophotolysis with a single-stage chloroplast-ferredoxin-hydrogenase reaction (38) or more complex two-stage systems (2). The chloroplast-ferredoxin-hydrogenase reaction has been the subject of several studies (39 - 45) since it is the simplest and most straightforward reaction that can presently be demonstrated. Most efforts have involved the search for an oxygen-stable hydrogenase (46), have attempted to create one through immobilization techniques (47,48), or have been directed at increasing the rate of the reaction or stability of the chloroplast system. However, the real problem, as has been pointed out originally (38), is that in this system "no oxygen is actually evolved into the gas phase along with the hydrogen." Although occasionally small amounts of oxygen were observed, they were far from stoichiometric and not reproducible (J. Benemann, unpublished observations). Since the stoichiometric production of hydrogen and oxygen is a fundamental requisite for any candidate biophotolysis system, this shortcoming is potentially fatal and must be resolved. Whether it is possible to achieve simultaneous hydrogen and oxygen with such a simple system coupling oxygen evolving photosynthesis with hydrogenase is very much in doubt. A recent Russian work indicates that only a two-electron acceptor (such as TPNH) could be stable enough to avoid oxidation in the presence of oxygen (49). However, this does not bear directly on the hydrogenase reaction and a final answer can only be given after the hydrogenase enzyme mechanism (44) and reaction rates with the electron carrier have been determined (45). At any rate, practical considerations dictate that any such in vitro system only be used as a model for any actual applied research. Thus, problems of system stability are secondary to a demonstration of a catalytic biophotolysis reaction. Another related approach that has been suggested (3) is the conversion of the primary electron acceptor (PEA) of photosystem II to a hydrogenase. The appeal of this idea is the fact that the PEA is oxygen stable in the reduced form (50); however, whether it is possible to convert it to a hydrogenase is yet to be demonstrated.

A few studies of hydrogen production by green algae have recently been reported (22,51). However, direct use of these algae in the biophotolysis systems suggested above still remain to be attempted. By contrast, the heterocystous blue-green algal hydrogen metabolizing reactions have been the subject of extensive research specifically directed toward biophotolysis applications (52-55 and also previously cited references). These studies are generally in agreement with the model presented in Figure 1 and the results previously obtained (12,13).

The most significant advance has been the demonstration of a sustained, catalytic biophotolysis reaction using heterocystous blue-green algae in an outdoor, rooftop converter (14). Earlier work in the laboratory had shown that a culture of Anabaena cylindrica would continuously produce hydrogen and oxygen at a light energy conversion efficiency of at least 0.4% when sparged with argon gas and occasionally provided with small amounts of ammonium chloride (13). (See also Figure 6 in last year's

presentation at this meeting) (11). Since then, the laboratory system has been improved by replacing the ammonia salt with a small (about 1%) amount of nitrogen in the gas phase (which increases the metabolic and mechanical stability of the heterocyst system), by carefully regulating gas flows and cell concentrations, and by testing other algal strains. This, together with a correction of an underestimate in previous measurements, has resulted in the achievement of a 2-5% conversion of (fluorescent) light into hydrogen energy.(8)

A typical result is shown in Figure 2 which also shows the hydrogen:oxygen stoichiometry obtained during different phases of the experiment. During the period of peak production, the photosynthetic efficiency was 2.2% and the average over the length of the experiment was 1.7%. During the initial period of nitrogen starvation, the hydrogen:oxygen ratio rapidly rose to over 4, reflecting the conversion of stored carbon compounds. Addition of 1% nitrogen stabilized subsequent hydrogen production, but also decreased the hydrogen:oxygen ratio to approximately one. Although this is below the desired ratio of 2:1, some oxygen evolution may be required for culture maintenance. It is expected that with better regulation of the nitrogen supply, hydrogen:oxygen ratios approaching two will be achieved. A similar system, consisting of simple glass tubes containing 1 liter of algal culture has been operated outdoors for over four weeks. Higher volume specific ratios of hydrogen production were achieved in the outdoor experiments, but conversion efficiencies were lower than in the indoor experiments (as would be expected since algal density, among other factors, has not yet been optimized). Experiments are in progress to extend these results, particularly to thermophilic blue-green algae (57). One major effect, which requires further study, is the peaking of hydrogen production observed after the initial hours of argon starvation (Figure 3) which has also been reported by others (55). This is apparently due to an inhibition of metabolism and, therefore, reductant supply, occasioned by nitrogen starvation.

Recently, the study of photosyntheticbacteria Rhodospirillum rubrum in photohydrogen fermentations was initiated (8). A long-term steady rate of hydrogen production could be obtained; however, the continual addition of substrate (malic acid) resulted in a pH drop and had to be regulated by a pH stat. Thus, this system is not self-regulating as are methane fermentations. Use of carbon compounds that are not directly metabolized by the photosynthetic bacteria such as glucose can be fermented in this system using a mixed heterotrophic-photosynthetic bacterial culture; however, rates of hydrogen production were low. The use of such a system, if it can be perfected, in the treatment and fermentation of wastes such as sugar beet processing wastes,is the next step in this research.

BIOENGINEERING CONSIDERATION

The many different distinct biophotolysis concepts discussed above, most of which are still to be demonstrated experimentally, have not yet been subjected to a comparative engineering and cost analysis. Of course, much of the information necessary for such an analysis is not yet available, but sufficient assumptions can be made to allow an estimate of the key engineering design problems and economic limitations. In regard to the latter, the value of the output of a biophotolysis system--hydrogen--can easily be

calculated from the incident solar energy and the conversion efficiency of the system. The latter value must, at present, be assumed while the former one depends on the location. A very favorable place in the southwest receives an average of about 1800 BTU/sq ft/day (4,900 Kcal/sq m/day). As previously discussed, a photosynthetic efficiency of 3% may be possible in practical systems. Thus, about 20,000 BTU (54,000 Kcal) of hydrogen may be produced per sq ft (sq m) per year. (This corresponds roughly to 7 liters/sq ft/day of hydrogen.) The value of this hydrogen gas may either be compared to present natural gas prices or assigned a higher value which would be dependent on remote siting applications, substitution for higher price fuels, small-scale commercial hydrogen supplies, use in electric fuel cells, or future real price increases.

The value of the hydrogen will also depend on its purity, concentration in the gas phase, and the presence of oxygen. Thus, the gas that may be produced by heterocystous blue-green algae--a stoichiometric mixture of hydrogen and oxygen at relatively low concentrations--would have a lower value than that produced by photosynthetic bacteria which are capable of producing hydrogen containing only carbon dioxide, which can be tolerated or easily scrubbed (similar to digester gas). Photosynthetic bacteria have the added advantage of not being limited by the efficiencies of green plant photosynthesis as they use already preformed organic molecules and the light energy is used only as an "assist" in their dissimilation. Although the exact energy requirements of the nitrogenase reaction are not yet known, they are likely to allow at least a ten-fold increase in the production rates per area. This makes such systems particularly attractive and their design of immediate interest. Basically, it would be designed as a shallow glass-covered reactor with a gas space to allow hydrogen to accumulate and be drawn off. Since photosynthetic bacteria are motile, mixing of the reactor can be minimal. The influent organic substrate would need to be fed at a rate that allows the bacteria to dissimilate it without undergoing pH stress. If, as is likely, a complex waste is to be dissimilated, the establishment and maintenance of a mixed bacterial culture would be required, as is done in the case of anaerobic digestion. Such a waste could not contain large amounts of suspended solids that block light. Cell growth would be controlled by influent nutrients. A high-strength waste would be preferred; however, if hydraulic detention time is greater than cell growth, cell recycle or cell immobilization would be required. As photosynthetic bacterial hydrogen production is most directly affected by the continuous presence of ammonia, rather than by fixed nitrogen as such, this nutrient must either be absent or specially mutated strains used. Materials and specific designs for such a system are yet to be chosen. However, the relatively high rates of hydrogen production expected should make this fairly simple.

This is not the case with microalgal biophotolysis systems which produce such relatively low rates of hydrogen per unit area that low-cost materials for building biophotolysis converters become the critical issue. Even at a value of $5/MBTU of hydrogen, only 10¢ worth would be produced per sq ft per year. In two-stage, two-chamber systems, the actual hydrogen generating stage could be relatively small, about one-tenth the size of the oxygen-generating stage. The advantages of such a hydrogen-producing stage are similar to those already discussed for photosynthetic bacteria. The larger oxygen-producing stage would be operated similarly to algal biomass production systems (57). The key difficulty becomes the pumping required between stages; however, this would not require large energy inputs.

The materials and design difficulties become severe in single-stage systems. Basically, a flat-plate glass collector-converter would be required to contain the algae and collect the hydrogen. The only material cheap enough to meet the stringent cost limitations described above appears to be thin-walled glass tubes such as those used in fluorescent tubes. The materials costs of a flat-plate collector using an array of such tubes is about $0.25/sq ft (one-half of which is for the glass) with a doubling of the price for assembly, installation, and connection to a grid for hydrogen collection. This may meet the capital costs limitations in biophotolysis. A conceptual design of such a flat plate, tubular glass collector is shown in Figure 3.

Before development of such a system may proceed, several difficult engineering problems need to be overcome. The rates of gas transfer are one limiting factor, unless sufficient mixing is provided. Similarly, settling of the algae, or clumping, must be overcome, again by mixing. One concept which may be applicable when the thermophilic blue-green algae are used is operation of a dual biophotolysis-thermal flat-plate collector. The tubular glass array would be covered by a second layer of either glass tubes or, preferably, flat glass. Trapped heat would be removed to a storage tank by using the algal cultures as the heat-absorbing working fluid. That such a concept could actually be demonstrated appears likely. However, it may be difficult to develop it in practice. These bioengineering aspects of biophotolysis are being subjected to detailed analyses (58).

Despite these problems, biophotolysis has several advantages in biological solar energy conversion. The process should be readily controllable, maximizing photosynthetic conversion efficiency, and fuel is obtained directly, minimizing any losses in the normal conversion process. Thus, this approach can, in principle, become the most efficient bioconversion system. Whether it can be developed so as to be economically competitive remains to be established.

FUTURE PROSPECTS FOR BIOPHOTOLYSIS

For an idea that has been experimentally explored for only five years, the history of biophotolysis has already been a turbulent one. After an initial flurry of activity and optimism, biophotolysis research has been severely curtailed. Thus, although the initial NSF-RANN program supported ten projects specifically for biophotolysis during FY 1972-75, and this research represented a major fraction of the overall budget for biological energy conversion, the successor--Fuels from Biomass Program (FFBP), now in the Department of Energy (DOE)--reduced this activity to a fraction of 1% of its budget. Recently, responsibility for this area was assigned to the newly formed Solar Energy Research Institute (SERI) at Golden, Colorado. Although, at present, there exists a hiatus, occasioned by this transfer, the prospects for biophotolysis research at SERI are much brighter than they were at DOE. SERI's in-house research program in biological energy conversion will place a major emphasis on the study of basic aspects of biophotolysis ( ) and, specifically, the production of hydrogen and generation of electricity with both *in vivo* and *in vitro* systems. SERI will also manage an extramural research contracts program in this area which will be directed toward biological approaches (rather than the photoelectric research that had been emphasized by the FFBP at DOE). Although only one extramural

biophotolysis project is presently supported, SERI is encouraging unsolicited proposals in biophotolysis. The relative balance or relationship between in-house and extramural research projects at SERI have not yet been defined. In any event, SERI will become the focus of much of the research activity and source of funding in biophotolysis in the foreseeable future.

A number of other federally supported projects exist in biophotolysis, including a NSF funded project dealing with marine algae (34) and basic research of heterocystous blue-green algae and hydrogenase. However, these activities represent a curtailed effort compared to recent years. Internationally, biophotolysis research has become a significant part of many biological solar energy programs, no doubt inspired by the earlier U.S. example. Thus, biophotolysis research has been carried out in England (39), the Soviet Union (60,61), Germany (32), Australia (30), and is starting up in other countries. The prospects for sustaining such research activities are uncertain, and their justification is often difficult in the overall context of specific national needs and opportunities. The prospects (e.g. probability) of successfully developing practical and economic biophotolysis systems capable of contributing a significant absolute or relative quantity of energy to the U.S.'s (or world's) energy supplies must be the ultimate criterion for justifying biophotolysis research and development budgets.

The above discussion presented the main approaches to biophotolysis and key technical and economic problems. It is clear that a few approaches could be made economically feasible in the near term, particularly the photosynthetic bacterial system for the photofermentation of, for example, sugar beet processing wastes. Technical difficulties appear minor and economic incentives significant, since sugar beet wastes are hard to treat by conventional waste treatment procedures and the sugar beet industry is very energy intensive, capable of using the hydrogen generated internally. This example could be extended to other low-nitrogen wastes and organic wastes in general, justifying a more vigorous effort at developing the still embryonic concept of photofermentations. Indeed, if it can be demonstrated practically, it may become an alternative, in some situations, to the anaerobic digestion of wastes.

If any of the proposed two-stage algal biophotolysis systems capable of generating pure hydrogen from water could be experimentally demonstrated, it would be a major step forward. Even if the stringent efficiency and economic requirements outlined above could not be met in the initial development of such systems, there may still be significant applications of such systems at remote sites which would allow a modest implementation. In this context, the potentially high efficiency for converting hydrogen gas to electricity in fuel cells and the ability to generate it on demand, would be a major factor in such applications which could compete with presently uneconomic photovoltaic systems for specialized, remote, or "marginal" applications. Just as in photovoltaics, where a market expansion induced lowering of production costs is being envisioned, so in biophotolysis the actual demonstration of even minor practical applications should give a great impetus to the development and refinements of the technology. Therefore, the most immediate approaches likely to be implemented need emphasis even though their ultimate potential in terms of national energy supplies may be limited.

Somewhat paradoxically, one of the longer term approaches has received the most attention--the heterocystous blue-green algal system of biophotolysis. Its poor prospects for near-term applications are primarily due to the nature of the hydrogen/oxygen mixture produced, restricting its use, for the present, in combustion and minimizing storage capabilities (unless expensive gas separation is used). Thus, this approach is likely to require development of a highly refined (e.g. high-efficiency, low-cost) system before applications are likely. Its attraction during the early stages of biophotolysis is understandable, as it could be readily demonstrated to fulfill the prerequisites of any practical biophotolysis system which are: (1) sustained and catalytic hydrogen and oxygen evolution for many weeks to months; (2) a stoichiometric (2:1) ratio of hydrogen and oxygen evolution; (3) high specific rates of hydrogen production and high photosynthetic efficiency; and (4) no limitations to scaling up of the system (13).

Heterocystous blue-green algae have the potential of becoming one of the lowest cost biophotolysis systems, as the process requires, in principle, relatively little operational inputs (e.g. pumping between stages). This justifies continued development without, however, exclusion of other alternatives. The question of the ultimate potential of biophotolysis as an energy resource must be left indeterminate at present. The potential advantage of biophotolysis over other biomass energy systems is the attainment of the practical limits of solar energy conversion efficiency. If this is realized, biophotolysis may become, in the long term, a significant fraction of the total biomass resource. However, it may be limited by the need for integration with buildings or other structures (to minimize space utilization) and the lack of integration into the food-feed-fiber waste recycling systems which characterize many other biomass energy alternatives. Those applications that may be made will be of a relatively small scale, reflecting the decentralized nature of this technology. Thus, a major role of biophotolysis in future energy systems cannot be predicted. Nevertheless, a balanced and focused research and development program focusing on parallel development of various biophotolysis approaches and related fundamental research can be justified on the basis of present knowledge.

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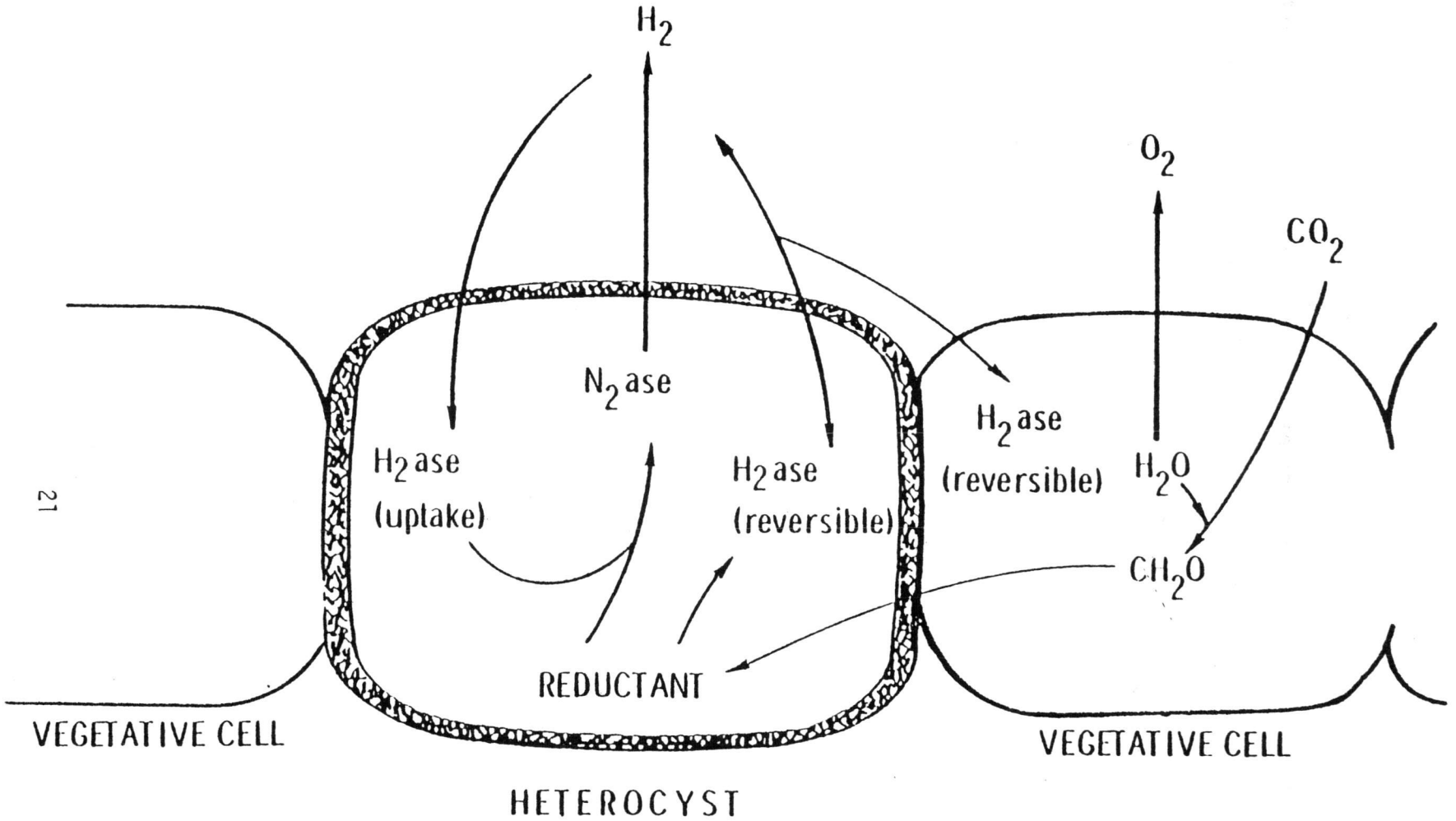

FIGURE 1. MODEL OF HYDROGEN METABOLISM BY HETEROCYSTOUS BLUE-GREEN ALGAE

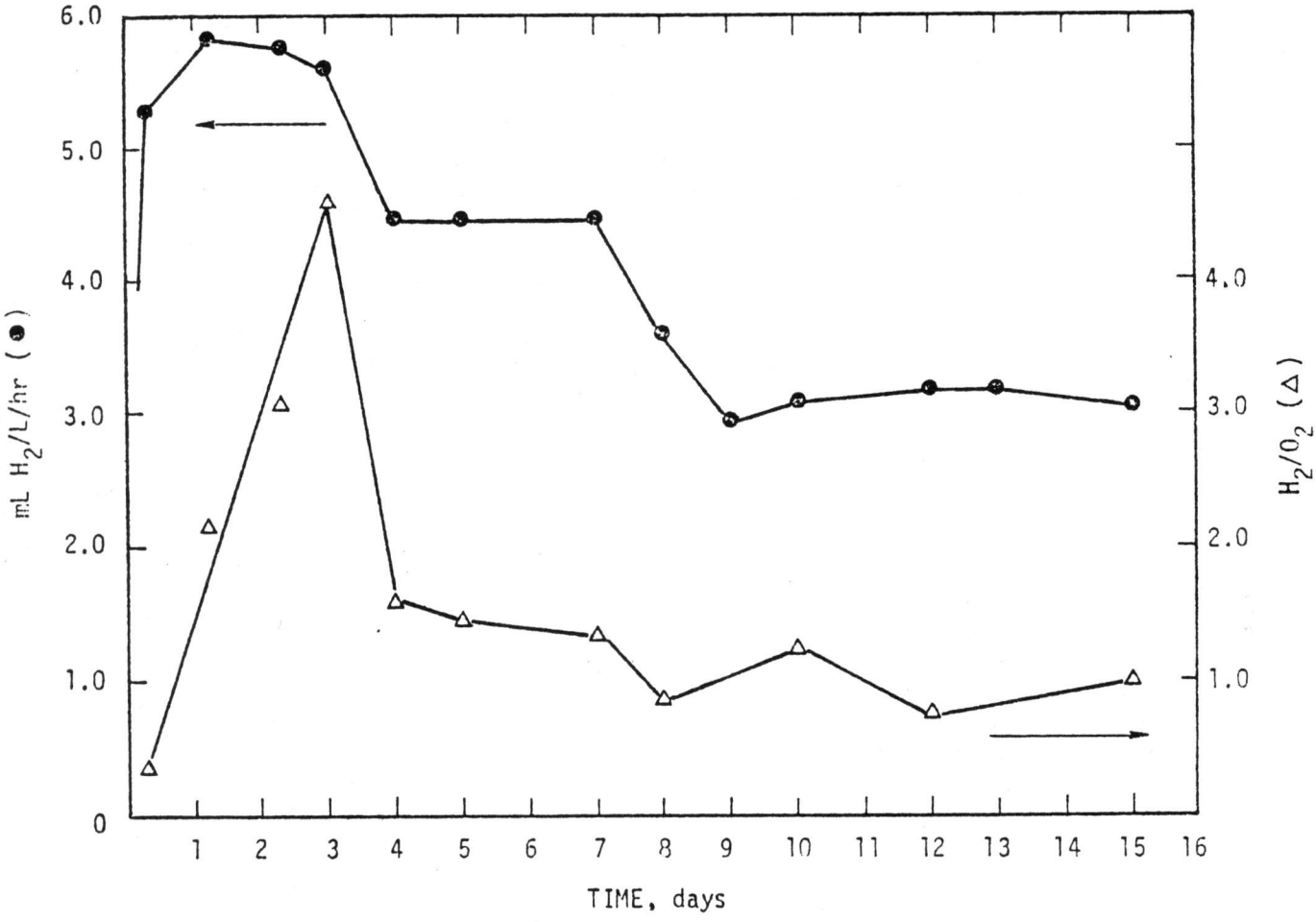

FIGURE 2. HYDROGEN AND OXYGEN PRODUCTION BY A 2 LITER CULTURE OF ANABAENA CYLINDRICA IN THE LABORATORY. (See text)

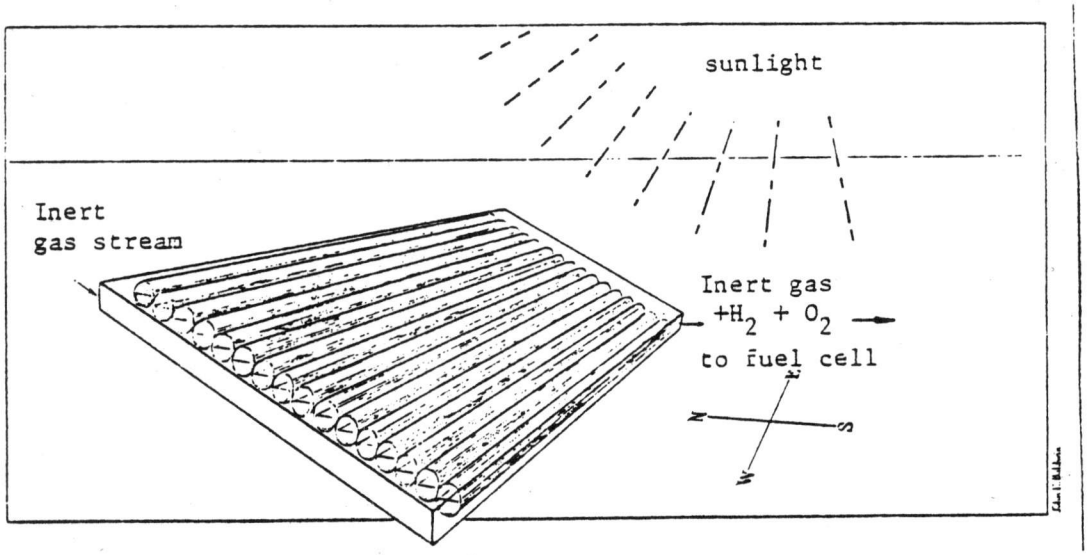

FIGURE 3. CONCEPTUAL DESIGN OF A BIOPHOTOLYSIS CONVERTER USING TUBULAR GLASS

III. THE EFFECTS OF NITROGEN SUPPLY ON HYDROGEN PRODUCTION BY CULTURES OF *Anabaena cylindrica*

INTRODUCTION

The production of hydrogen (and oxygen) from water and light by photosynthetic biological catalysts is known as biophotolysis. Biophotolysis has received attention as a potential method for the conversion of solar energy (1). Among the different biocatalysts proposed (2), the only biophotolysis system that has been demonstrated to sustain hydrogen evolution, for up to four weeks, involves heterocystous blue-green algae (3-9). In this system, the blue-green algae are maintained as nitrogen limited cultures under an inert gas (argon) atmosphere which allows them to simultaneously produce oxygen, in vegetative cells, and hydrogen in the heterocysts. Many technical difficulties remain in developing a practical biophotolysis system. One is to increase the metabolic activity and life span of the cultures. Weissman and Benemann (4) have reported that both the ratio of oxygen to hydrogen evolution and total hydrogen production of *Anabaena cylindrica* cultures could be increased by intermittent additions of 0.1 to 0.5 mM ammonium, and Hallenbeck *et al.*, (8) used 1% gaseous nitrogen (in Ar/CO_2) and obtained sustained but variable hydrogen production rates both under artificial light and outdoor sunlight. Here we report the effects of nitrogen supply, at various controlled levels, on hydrogen production by *A. cylindrica*.

MATERIALS AND METHODS

Anabaena cylindrica 629 was grown as described previously (4). When the desired algal densities (0.3 ∿ 0.4 mg/ml) were reached during exponential growth phase, cultures were transferred into 1-liter column converters (5 cm in diameter, with 0.8 liters of working volume) illuminated by 8 UHO Vita-Lite fluorescent lamps placed 30 cm from the line of converters. A 7 cm thick transparent water tank was set in the light path as a heat filter. The average light intensity on the side of columns was 3.3×10^4 erg/cm^2/sec. Temperature was 25 ± 1°C and pH 8.3 ± 0.5.

The effluent gas lines of the column converters were connected via a gas sample valve to an Aerograph (Model A-90-P3) gas chromatograph equipped with a 6' x 1/8" stainless steel column (packed with a molecular sieve 5-A) and a thermal conductivity detector. Hydrogen, oxygen, and nitrogen could be measured simultaneously using argon as the carrier gas.

The volumetric transfer coefficient ($k_L a$) for oxygen was determined as a function of gas flow rate. Columns were sparged with argon until free of dissolved oxygen. Then aeration was started at the appropriate gas flow rates and the increase in dissolved oxygen was followed with a Beckman 123303 Dissolved Oxygen Monitor.

RESULTS

Mass Transfer in the Vertical Converter

A volumetric oxygen transfer coefficient in 1-liter vertical column converters used in this and previous (8) studies was measured as a function of gas flow rate. Results obtained for four similar columns show no significant difference between columns, and the transfer coefficient could be correlated with gas flow rate as shown in Figure 1. Based on the experimental results for oxygen, the transfer coefficient for nitrogen was estimated by

using the following equation:

$$\frac{K_L^{N_2}}{K_L^{O_2}} = \left(\frac{D_L^{N_2}}{D_L^{O_2}}\right)^{1/2}$$

where $D_L^{N_2}$ and $D_L^{O_2}$ are the diffusivities in water of N_2 and O_2, respectively. This relationship between K_L's and P_L's has been experimentally verified for aeration systems that produce fairly large bubbles (>2.5 mm diameter)(10), which is similar to the aeration system used in the present study. At the relatively low gas flow rates, which are employed in biophotolysis experiments both to increase the partial pressure of hydrogen and to minimize filament breakage, values of the mass transfer coefficient were rather small ($k_L a$ for nitrogen = 1.5 ∿ 6 hr^{-1}; corresponding to gas flow rate = 2 ∿ 14 l/hr).

Growth and Hydrogen Production of Nitrogen Limited Cultures

Hydrogen production was initiated by transfering actively growing cultures of A. cylindrica from a 2-liter culture vessel into 1-liter column converters. The nitrogen supply to the cultures was controlled by varying both the nitrogen percentage of the sparging gas mixture and the gas flow rate: N_2 from 0% to 1.4%, and gas flow rate from 2 to ∿ 14 l/hr.

Typical patterns of growth and hydrogen evolution under these conditions of nitrogen starvation are shown in Figure 2. Even in strictly nitrogen starved cultures, the cell density increased during the initial 24 hours of starvation, probably due to the utilization of a nitrogen pool accumulated during the previous exponential growth. Thereafter, the nitrogen starved culture remained at a constant density (Figure 2A). However, with the cultures which received nitrogen in the gas phase, linear growth was observed after a 2 to 3 day lag period (Figure 2A). The linear growth rate could be correlated with maximum possible nitrogen supply ($k_L aC^*$), as shown in Figure 3. The convex curve obtained suggests that actual nitrogen consumption was lower than

the corresponding $k_L aC^*$ at higher values of $k_L aC^*$.

Hydrogen evolution was initiated by changing the gas phase to a nitrogen limited mixture, and usually reached its maximum value 2 to 3 days afterwards. Figure 2B compares the hydrogen production patterns of completely nitrogen starved and optimally nitrogen supplied cultures. Even with optimal nitrogen supply, the specific production rate of hydrogen decreased monotonically after reaching peak production. However, the volumetric production rate was rather stable during the first week and then decreased gradually. This decrease can be attributed to light limitation caused by continued algal growth.

As a first approximation, the half-life period of the volumetric hydrogen production, which was defined as the period during which the hydrogen production decreases from its peak rate to half of the peak rate, was chosen as an indication of the stability of a hydrogen producing culture. The half-life is plotted as a function of $k_L aC^*$ in Figure 4. The optimum supply was rather broad, with $k_L aC^*$ value of between 15 to 35 μmole N_2/l·hr (or 0.7 ∿ 1.6 μg-N/mg·hr assuming 0.6 mg/ml of algal density) supporting stable hydrogen production. The general shape of this curve can be explained as follows: At very low rates of nitrogen supply, not enough nitrogen is available for cell maintenance (protein turnover) and filament breakage occurs (8). At high rates of nitrogen supply, sufficient nitrogen is available to support rapid growth, and hydrogen evolution is curtailed since the cultures rapidly become light limited. At intermediate rates of nitrogen supply, sufficient nitrogen is available for cell maintenance while growth is still limited. The slow growth observed under these conditions maintains the cultures in a nitrogen limited state by increasing the demand for nitrogen, thus accounting for the rather broad optimum.

DISCUSSION

The range of nitrogen supply determined here was higher than that calculated from the ammonium addition data previously reported by Weissman and Benemann (4), which was, averaged over the length of the experiment, about 0.15 μg-N/mg.hr. However, in this particular case, algal density decreased about 20% during the 18-day experimental period; so this value of nitrogen supply can be taken to be less than the minimal requirement for cell maintenance. Indeed, increases in algal density were observed when ammonia was added in larger amounts. Jeffries, et al. (7) have also reported on the effect of nitrogen addition to cultures of A. cylindrica and observed that additions of 0.2 mM ammonium at 2-day intervals resulted in shortening the periods of hydrogen evolution, when compared with that without any added ammonium. The reason for the inconsistency between their and our results on nitrogen addition is not clear at present. Their addition of ammonium might be somewhat in excess, since re-greening of cultures was observed after the third or fourth addition (7). Although a very stable (30 days) hydrogen production under dim light (6 watt/m^2) was reported without any added ammonium, this was obviously attained at the expense of total hydrogen production, i.e. photosynthetic conversion efficiency. In the present study, stable hydrogen production was obtained without sacrificing efficiency by supplying nitrogen in the gas phase.

The conversion efficiency of light energy to free energy of hydrogen was calculated for the best cultures shown in Figure 2. The efficiency of 3.0 % was attained at the highest production period, and the average efficiency was 2.5 % over a 15 day experimental period. The first observation on the efficiency in this system is found in the report of Weissman et al. (4); the efficiency was a somewhat low value of 0.4 % , which might be due to the rather high light intensity employed (2.2 - 4.0 x 10^5 erg/cm^2·sec). Hallenbeck

et al. (8) observed very low efficiency (0.1 to 0.2%) in their outdoor biophotolysis experiments, though they obtained much higher efficiency (1.5%) under lower artificial illumination. Thus, elucidation of the main factor to which the low efficiency under high light intensities is attributable would have first priority in further development of a practical biophotolysis system.

The rate of oxygen evolution decreased drastically after the initiation of argon sparging, and after 2 to 3 days of nitrogen limitation, oxygen evolution rates were comparable to hydrogen production rates, which means that H_2/O_2 ratio was about unity for most of the period of hydrogen production. Similar results have been reported in previous experiments on the effects of ammonium addition on hydrogen producing cultures of Anabaena cylindrica (4): the H_2/O_2 ratio decreased from 4.0 (near the beginning) to 1.1 (2 days after the second addition of ammonium). Thus, the supply of nitrogen, either as ammonium or as nitrogen gas, fails to achieve a completely stoichiometric ratio of hydrogen to oxygen. The reason for this effect is not clear at present, although some of the photosynthetically derived reductant is necessarily used to support the low rate of nitrogen fixation and growth and is thus not available for hydrogen production. Storage of organic metabolites may also be involved.

In summary, both the stability and efficiency of hydrogen production by cultures of the blue-green algae, Anabaena cylindrica, can be improved by controlled nitrogen supply through pre-determination of the mass transfer characteristics of vertical column converters. Biophotolytic hydrogen production by nitrogen limited cultures is not strictly catalytic (H_2/O_2 ratios of around unity were obtained) since a small amount of growth is associated with this process.

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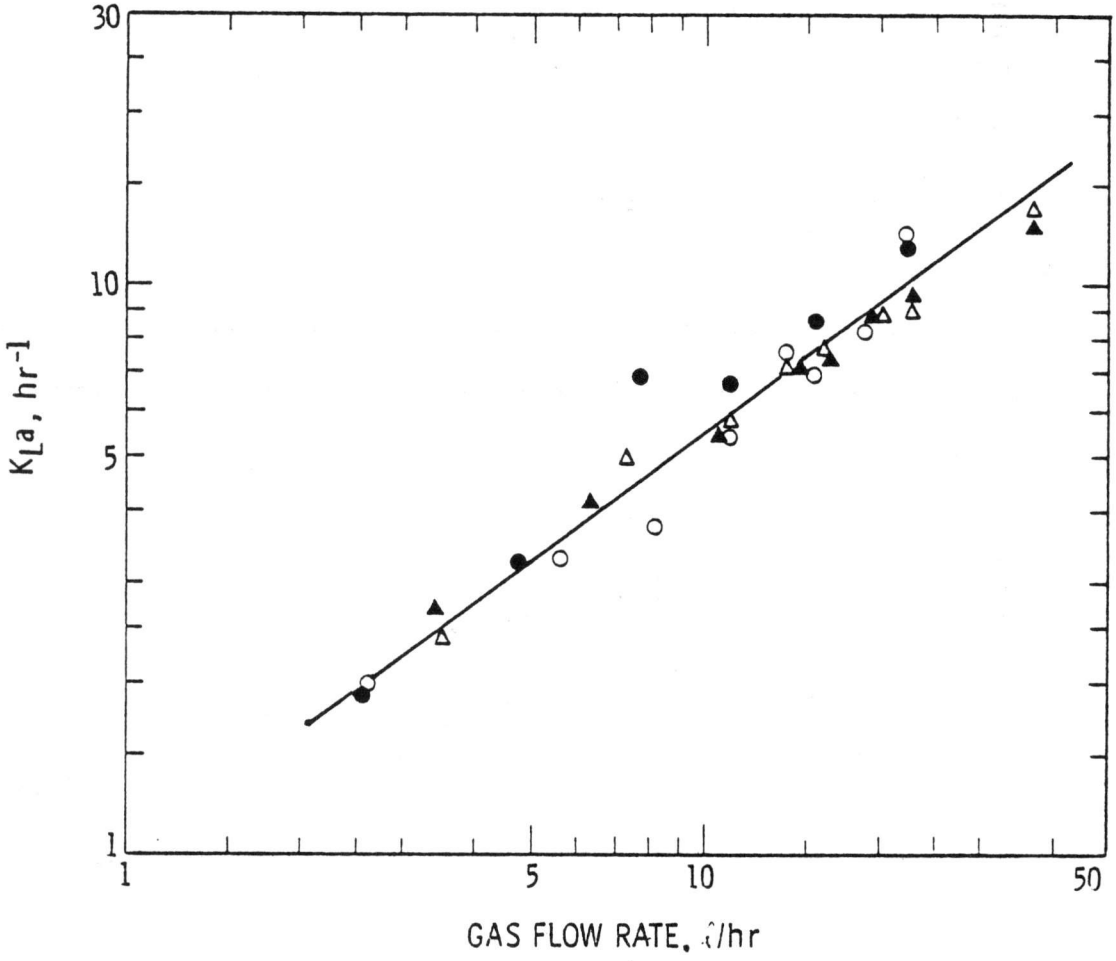

FIGURE 1. Correlation Between k_La and Gas Flow Rate in 1-Liter Vertical Column Converters (Air in Water)

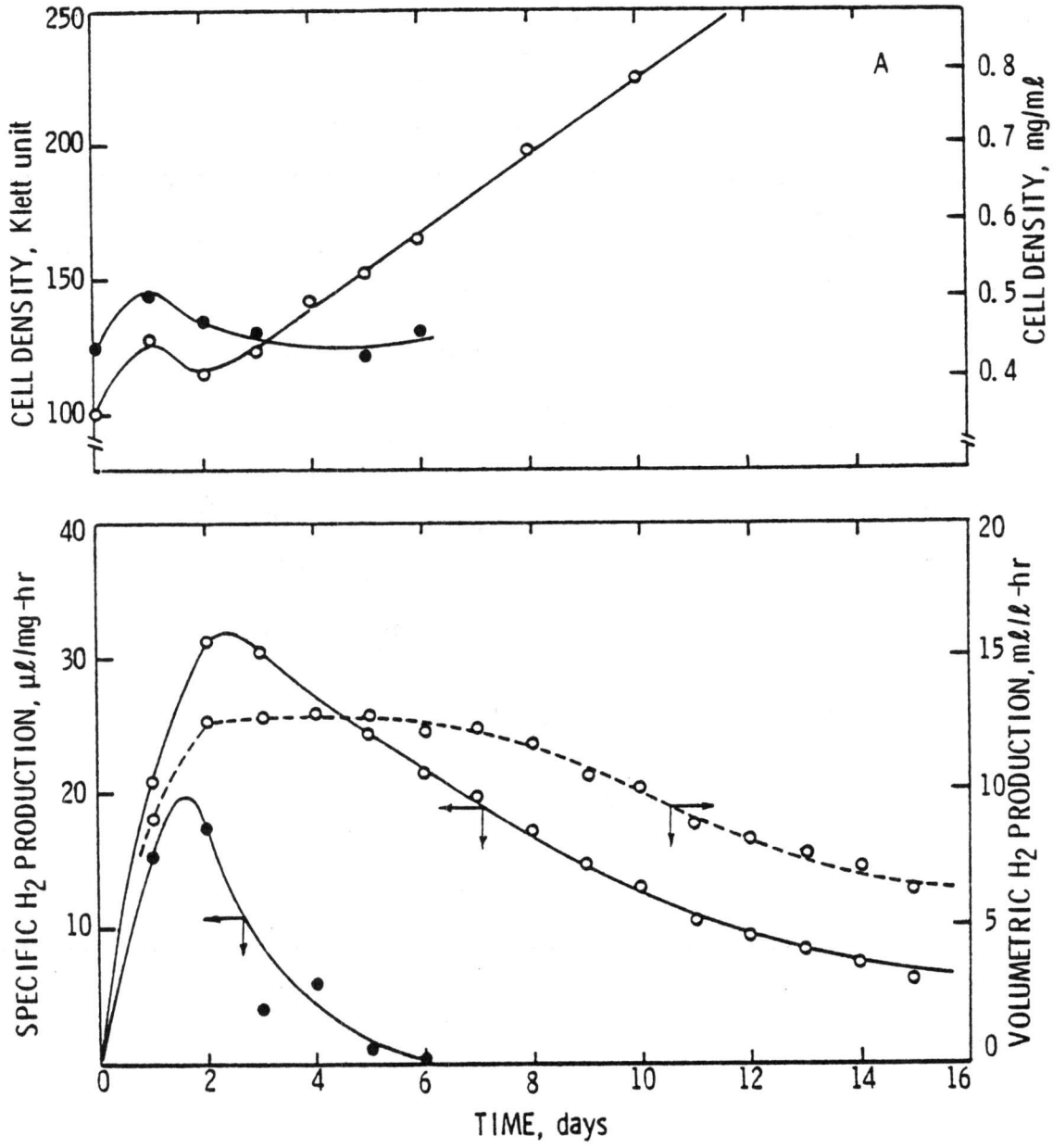

FIGURE 2. Growth (Upper) and Hydrogen Production (Lower) of nitrogen-Limited Cultures of A. cylindrica.

A. cylindrica grown in 2-liter bottles was transferred into column converters and sparged with $Ar/N_2/CO_2$ at time 0. Temperature was 25 ± 1°C and pH 8.3 ± 0.5. Changes in algal cell density during the hydrogen production phase are shown in a dry weight per volume base in the upper figure. In the lower part, hydrogen production is shown in specific production rate (solid lines) and in volumetric production rate (broken line). Symbols: o, for a culture sparged with a nitrogen-containing gas mixture ($Ar/N_2/CO_2$ = 98.7 to/0.8%/0.5%) at a rate of 10 l/hr; ●, for a completely nitrogen-starved culture spared with Ar/CO_2 (99.5%/0.5%) at a flow rate of 3 l/hr.

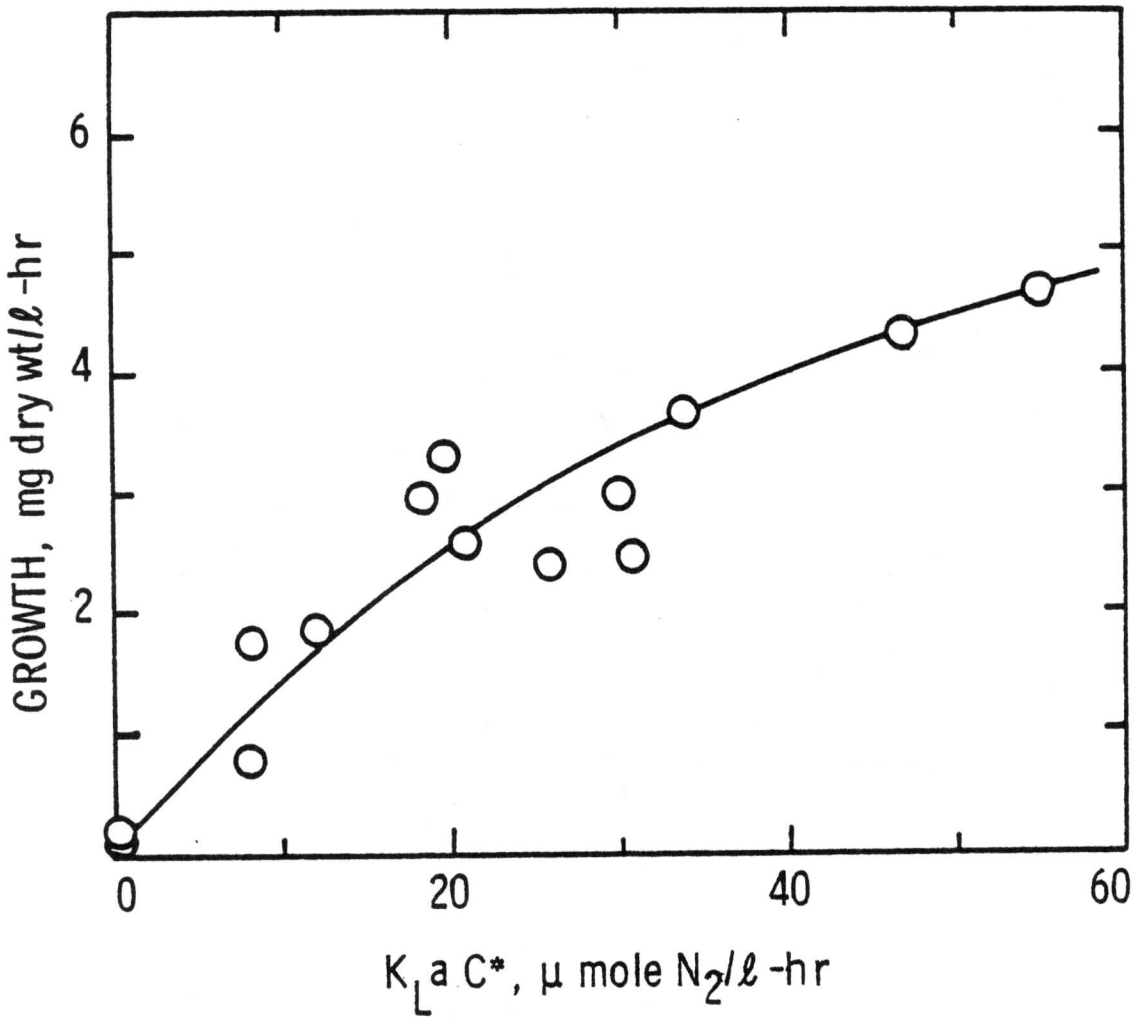

FIGURE 3. Growth in Hydrogen Production Phase as a Function of $k_L aC^*$.

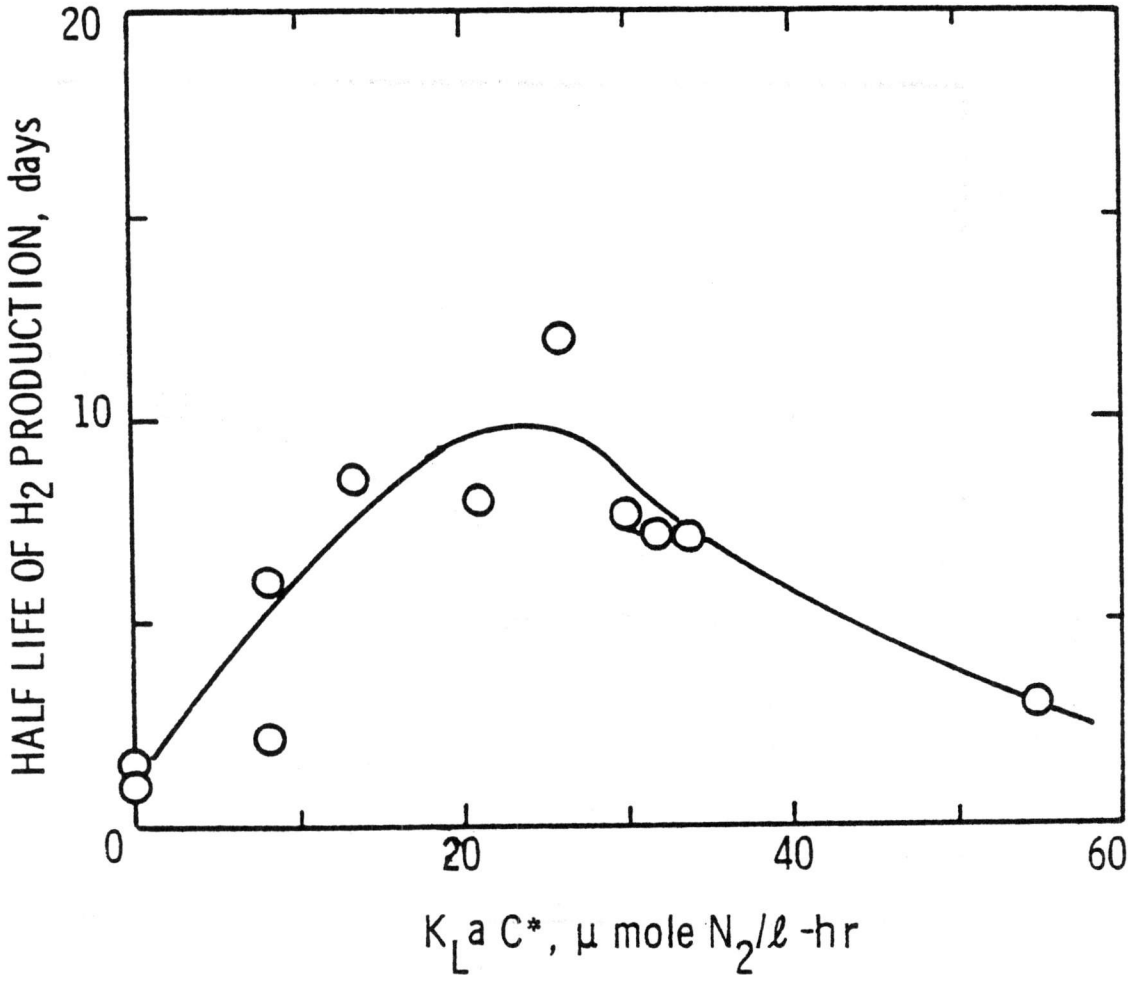

FIGURE 4. Half Life of Hydrogen Production as a Function of $k_L aC^*$.

IV. HYDROGEN EVOLUTION CATALYZED BY HYDROGENASE IN CULTURES OF CYANOBACTERIA

ABSTRACT

Cultures of Anabaena cylindrica, grown on media containing 5 mM NH_4Cl (which represses heterocyst formation), evolved hydrogen after a period of dark incubation in argon. This hydrogen production was not due to nitrogenase activity, whose biosynthesis was almost completely inhibited, but can be attributed to hydrogenase. Cultures were also grown on media with tungsten substituted for molybdenum, resulting in a high frequency of heterocysts (15%), but yielding inactive nitrogenase after nitrogen starvation. These cultures exhibited an enhanced evolution of hydrogen due to hydrogenase. The effects of oxygen inhibition on hydrogenase suggest that two pools of hydrogenase exist--an oxygen-sensitive hydrogenase in the vegetative cells and an oxygen-resistant hydrogenase in the heterocysts. Inhibition by oxygen of hydrogen evolution was rapidly reversed upon removal of oxygen, indicating that this hydrogenase reaction is reversibly inactivated. Light had an inhibitory effect on hydrogen evolution. Cell-free hydrogenase assays gave much higher rates of hydrogen production in vitro than in vivo, indicating that the cyanobacterial cultures were not fully activated or that endogenous reductant-generating mechanisms were not able to drive the hydrogenase at its maximal rate. When incubated at relatively low pH_2's, these cultures catalyzed a net uptake of hydrogen.

INTRODUCTION

Some species of green algae have been shown to possess a hydrogenase which, after a period of dark anaerobic incubation, catalyzes the evolution of hydrogen both in a dark, fermentative reaction, and in a light-dependent reaction. The physiology and biochemistry of this enzyme have been reviewed in detail (14,20). The following generalizations about this hydrogenase may be made: Although protein synthesis is probably not required, hydrogen evolution commences only after the algae have been anaerobically adapted in the dark for 2-30 hours. The period of peak production is only a few hours long and hydrogen production ceases after one day. Gas phase concentrations of over 0.2% oxygen are inhibitory. Under low light intensities hydrogen evolution is usually several-fold higher than in the dark; however, moderate to high light intensities tend to inhibit the reaction probably because of increased photosynthetic oxygen production.

Hydrogen evolution by nitrogen-fixing, filamentous cyanobacteria has been previously demonstrated (4,9,19) and studied in some detail with nitrogen-starved cultures of Anabaena cylindrica (18,21,33). Under the conditions used in these studies, the majority of the hydrogen evolution activity is due to nitrogenase, as evidenced by the requirement for the absence of fixed nitrogen, insensitivity to carbon monoxide, and inhibition by nitrogen or acetylene (4,7,9,19,21).

In reductant depleted cultures, hydrogen can support nitrogenase activity (3,6,10,29). Both isolated heterocysts (24,25,26) and intact filaments (6,8,10,29) have been found capable of consuming hydrogen in an oxy-hydrogen reaction. In addition, some cyanobacteria photoassimilate CO_2 using molecular hydrogen as an electron donor (2). This hydrogen consumption activity has been ascribed to an uptake hydrogenase, a membrane bound enzyme that is saturated at a relatively low pH_2 (3,30), similar to the Azotobacter

hydrogenase (17). In nitrogen-fixing cyanobacteria this hydrogenase may act to increase the efficiency of nitrogen fixation by recycling reductant lost in the nitrogen fixation process (7,29) and indirectly by scavenging oxygen (7,8).

The presence of an additional hydrogenase activity (hydrogen evolution) has been implied from whole cell studies (4,5) and clearly demonstrated in crude extracts (13,30,31). Whereas hydrogen consumption activity appears to be membrane bound and localized primarily in the heterocyst (26,30), the soluble hydrogen evolution activity is thought to be equally concentrated in both cell types (10,30). Further evidence that two distinct hydrogenases may exist is provided by the differential sedimentation of hydrogen consumption and hydrogen production activities in fragmented, isolated heterocyst preparations (30). However, little is known about the physiology of this additional hydrogenase activity, partially because any possible hydrogen evolution by this enzyme is usually masked by nitrogenase catalyzed hydrogen evolution.

Here we describe hydrogen evolution by hydrogenase in vivo and in vitro and describe some of the characteristics of this enzyme. To unequivocally attribute the observed hydrogen evolution to hydrogenase it was considered necessary to use cultures in which nitrogenase activity is absent, either by inhibition of biosynthesis through ammonium repression, or through inactivation by substitution of tungsten for molybdenum in the growth medium. The properties of this hydrogenase indicate that its relation to the metabolism of the cell is different than the hydrogenase of green algae.

MATERIALS AND METHODS

ORGANISM AND CULTURE METHODS

5 mM NH_4Cl or Nitrogen-Fixing Batch-Grown Cultures

Anabaena cylindrica (629), Nostoc muscorum (6719), Phormidium luridium (426), and Chloroglea fritschii (6912) were grown in 2-liter vessels on modified Allen and Arnon media (33) to which NH_4Cl was added, when desired, to give a concentration of 5 mM. Growth was at 25 to 30°C with a gassing mixture of 0.3% CO_2 in air to maintain the pH between 7.8 and 8.2 (in conjunction with 5 mM $NaHCO_3$ in the media). Lighting was as previously described (33). Nitrogen-starved cultures with active nitrogenase were prepared as previously described (33).

Growth was routinely measured with a model 800-3 Klett-Summerson photoelectric colorimeter with a No. 66 red filter. When the Klett reading of ammonia grown cultures reached 150-200, NH_4Cl was depleted and an additional aliquot of NH_4Cl (to bring the media to 5 mM NH_4Cl) was added sterily. Dry weight determinations were made by filtering portions of culture on preweighed filters and drying to constant weight at 80°C. The conversion factor was .0035 mg of dry weight per ml equals 1 Klett unit. Bacterial contamination was checked by streaking nutrient agar plates and incubating these plates at 28°C for ten days. Heterocyst frequencies were determined as described previously (33).

Tungsten-Grown Cultures. A. cylindrica was axenically grown and measured as above. Growth was in 8-liter vessels containing Allen and Arnon media from which molybdenum had been omitted and sufficient 1 M sodium tungstate has been added to give 8 ppm tungsten. NH_4Cl concentration of 1 mM provided fixed nitrogen. When the ammonia was depleted, the cultures yellowed, due to phycocyanin degradation induced by nitrogen starvation (22). The cultures were used when the heterocyst frequency had increased to 15% Any

nitrogenase that was synthesized under these conditions was inactive, since these cultures had very low acetylene reduction activities.

ASSAY PROCEDURES

Cultures were concentrated to a Klett turbidity range of 1000-3000 Klett units by settling. The concentrate was then made anaerobic by sparging with argon: CO_2 (99.7:0.3) for 10 minutes before injection into assay flasks.

H_2 Production and Consumption Assays

Two ml aliquots of the concentrated culture were injected into 6.4 ml serum stoppered Fernbach flasks which had been previously flushed with argon. Dark incubation was carried out by covering the flasks with aluminum foil. Lighting, when required, was via a bank of 30 w tungsten spotlights which gave a light intensity of 6.0×10^4 ergs/cm^2 per s. The flasks were incubated on a thermostated (25°C) shaker for various time periods. Assays were terminated with the injection of 0.20 ml of 25% trichloracetic acid. Gas samples were withdrawn from the assay flasks with a 100 ml gas-tight syringe and injected into a Varian model 3700 gas chromatograph equipped with a 1/8" x 6' stainless steel column packed with molecular sieve 5A and a thermal conductivity detector. Argon was used as the carrier gas. When correlated with a H_2 standard curve, hydrogen volumes were obtained. The minimum volume of hydrogen measurable in the 6.4 ml assay flasks was about 0.01 µl H_2/flask. Mass specific rates were calculated from volume rates, Klett, and the Klett dry weight conversion factor. Hydrogen consumption was measured by gas chromatography as the decrease in hydrogen concentration in the assay flasks (6), which had previously been made up to 2% H_2 in argon. Leakage of hydrogen was negligible.

Acetylene Reduction Assays

6.4 ml serum-stoppered Fernbach flasks were prepared by flushing with argon and then were injected with 1 ml of acetylene. After venting the flasks

to atmosphereic pressure, the gas phase was 15% acetylene (by volume). Two ml portions of anaerobic concentrate were injected into assay flasks with an 18-gauge needle and a 3 ml syringe. This procedure minimized O_2 contamination. Flasks were incubated in the dark on a shaker at 25°C. Assays were terminated by the injection of 0.20 ml of 25% trichloroacetic acid. Ethylene production was measured by injection of 100 µl gas samples into a Varian model 3700 gas chromatograph equipped with a 5' x 1/8" stainless steel column packed with Poropak R, and a flame ionization detector. This procedure gives an upper limit to the possible contribution of nitrogenase to the observed hydrogen evolution since rates of hydrogen evolution by nitrogenase are usually less than rates of acetylene reduction (7,33).

Additions to Assay Flasks

Some experiments involved the addition of various liquid or gaseous chemicals to assay flasks. Any additions to the flasks were made before the culture sample was added. Final concentrations of different substrates were: 2×10^{-5}M DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), 5 mM glucose, 5 mM isocitrate, 5 mM succinate, 50 µg/ml chloramphenicol, 50 µm CCCP (carbonyl cyanide m-chlorophenyl hydrazone), 0.5 mM methyl viologen, and 2.5 mM dithionite. O_2 inhibition experiments were performed by injecting varying amounts of oxygen into argon flushed flasks to give a final O_2 percentage of from 0.1% to 30%.

Cell-Free Extract Preparation and Assays

Cell-free extracts were prepared by injecting anaerobic incubated culture concentrate into an anaerobic, serum-stoppered sonicator cell. The suspension was disrupted at 4°C for 6 minutes with an Heat Systems Ultrasonics model W200 sonicator set at medium power and pulsed with a 65% duty cycle. This procedure destroyed all vegetative cells and 80-90% of the heterocysts. 0.75 ml extract were injected into serum-stoppered, argon-flushed 6.4 ml

Fernbach flasks. 0.20 ml of sodium dithionite was added. (Sodium dithionite solution was made as follows: 9.65 ml of H_2O, 0.05 ml 1M NaOH, 0.3 ml 1M HEPES (N-2-hydrozyethylpiperazine-N-2ethanesulfonic acid) pH 7.75 were placed in a 10 ml flask which was fitted with a serum stopper and made anaerobic by flushing with argon. 43 mgs of sodium dithionite was rapidly added to the flask which was again made anaerobic.) 50 µl of a 20 mM solution of methyl viologen was added to start the reaction. Assays were run for 20 minutes, terminated with the injection of 0.2 ml of 25% trichloroacetic acid, and the H_2 produced was measured as in whole cell assays (described above). Results are expressed as µl H_2 evolved per mg dry weight (of the original culture concentrate) per h, since the volume change during disruption was negligible. This allows direct comparison between *in vitro* and *in vivo* assays.

RESULTS

Ammonia-grown (5 mM NH_4Cl) and nitrogen-starved (tungsten) cultures showed similar time courses for the development of hydrogenase activity *in vivo*, with maximum rates of hydorgen production being achieved within one to three hours of the onset of dark anaerobiosis (Fig. 1). After reaching a maximum, rates of hydrogen evolution were constant for at least seven hours (i.e. hydrogen production was linear with time). The maximum rates of hydrogen production (per mg dry weight) obtained with nitrogen-starved (15% heterocysts), tungsten-poisoned cultures were three to four-fold higher than with ammonium gorwn cultures (< 0.1% heterocysts). These results suggest that the heterocysts contain a proportionately larger amount of hydrogenase, or that the hydrogenase present in heterocysts is more active.

To rule out the possibility of indirect effects of tungsten or ammonium on hydrogenase activity, the *in vitro* hydrogenase activity of different culture types was examined (Table 1). Since added reductant is in excess in these *in vitro* assays, rates of hydrogen evolution are limited only by the amount of active enzyme present, and thus these assays measure the maximum rate of hydrogen evolution attainable for a given amount of active enzyme. In addition, this procedure gives some indication of the hydrogenase content of nitrogen-fixing cultures, whose *in vivo* hydrogen evolution by hydrogenase cannot be directly measured due to interfering hydrogen evolution by nitrogenase (4,9,19,19,21,23). As Table 1 shows, *in vitro* hydrogenase activity was correlated to some extent with heterocyst frequency. Cultures that had been grown on 5 mM NH_4Cl had practically no heterocysts (< 0.1%) and possessed a small but significant amount of *in vitro* hydrogenase activity. The N_2-grown culture (with 5% heterocysts) had an *in vitro* hydrogenase activity that was intermediate between the activity of ammonium-grown cultures and nitrogen-starved (15% heterocysts) cultures. The higher *in vitro* activity

of the nitrogen-starved culture with Mo versus the nitrogen-starved culture with W may indicate that W was somewhat inhibitory.

The requirement for a period of dark anaerobiosis for hydrogen production by cyanobacteria is similar to that found for hydrogen production by green algae (14,20). Dark anaerobiosis may serve to permit the synthesis of hydrogenase enzyme, or the adaptation of an inactive form. The cyanobacterial hydrogenase appears, at least to a large extent, to be constitutive. A cell-free extract prepared from an ammonium-grown culture that had not been adapted evolved 1.5 µl H_2/mg dry wt per h. After adaptation, the rate of hydrogen evolution in a crude extract had almost doubled (2.7 µ H_2/mg dry wt per h. Table 2). Addition of chloramphenicol (an effective inhibitor of protein synthesis by A. cylindrica [27]) during this adaptation period is without effect indicating that activation of inactive, preformed enzyme is occurring rather than de novo synthesis. Adaptation under hydrogen (for periods up to 34 hours) did not result in significantly higher activities than incubation under argon.

That the increased rates of hydrogen production by nitrogen starved, tungsten poisoned, cultures is due to disproportionately higher activity in heterocysts is also suggested by the inhibitory effects of various oxygen concentrations (Figure 2). Hydrogen evolution by heterocyst-free cultures was almost completely abolished by 5% oxygen. At 5% oxygen, hydrogen production by cultures with 15% heterocysts showed the same decrease in absolute hydrogen production. The remaining hydrogen evolution activity ($\sim$ 2/3 of the original) was fairly oxygen resistant, with 50% inhibition at 15% oxygen in the gas phase. Heterocystous cyanobacteria also contain the extremely oxygen labile enzyme, nitrogenase, and indirect evidence has led to the hypothesis that nitrogenase is localized in the heterocyst (11), and therefore is resistant to oxygen inactivation (33). The results shown in

Figure 2 suggest that a large proportion of the hydrogenase activity in the nitrogen-starved cultures is contained in the heterocyst and thus protected from the inhibitory effects of oxygen. However, oxygen inhibition is almost completely reversible (Figure 3). Likewise, when cell-free extracts were incubated in the presence of 20% oxygen for one hour and then made anaerobic and assayed for hydrogenase activity, more than 85% of the original activity remained. These results are consistent with either oxygen acting directly, in a manner which is readily reversible upon removal of oxygen from the gas phase, or oxygen acting indirectly by competing for some reduced compound which drives hydrogen evolution.

Light also had an inhibitory effect (Figure 4). At the lowest light intensity tested (6.0×10^3 ergs/cm^2 per s), hydrogen evolution was already inhibited fifty percent over that of the dark control. Higher light intensities were increasingly inhibitory. In green algae, high light intensities are thought to inhibit hydrogen evolution indirectly by the inhibitory effect of photosynthetic oxygen evolution. However, unlike the case with green algae, where addition of DCMU alleviates inhibition by high light intensities (15), addition of 2×10^{-5}M DCMU had little effect on activity at the highest light intensity tested. At present, the cause of this inhibition is unknown. It is possible that illumination stimulates a reaction which competes with hydrogenase for the reduced substance which is a primary electron donor.

Broken cell preparations of Anabaena cylindrica can evolve hydrogen from some electron donors, such as glucose, glucose-6-phosphate, isocitrate and dithionite when supplied with an artificial electron carrier (13,20). We found that glucose, succinate, and isocitrate (all 5mM), when exogenously supplied, had no effect on in vivo hydrogenase activity in Anabaena cylindrica (Table 3). This result could reflect the general inability of Anabaena

cylindrica (which is considered an obligate photoautotroph [1]) to assimilate and utilize exogenous carbon compounds. However, it is likely that glucose, and to a lesser extent, isocitrate and succinate were assimilated, but inactive, in supporting hydrogenase acticity since one Anabaena species has been shown to incorporate and metabolize these compounds (16,23). Paradoxically, addition of 50 μM CCCP caused a two-fold stimulation of hydrogen evolution. When cultures were incubated in the presence of 2.5 mM dithionite and 0.5 mM methyl viologen a ten-fold stimulation in activity was observed (Table 3).

Indeed, in vitro hydrogenase activity was invariably found to be much higher than in vivo hydrogenase activity (Table 1). Hydrogen evolution by cultures was only 3-7% of the activity obtainable in vitro indicating that in vivo activity is severely limited by the supply of an active source of reductant. Similar results were obtained with Nostoc muscarum as shown in Table 4, which also includes data obtained in experiments with Phormidium luridum and Chlorogłea fritschii. The last two cultures gave no hydrogen evolution, either in vivo, or in vitro, that could be attributed to reversible hydrogenase. Thus, the ability to evolve hydrogen, either in vivo, or in vitro, is not universal among the cyanobacteria.

Cultures of Anabaena cylindrica with (tungsten poisoned) and without (5mM NH_4Cl media) heterocysts catalyzed a net uptake of hydrogen at relatively low hydrogen concentrations (1%-2%) as shown in Table 5 . This result is surprising since it has been previously suggested that hydrogen consumption is associated primarily with the heterocyst (26,30). The relatively high rates of consumption (about three times the rate of hydrogen evolution observed) at these low pH_2's suggest that an uptake hydrogenase may be at least partly responsible for this activity. However, further work is needed to clarify this point. Rates of hydrogen consumption declined after three to six hours, probably due to the reduction of the available pool of endogenous

electron acceptors. The rates of hydrogen consumption observed in this study; 0.14-0.40 µl/mg dry wt per h, or 1.1-3.3 µ moles/mg chlorophyll per h (assuming 5 µg chlorophyll per mg dry wt (33))are similar to those previously observed with aerobically grown, nitrogen-fixing cultures of Anabaena cylindrica, (0.6 to 1.1 µ moles/mg chlorophyll per h) under conditions where the reduction of endogenous electron acceptors is taking place (6,30). The effects of incubating the cultures in an atmosphere that contained 5% oxygen were variable, but in general a slight stimulation was observed. As previously noted for hydrogen consumption by vegetative cell fragments (30), addition of methylene blue did not have a significant effect on hydrogen consumption by the cultures used here.

DISCUSSION

The results presented above give evidence for *in vivo* hydrogen evolution by cyanobacteria that can be attributed to a reversible hydrogenase, hitherto only characterized *in vitro* (10,11,13,30,31). This hydrogenase appears to be unlike the hydrogenase of green algae in several respects. Illumination (within the range tested) did not lead to a photo-induced evolution of hydrogen, rather, a photoinhibition was found, and DCMU failed to alleviate this inhibition. Thus, at least in *Anabaena cylindrica*, reductant generated directly by photosynthesis does not seem to be available to hydrogenase. This is consistent with the lack of interaction between the *A. cylindrica* plant type ferredoxin and this hydrogenase *in vitro* (13).

The metabolic pathways leading to hydrogen evolution in the dark are unknown at present. Addition of several carbon compounds, glucose, isocitrate, and succinate, failed to stimulate hydrogenase activity. The *in vivo* rates of hydrogen evolution without added electron donors were only one tenth or less than the rates obtained *in vitro* or *in vivo* with dithionite and methyl viologen. (*In vitro* dithionite-driven hydrogen evolution is about twice that obtained *in vivo* possibly indicating some difficulty in transfer of the artificial carrier and donor through the membrane.) These results indicate that hydrogenase activity *in vivo* is severely limited by reductant supply.

Heterocyst containing cultures invariably gave higher rates of hydrogen evolution than did cultures grown on ammonia which repressed heterocyst formation. It is possible that this effect is partially due to some general inhibitory effect of ammonia, or to a stimulation by tungsten, but it is more likely that heterocysts actually contained a larger amount of active hydrogenase. This conclusion is based on the following observations: heterocyst containing cultures were more resistant to oxygen inhibition than cultures without heterocysts, and the magnitude of the decrease in hydrogen evolution

by heterocystous cultures at low oxygen concentrations is the same as that observed with heterocyst-less cultures. Perhaps more significant is the three to four-fold higher *in vivo* rates of hydrogen evolution by the 15% heterocystous cultures, and the higher rates of *in vitro* hydrogen evolution that were roughly correlated with heterocyst frequency. Since *in vitro* assays are a direct measure of the amount of active hydrogenase present, these results are consistent with heterocysts containing a proportionately larger amount of hydrogenase. While these arguments are admittedly indirect, at least some other methods of localization appear to be equally equivocal. Previous studies with isolated heterocysts and vegetative cell fragments have indicated that hydrogen evolution is equally concentrated in both cell types (10,30). However, isolated heterocysts probably leak some of their soluble contents (12,34), and thus these previous studies may be consistent with the findings reported here if hydrogen evolution activity,which is soluble (13,30),is readily lost from isolated heterocysts.

In conclusion, the results presented here have established the existence of *in vivo* hydrogen evolution catalyzed by hydrogenase, and demonstrated that some of the characteristics of this activity are unlike the hydrogenase activity that has been characterized in green algae. The *in vivo* rates of hydrogen evolution reported here are much lower than rates of hydrogen evolution by nitrogenase (4,9,18,19,21,23), but are significant both in the sense of being well above the limits of detection, and in indicating a form of cyanobacterial metabolism that has not been previously described. Maximizing *in vivo* hydrogenase activity will depend on gaining information on activating the supporting reductant generating mechanisms.

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Table 1. Comparison of *In Vivo* and *In Vitro* Hydrogenase Activity of *Anabaena cylindrica* Grown on Various Media

Culture Media[a]	Heterocyst Frequency (%)	*In Vitro* Hydrogen Evolution (μℓ/mg dry wt-h)	*In Vivo* Hydrogen Evolution (μℓ/mg dry wt-h)	Dark Acetylene Reduction Activity (μℓ C_2H_4/mg-h)
Tungsten (-Mo)	15	1.18	0.057	0.0008
N_2Grown (nitrogen-starved)	15	1.86	b	b
N_2Grown	5	0.83	b	b
5 mM NH_4Cl	0.1	0.30	0.009	0.0007

[a] Cultures were grown and assayed as described in Materials and Methods. The results shown for tungsten and ammonium chloride cultures are the average of three experiments for each culture type. The N_2 grown culture was a single culture from which an aliquot was anaerobically adapted and then assayed for hydrogenase *in vitro*. The remaining culture was nitrogen-starved, which increased the heterocyst frequency to 15%, anaerobically adapted, and assayed for hydrogenase *in vitro*.

[b] It was not possible to assay for *in vivo* hydrogen evolution by these cultures due to interferring hydrogen evolution by nitrogenase.

Table 2. Effect of Dark Argon Incubation on *In Vitro* Activity

Conditions[a]	Additions	μℓ H_2/mg dry wt per h (*in vitro*)
Before argon incubation	-	1.47
After argon incubation	50 μg/mℓ Chloramphenicol	2.61
After argon Incubation	-	2.70

[a] A heterocyst-free (>0.1%) culture of *A. cylindrica* was grown on 5 mM $NH_4Cℓ$ to 400 Klett and concentrated to 970 Klett. From an aliquot, a crude extract was prepared and assayed for hydrogenase activity. The remaining whole cell concentrate was subjected to dark, anaerobic incubation with and without 50 μg/mℓ chloramphenicol and *in vitro* hydrogenase activity measured again after 24 hours.

Table 3. The Effect of Various Electron Donors and An Uncoupler On *In Vivo* Hydrogenase Activity[a]

Additions	*in vivo* Hydrogenase Activity μℓ H_2/mg dry wt per h
-	0.045
50 μM CCCP	0.090
2.5 mM dithionite + 0.5mM methyl viologen	0.61
5 mM glucose	0.045
5 mM isocitrate	0.045
5 mM succinate	0.045

[a] A Tungsten grown culture of *Anabaena cylindrica* was concentrated, anaerobically adapted (3 hours) after which any additions were made. Aliquots of the culture concentrate were then assayed for hydrogen evolution for one hour. The dark acetylene reduction activity was 0.001 μℓ C_2H_2/mg per h.

Table 4. Hydrogenase Activity in Various Species of Cyanobacteria

Species[a]	In Vitro Activity ($\mu\ell$ H_2 evolved/mg dry wt per h)	In Vivo Activity ($\mu\ell$ H_2/mg dry wt per h)	Dark Acetylene Reduction Activity ($\mu\ell$ C_2H_4/mg-h)
Anabaena cylindrica	0.30	0.01	.0008
Nostoc muscorum	0.16	0.004	.0002
Phormidium luridum	0	0	0
Chloroglea fritschii	0	0	0

[a] Cultures were grown on media containing 5 mM NH_4Cl, and assayed for in vivo and in vitro activity as described in Materials and Methods.

Table 5. Hydrogen Consumption Activities

Culture	Hydrogen Consumption Activity[a] (μℓ/mg dry wt per h)		
	Time (h)	2% H_2	2% H_2 + 5% O_2
Tungsten Grown (15% Heterocysts)	2	.14	.22
	3.5	.20	-
	6	.19	.22
Ammonia Grown (<0.1% Heterocysts)	1.5	.40	-
	2.5	.30	-
	3.5	.30	-

[a] Assays were performed in an atmosphere of 2% H_2 in argon, or 2% H_2 , 5% O_2 in argon. Leakage of hydrogen from control flasks was negligible.

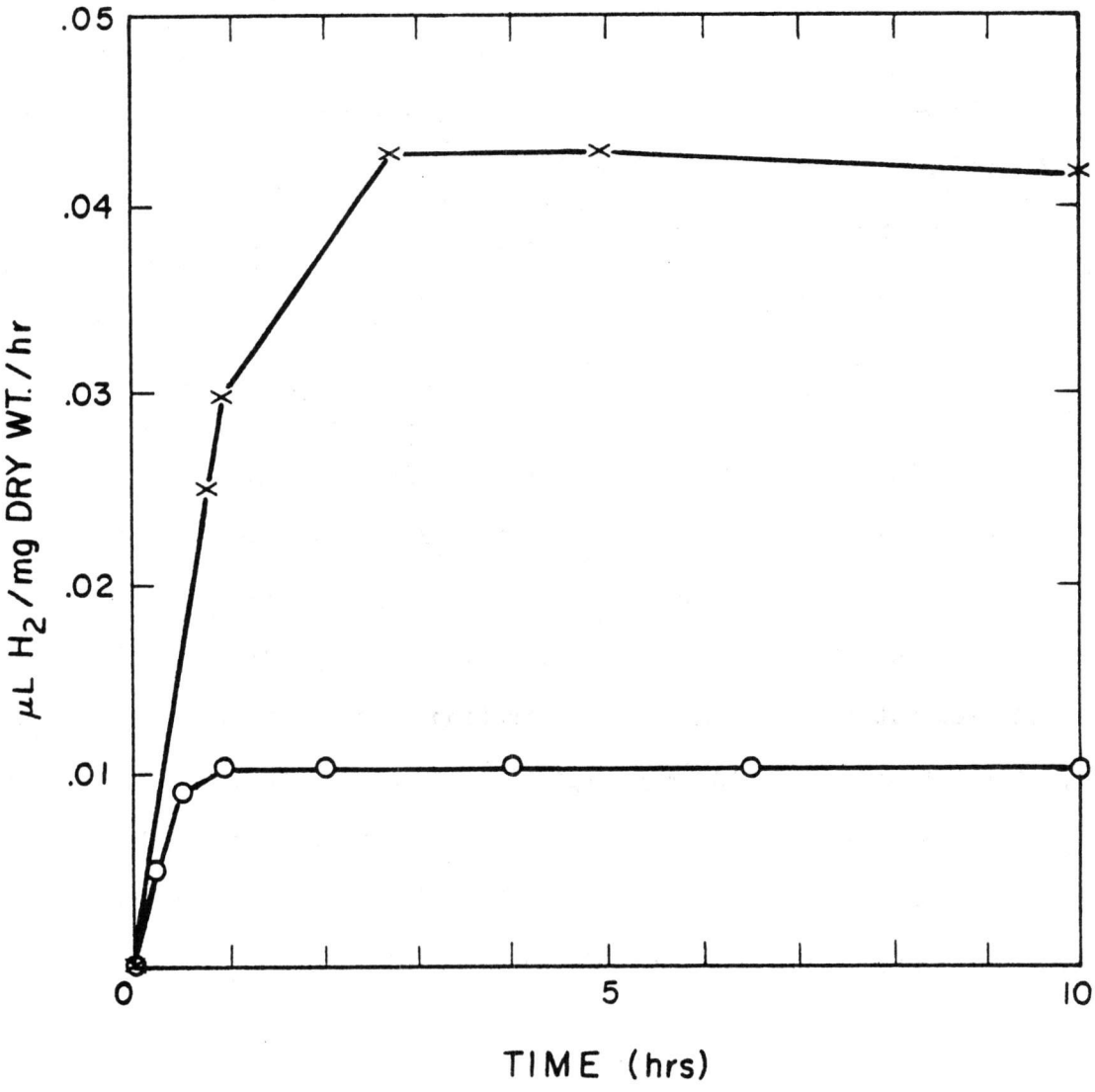

FIG. 1. Time course of dark activation of hydrogenase in A. cylindrica.

FIG. 1. Time course of dark activation of hydrogenase in A. cylindrica. Symobols: o - 5 mM NH_4Cl batch-grown culture (<0.1% heterocysts). The results shown are the average of three cultures. The observed variation in peak hydrogen production was 0.009 - 0.013 μℓ H_2/mg dry wt-h. X - Tungsten-grown culture (15% heterocysts). The results shown are the average of two cultures. The observed variation in peak hydrogen production was 0.037 - 0.043 μℓ H_2/mg dry wt-h. Dark acetylene reduction rates for both cultures were about 0.001 μℓ C_2H_4/mg dry wt-h.

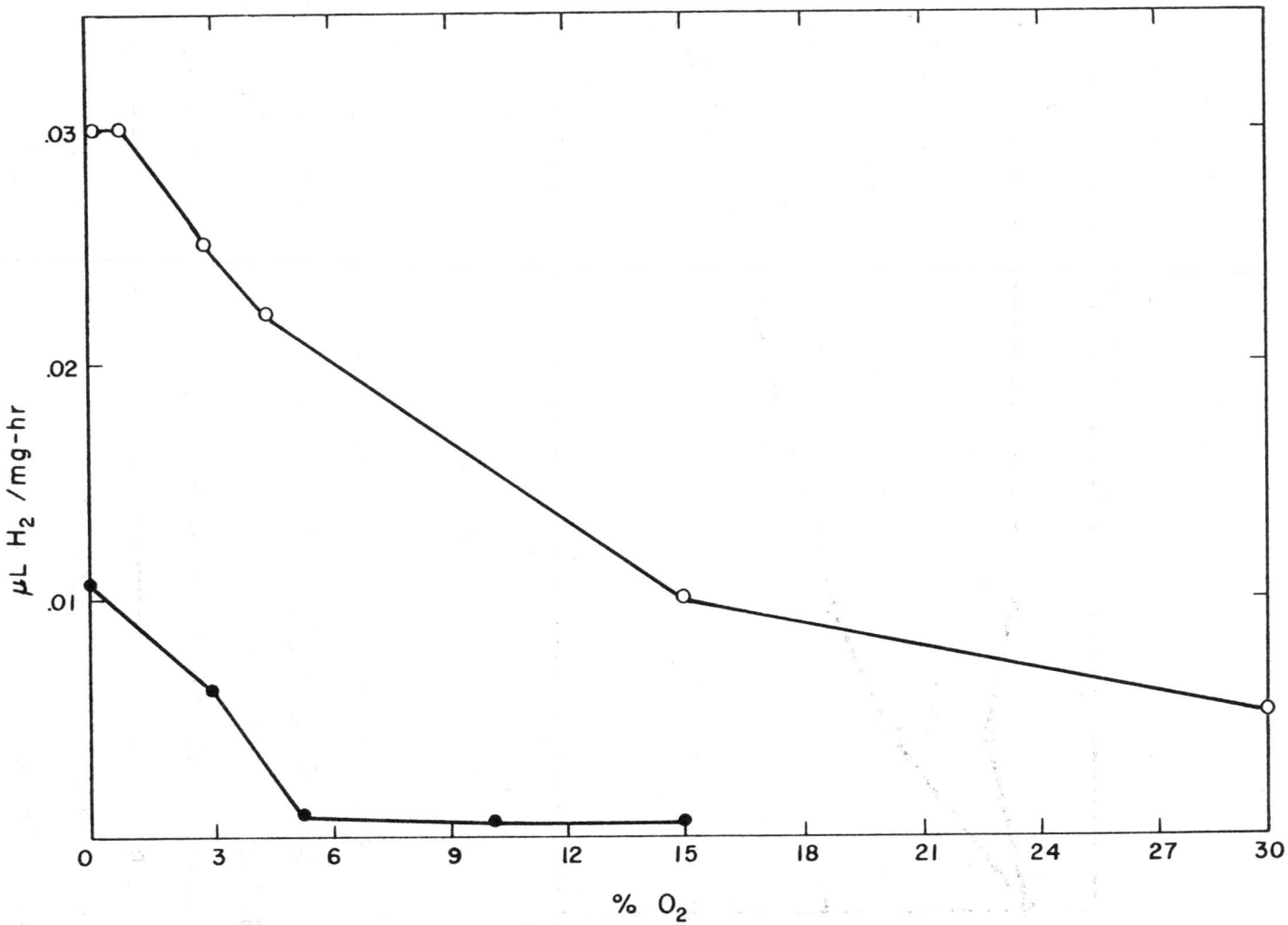

FIG. 2. Oxygen inhibition of hydrogenase activity in A. cylindrica. Symbols: ●—● NH_4Cl batch-grown culture (0.1% heterocysts). Average of two experiments (peak hydrogen production was .010 and 0.015 μℓ H_2/mg dry wt-h). Dark acetylene reduction activity = .0006 μℓ C_2H_4/mg-h. o—o Tungsten-grown culture (15% heterocysts). Average of two experiments (peak hydrogen production was 0.033 and 0.038 μℓ H_2/mg dry wt-h). Dark acetylene reduction activity = .001 μℓ C_2H_4/mg-h.

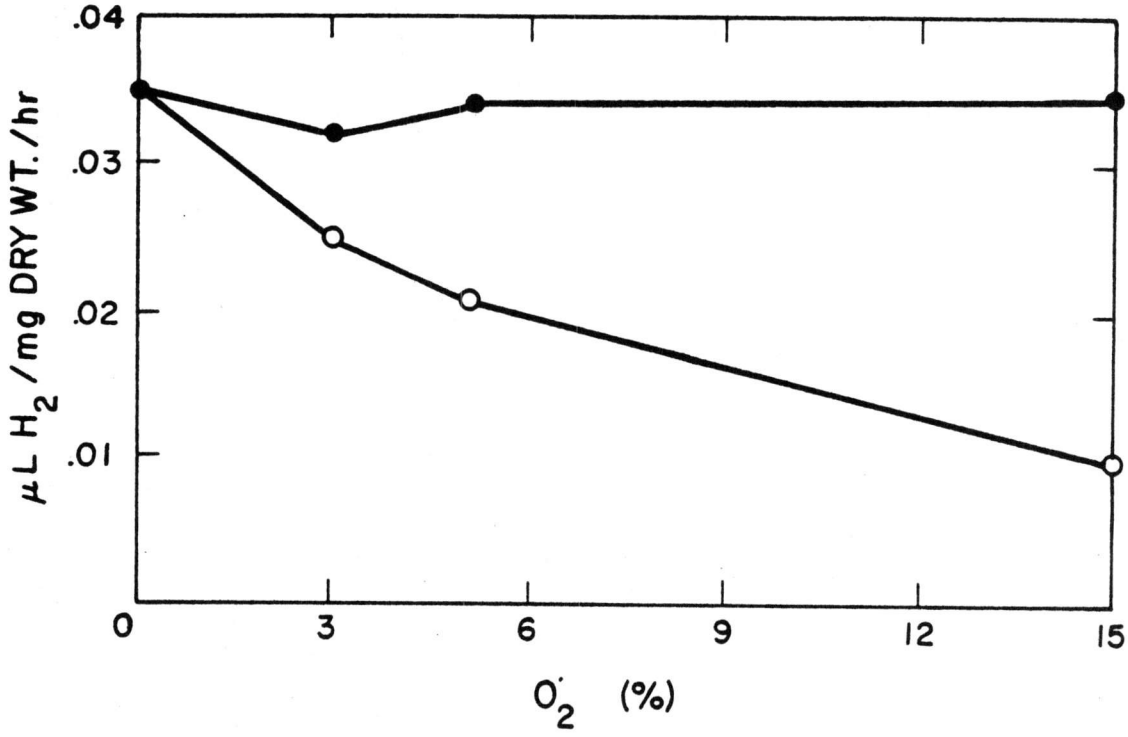

FIG. 3 Reversibility of oxygen inhibition on hydrogenase activity in whole-cell assays of A. cylindrica. The culture used was a nitrogen-starved culture (15% heterocysts) grown on tungsten media. Symbols: —o. The culture was incubated in varying oxygen concentrations for three hours and then assayed for H_2 production by withdrawing a 0.1 ml gas sample. ——●
The same vials were then flushed with argon for 10 minutes and assayed again after one hour. The dark acetylene reduction activity equaled 0.001 μℓ C_2H_4/mg dry wt-h.

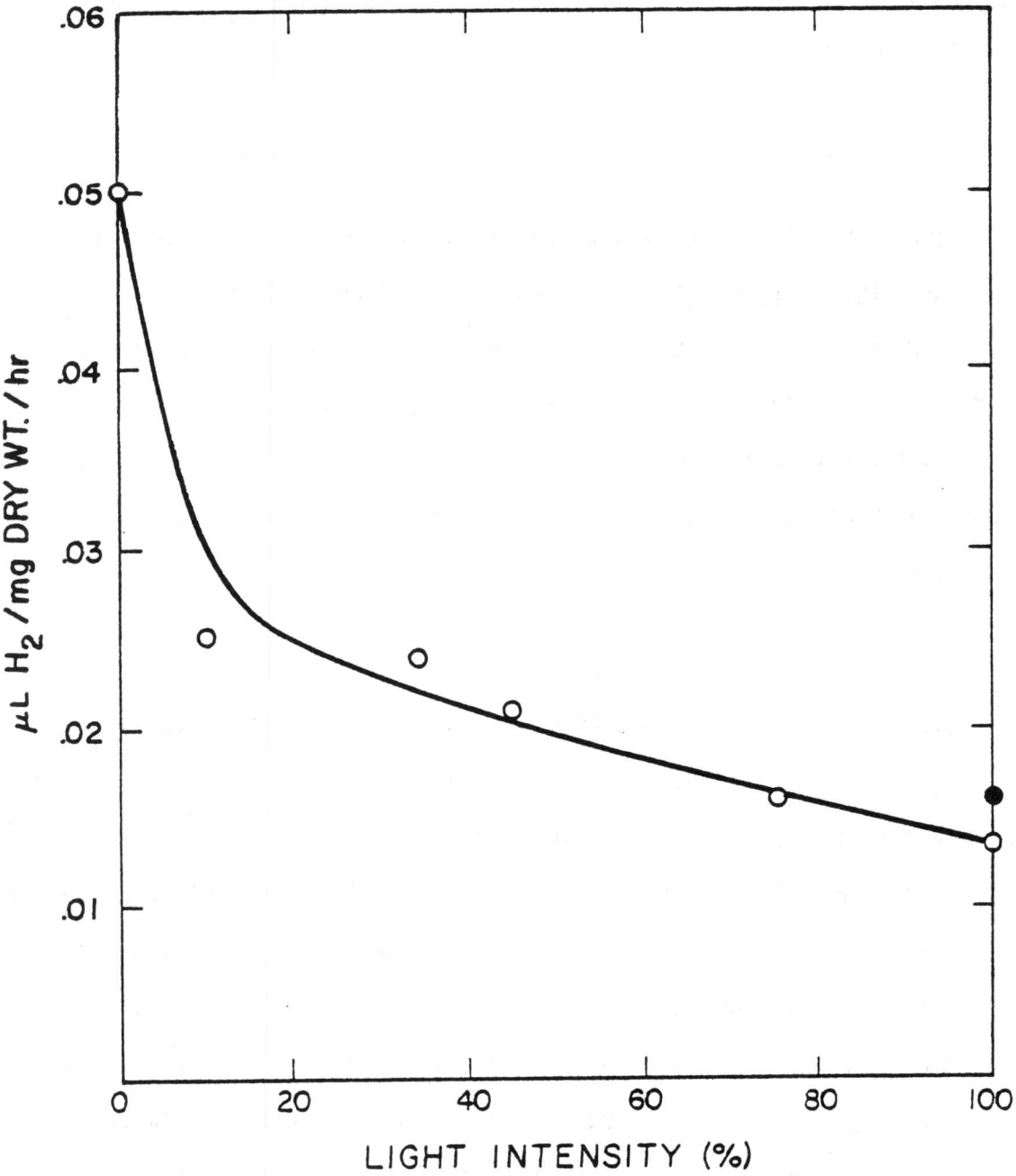

FIG. 4. Effects of varying light intensities on reversible hydrogenase activity in A. cylindrica. Symbols: O—Tungsten grown culture with dark acetylene reduction activity of .0005 μℓ C_2H_4/mg-h . One-hundred percent light intensity equaled 6 x 10^4 erg/cm^2/sec. ● + 2 x 10^{-5} M DCMU.

V. HYDROGEN PRODUCTION BY THE THERMOPHILIC ALGA, *Mastigocladus laminosus*: EFFECTS OF NITROGEN, TEMPERATURE, AND INHIBITION OF PHOTOSYNTHESIS

ABSTRACT

Hydrogen production by nitrogen-limited cultures of a thermophilic blue-green alga, *Mastigocladus laminosus*, was studied in order to develop the concept of a high-temperature biophotolysis system. Biophotolytic production of hydrogen by solar radiation was also demonstrated. Hydrogen consumption activity in these cultures was relatively high and is the present limiting factor on both the net rate and duration of hydrogen production.

INTRODUCTION

Biophotolysis--the production of hydrogen from water and sunlight by biological catalysts--has been the subject of numerous studies in recent years. Among the systems being developed, heterocystous blue-green algae have been the most intensively investigated (3, 11, 13-17, 20, 23). Under conditions of N_2-limitation, these algae can simultaneously produce oxygen (in vegetative cells) and hydrogen (in heterocysts) (23). Although there are both advantages and disadvantages to this approach (2, 5), it is the only biophotolysis system with sustained (up to four weeks) catalytic light conversion with conversion efficiencies of 0.1-0.2% outdoors and about 1.5% in the laboratory (11).

Development of a practical biophotolysis system will require the design of a low-cost culture and H_2 collector system, as well as selection of the most suitable strain of algae. *Anabaena cylindrica*, the alga used in

most previous studies, may not be the most appropriate organism for practical biosolar converters where the effects of natural conditions must be considered. One requirement of practical biosolar converters may be the resistance of a particular alga to high temperatures. Because of the lack of evaporative cooling and inadequate convective cooling, this would be an important consideration in the operation of a closed biophotolysis system.

Another reason for interest in thermophilic algae is their possible application in a dual converter--combining biophotolysis with water heating (with the algal culture as the working fluid), thus appreciably increasing the overall efficiency of the system. For the above reasons, hydrogen production by the thermophilic blue-green alga Mastigocladus laminosus was studied under both natural insolation and artificial light.

In our previous study (17), temperature effects on growth and nitrogen fixation were investigated, and hydrogen production by this organism was demonstrated.

In these initial experiments the sparged gas was switched at various growth phases, from air/CO_2 (99.5%, 0.5%) to various mixtures of argon/N_2/CO_2. Two to three days afterwards, peak rates of hydrogen evolution were observed. After reaching peak productivity, rates of hydrogen evolution usually declined. The peak rates obtained at various growth phases are shown in Figure 1 as a function of algal density. The rate of hydrogen evolution was roughly proportional to the algal density when exponentially growing cells were used for hydrogen production, and the volume specific rate reached its highest value when late exponential growth phase (Klett around 150) cultures were used. The conversion efficiency of light energy to hydrogen gas was calculated as 2.7% at the highest production rate.

During exponential growth, nitrogenase specific activity is maximal and rather constant. Decreased rates of hydrogen production at algal densities greater than Klett 150 reflect a lower *in vivo* specific activity of nitrogenase due, in part, to light limitation on energy supply to nitrogenase.

To investigate the environmental factors affecting hydrogen production, experiments on the effects of temperature, light, and the photosystem II inhibitor, DCMU [3-(3,4-dichlorophenyl)-1,1 dimethylurea] were carried out. Hydrogen production under outdoor conditions was also demonstrated. The results of these studies indicate that hydrogen consumption activity in these cultures is the present limiting factor on the net rate of hydrogen production.

MATERIALS AND METHODS

Organisms and culture methods. *Mastigocladus laminosus* NZ-86-m was obtained from Professor R.W. Castenholz, University of Oregon, Eugene, Oregon. The alga was grown on modified Allen and Arnon (1, 17) medium at 45°C ± 1°C. Cultivation vessels used were 1.7 l water jacketed columns, and culture methods were as previously described (17) except for illumination, which was supplied by eight UHO Vita-Lite fluorescent lamps through a transparent heat filter. The average light intensity on the side of the cultivation vessels was 3.0×10^4 erg/cm^2/sec.

Hydrogen production. For laboratory studies, *Mastigocladus laminosus* was grown initially on air/CO_2 until a density of around 0.2 mg/ml was reached. H_2 production was then initiated by N_2-limitation, i.e. sparging the cultures with a gas mixture (argon and CO_2) containing 0.2 to 0.4% N_2 at a gas flow rate of about 3 l/hr. Rather low algal densities were used in laboratory experiments to eliminate effects due to light limitation

(17). H_2, O_2, and N_2 were measured with a Model A-90-P3 Aerograph gas chromatograph equipped with a thermal conductivity detector and a column packed with molecular sieve 5-A. Argon was used as the carrier gas. The effluent gas lines of the culture vessels were connected via a gas sample valve to the gas chromatograph; this allowed the study of transients in H_2 evolution rates after changes in culture parameters.

Light and temperature shifts and DCMU addition. Light intensity was decreased by covering the culture vessel with gray filter films (Berkey Colortran, Berkeley, Calif.). This filter reduced the light intensity by two-thirds without any significant change in spectrum. A new steady-state temperature in the culture media was reached within 30 min after changing the circulating water temperature in the water jacket to the desired temperature. Where indicated, DCMU (3-(3,4-Dichlorophenyl)-1, 1-dimethylurea) 2×10^{-2} M in ethanol, was injected into the culture to bring the final concentration of DCMU to 2×10^{-5} M.

Acetylene reduction assays. Two ml samples from the algal cultures were injected into 6.9 ml serum-stoppered Fernbach flasks which had been previously flushed with argon. Flasks were vented to atmospheric pressure, preincubated in the light at 45°C for 10 min, and injected with acetylene gas (0.65 ml) after which they were again vented, rendering the gas phase 11% acetylene by volume. Where indicated, H_2 gas was injected into the flasks to a concentration of 20% by volume prior to preincubation. The flasks were then incubated in the light (3.3×10^4 erg/cm^2/sec) at 45°C for 20 min on a reciprocal shaker (Gilson Differential Respirometer, 160 rpm). Assays were terminated by the injection of 0.25 ml of 25% trichloroacetic acid solution. The ethylene produced was measured by injection of 100 µl gas samples into a Varian model 3700 gas chromatograph equipped with flame ionization dector and a 5' x 1/8" stainless steel column packed with Poropak R.

In situ acetylene reduction was also measured by sparging the cultures with a gas mixture containing 11% acetylene. 100 µl gas samples were withdrawn from the effluent gas tube and assayed as described above. One hour after switching the gas phase, a steady-state of ethylene production was observed. Ethylene volumes, together with gas flow rates, were used to calculate *in situ* acetylene reduction activity.

Hydrogen consumption assays. Two ml culture samples were injected into 6.9 ml serum-stoppered Fernback flasks which had been previously flushed with argon. After venting the flasks to atmospheric pressure, they were preincubated in the light at 45°C for 10 min, then 50 µl of H_2 was injected to initiate the assays. At 5 and 25 min after H_2 injection, gas samples were withdrawn from assay flasks with a 50 µl gas-tight syringe and injected into a Varian Model 3700 gas chromatograph equipped with a thermal conductivity detector and a 6' x 1/8" stainless steel column packed with a molecular sieve 5A. Lighting and shaking conditions were the same as described in the acetylene reduction assays.

Operation of the outdoor converter. Biophotolysis converters used for outdoor studies were one-liter capacity glass tubes of 5 cm diameter, placed at a 65° angle to the horizontal and facing southwest, and maintained at a constant temperature by a double-glass tube heat exchanger and a recirculating water bath. The cultures were initially grown outdoors on air/CO_2 until a density of 0.7 to 1.2 mg/ml was reached. H_2 production was initiated by sparging the cultures with argon/N_2/CO_2 (98.7%, 1.0%, 0.3% respectively). H_2 samples were collected by withdrawing 1.0 ml of gas from the effluent tube of the converter and were quantified by use of a gas chromatograph equipped with a thermal conductivity detector.

RESULTS

Effect of nitrogen supply. Figure 2 shows the typical effects observed when the N_2 supply to cultures of M. laminosus was changed. The culture was initially sparged with an argon/CO_2 gas mixture of fairly high N_2 content (0.7%). During this period, maximum H_2 production was observed. However, at the same time, considerable growth occurred, and the rate of H_2 production decreased significantly on the fourth day. The increase in algal cell density showed that N_2 supply was in excess. After the N_2 percentage of the gas mixture was decreased on the fourth day to 0.1%, growth creased and H_2 production was temporarily restored. After reaching a new peak of H_2 evolution on the sixth day, production decreased. Increasing the N_2 supply on the eighth day caused re-growth and a slight increase in O_2 evolution but not in H_2 evolution. This observation, confirmed by duplicate experiments, shows that N_2 supply has a subtle effect on the stability of H_2 production. In the subsequent experiments reported below, the N_2 concentration in the gas phase was held constant throughout the experimental period with either 0.2%, 0.3%, or 0.4% N_2 in argon, CO_2 (0.3% CO_2, balance argon). N_2 supply was empirically optimized for the present study in this manner. A typical H_2 evolution pattern for a culture of M. laminosus, maintained under a constant degree of N_2-limitation, is shown in Figure 3.

Effects of light and temperature shifts. In situ acetylene reduction activity was similar to activity obtained using the standard acetylene reduction procedure (which is carried out under saturating light). Thus, as shown in Table 1, light is not a limiting factor in the present H_2-producing system. However, when the light intensity was decreased to one-third of full intensity (3.0×10^4 erg/cm^2·sec), both H_2 and O_2 evolution rapidly decreased (to 40% and 20% of initial values, respectively) and new steady-states of gas evolution were reached within one hour.

The effects of cultivation temperature on growth rate and *in vivo* nitrogenase activity of *M. laminosus* were previously studied (17). Both growth rate and nitrogenase activity were found to be highest at around 45°C. The results of temperature shift experiments on H_2 evolution are shown in Figure 4. A shift from 45°C (normally used temperature) to 49°C increased H_2 production two-fold, while a shift to 54.5°C (just below the maximum tolerable temperature) first sharply decreased H_2 production and then resulted in a 50% increase over the initial production rate. At all temperatures below 45°C, H_2 production decreased, reaching a new steady-state production rate after about three hrs. Oxygen evolution decreased in most experiments, even at temperatures greater than 45°C (the new steady-state O_2 production rate at 54.5°C was only 50% of initial activity).

Effects of DCMU and carbon monoxide. The results of the temperature shift experiments described above suggested that increased H_2 evolution at elevated temperatures may be due to decreased O_2 evolution (and, hence, dissolved O_2 concentration) at those temperatures. To establish if decreasing O_2 concentration at the same temperature would also affect H_2 evolution, the effect of DCMU (3-(3,4-dichlorophenyl)-1, 1 dimethylurea), a photosystem II inhibitor, was studied.

DCMU was added to H_2-producing cultures at various times following the initiation of N_2-limitation. At the early stages of H_2 production (Fig. 5 A,B), DCMU stimulated H_2 evolution 100%, and even at the maximum production stage (usually 2-3 days after the initiation of N_2 limitation), H_2 production was stimulated by 50% (Fig. 5 C). Stimulation was much higher during the declining production phase (after 4 days) (Fig. 5 D). In all cases, DCMU stimulation was a relatively short-term phenomenon, with a significant decrease in H_2 production after 24 hrs, indicating that endogenous reductant pools are depleted within that time.

As expected, O_2 evolution was drastically decreased at all stages of the H_2 production phase by the addition of DCMU. Decreasing the O_2 concentration could stimulate nitrogenase activity by eliminating the competition between nitrogenase and O_2 scavenging processes for reductant (23). Alternatively, elimination of dissolved O_2 would decrease H_2 consumption activity which is dependent upon O_2 for maximal activity (7, 19, 22). The latter mechanism appears to be the most important. Acetylene reduction assays consistently gave a several-fold higher nitrogenase activity than did net H_2 production (Table 1), indicating the presence of an uptake hydrogenase. In addition, acetylene reduction activity of the cultures was not affected by short-term treatments with DCMU (Table 1).

Carbon monoxide inhibits most hydrogenases (18), including that of Anabaena cylindrica (12), but does not affect H_2 production by nitrogenase (3). A culture actively producing H_2 after two days of N_2-limitation was sparged with 11% carbon monoxide (balance argon, N_2, CO_2, 88.1, 0.3, 0.6%, respectively). This treatment, however, did not stimulate H_2 production; instead, a slight inhibition was observed (Fig. 6). Moreover, addition of DCMU in the presence of CO stimulated net H_2 evolution. Thus, the uptake hydrogenase activity in this alga appears to be carbon monoxide insensitive.

H_2 consumption was measured in 6.9 ml Fernbach flasks with 1% H_2 in argon (Table 2) as described in Materials and Methods. Under the light intensities used, H_2 was consumed. However, DCMU-treated (2 hr) samples evolved H_2. This is consistent with the stimulation of H_2 evolution observed in other experiments (Figure 5). With 5% O_2 in the gas phase, DCMU-treated samples also consumed H_2, but at a lower rate than non-treated samples, suggesting that this amount of O_2, added exogenously, was suboptimal. Thus, the net H_2 evolution observed in situ is the result of dynamic balance between H_2 evolution (by nitrogenase) and H_2 consumption (by an uptake hydrogenase).

by a culture of Mastigocladus laminosus NZ-86-m maintained at 33-47°C in an outdoor biophotolysis converter receiving natural insolation. Daily insolation data were obtained by integrating the output of an Eppley Model 848 pyranometer. Average insolation during the period of the experiments was 4.4×10^5 erg/cm^2·sec (or 501 Langleys/day). The photosynthetic efficiency of conversion of incident solar energy to free energy of H_2 was 0.17%. At gas flow rates presently being used (average rate of 6.5 l/hr), the effluent gas phase contained about 0.16% H_2.

Average rates of H_2 production, either per liter of culture (10.0 to 16.3 ml/l·hr) or per mg dry wt (6.4 to 13.2 µl/mg·hr) were about one and one-half-fold higher than previously obtained in similar experiments with Anabaena cylindrica (11). However, the daily H_2 evolution data were rather erratic due to considerable changes in operational variables such as temperature, pH, insolation, and, particularly, gas flow rate.

DISCUSSION

The low conversion efficiency (0.1%-0.2%) observed in outdoor experiments is the limiting factor in the development of a practical biophotolysis system. Although the efficiency was 2.7% under laboratory conditions (17), H_2 evolution was still only 20% of the acetylene reduction activity of this alga. If the acetylene reduction activity, shown in Table 1, could be actualized as net H_2 produced, then two liters of H_2 per m^2 of converter area per hr would be produced. The laboratory experiments described here were carried out to elucidate in more detail the causes of low H_2 production.

It has been speculated that reductant supply to nitrogenase may limit nitrogenase activity in slowly growing ("old") cultures (4) and also in N_2-starved cultures (23) of Anabaena cylindrica. With N_2-limited cultures of M. laminosus, reductant limitation was not the critical limitation because The presence of O_2 shifts this balance towards increased consumption of H_2, thereby lowering net H_2 evolution.

Outdoor hydrogen production. Figure 7 and Table 3 show H_2 production

1) in the short term, the photosystem II inhibitor, DCMU, stimulated H_2 evolution instead of inhibiting it (Figures 5 and 6); 2) acetylene reduction activity of N_2-limited cultures of M. laminosus was not enhanced in the presence of 20% H_2 in argon. Thus, when the cultures were N_2-limited (i.e. supplied with a low, continuous amount of N_2), the supply of reductant did not limit H_2 production. The response of H_2 and O_2 evolution to a decrease in light intensity was, however, substantial, indicating that H_2 evolution may be limited by ATP produced via photophosphorylation.

Addition of DCMU to N_2-limited cultures significantly increased the net H_2 evolved, as has been noted for Nostoc muscorum (20). There are several possible explanations for this effect: Lowering the O_2 tension could increase H_2 evolution by relieving O_2 inhibition of the nitrogenase reaction. However, this is not a significant factor since acetylene reduction activities were not changed by DCMU additions. H_2 recycling occurs in Anabaena cylindrica and some other blue-green algae (7, 8, 9, 21). Thus, DCMU additions may stimulate H_2 evolution by directly inhibiting H_2 consumption or by limiting (through decreased O_2 levels) the oxy-hydrogen reaction catalyzed by hydrogenase. DCMU is probably not directly inhibitory to H_2 consumption since the DCMU effect could be partially relieved by the addition of O_2.

The large discrepancy between acetylene reduction and net H_2 production (Table 1) indicates an active H_2 consumption by M. laminosus. This difference cannot be attributed to differences in light intensities and other variables between the two assays since in situ acetylene reduction was also five-fold higher than net H_2 evolution. (As expected, in situ H_2 evolution was inhibited by acetylene, declining to one-fourth of the initial value.) It is possible that the low concentration of N_2 in the gas phase resulted in part of the photosynthetically produced reductant being consumed in N_2 fixation. However, even completely N_2-starved cultures showed the same response to DCMU additions,

and H_2 evolution by these cultures was not higher than that by N_2-limited cultures.

H_2 production by N_2-limited cultures of Anabaena cylindrica has been previously reported (11, 14, 23). To compare the H_2 consumption activity of this alga with M. laminosus, DCMU was added to H_2-producing cultures of A. cylindrica in similar experiments to the ones given in detail here. In these cultures DCMU stimulated H_2 production by at most 25%, in contrast to the 50 to 250% increase observed with M. laminosus cultures. Also, the ratio of acetylene reduction to H_2 production is close to unity in A. cylindrica (23), whereas this ratio is five to one in M. laminosus. Thus, there is a significant difference in the H_2 consumption activity of the two algae grown and induced under the same culture conditions. H_2 consumption activity may also vary with culture conditions such as algal density, light intensity, and degree of N_2-starvation. Thus, under some conditions, A. cylindrica may also exhibit high rates of H_2 consumption and, therefore, low net H_2 production (6).

Experiments with a biophotolysis reactor receiving natural insolation resulted in low photosynthetic conversion efficiencies and very variable H_2 production, probably because of poor control of operational variables (Figure 7 and Table 3). Some of the occasional decreases in H_2 evolution were attributable to algal clumping due to poor mixing (decreased gas flow) or to changes in culture temperature, with lower rates at lower temperatures as confirmed by temperature shift experiments (Figure 4). Other variables, such as N_2 supply, pH, and algal density affect both the rate and stability of H_2 production. However, these variables (except for natural insolation) are subject to better control and more stable production can be expected. In fact, less variable production data were obtained in the laboratory studies with M. laminosus reported here.

A several-fold improvement in conversion efficiency of H_2 production can be expected if the H_2 consumption activity is regulated or inhibited. Since bacterial hydrogenases (18) and the hydrogenase from A. cylindrica are inhibited by carbon monoxide (12), CO was used with a N_2-limited culture of M. laminosus in an attempt to obtain an enhancement in net H_2 production. However, 11% CO did not increase H_2 evolution (Figure 6), and DCMU stimulation was also observed in the presence of CO. Lower concentrations of CO also had no effect in H_2 consumption experiments. To increase H_2 production by this alga, another method of controlling H_2 consumption activity must be found.

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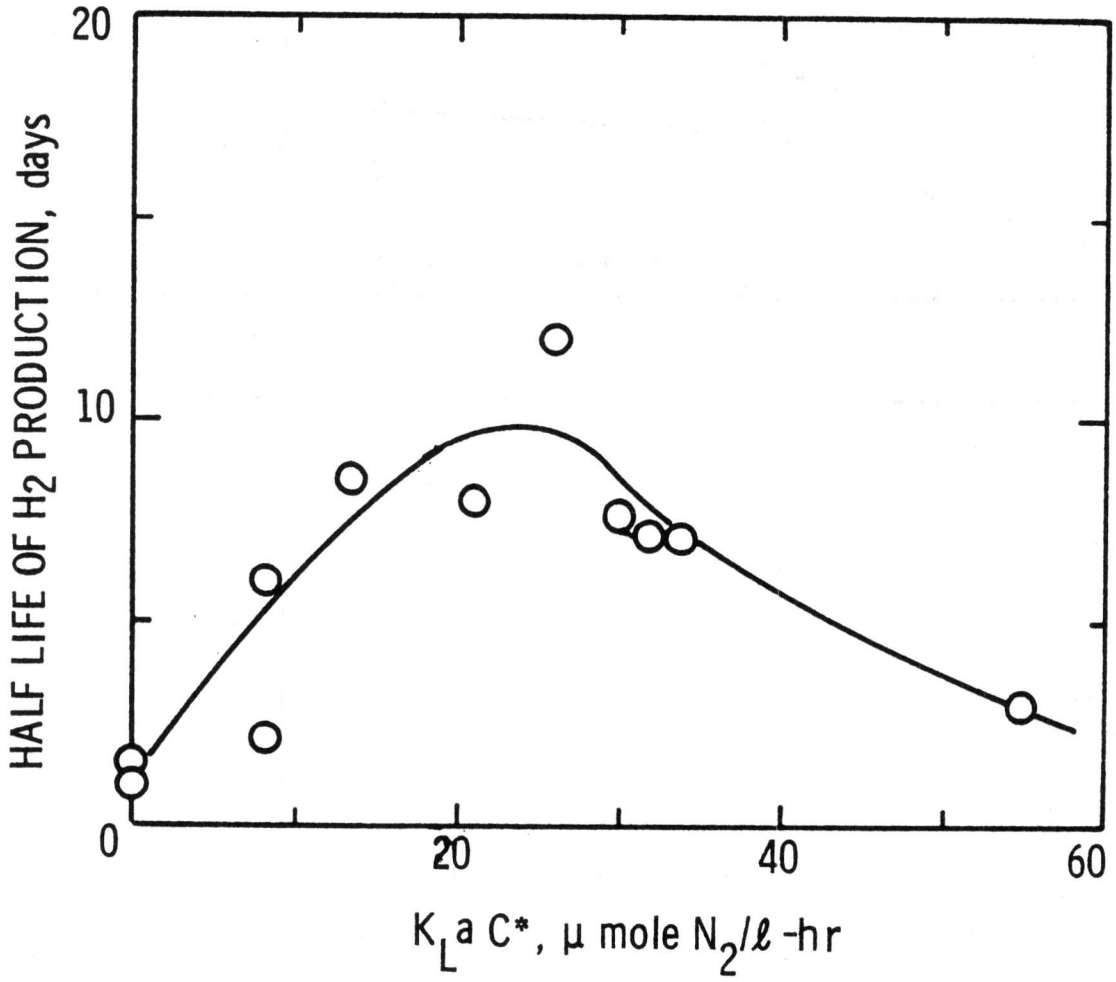

FIGURE 1. HYDROGEN PRODUCTION BY M. laminosus NZ-86-m AT DIFFERENT CELL DENSITIES.

Algal cells grown at an increased light intensity (3.0×10^4 erg/cm^2.sec) were sparged with various mixtures of argon/N_2/CO_2 at various growth phases. Percentages of N_2 were varied from 0.1 to 1.0%, while CO_2% was 0.5%. Peak rates of hydrogen evolution were observed 2 to 3 days after the initiation of nitrogen limitation and are shown as a function of algal density at that time

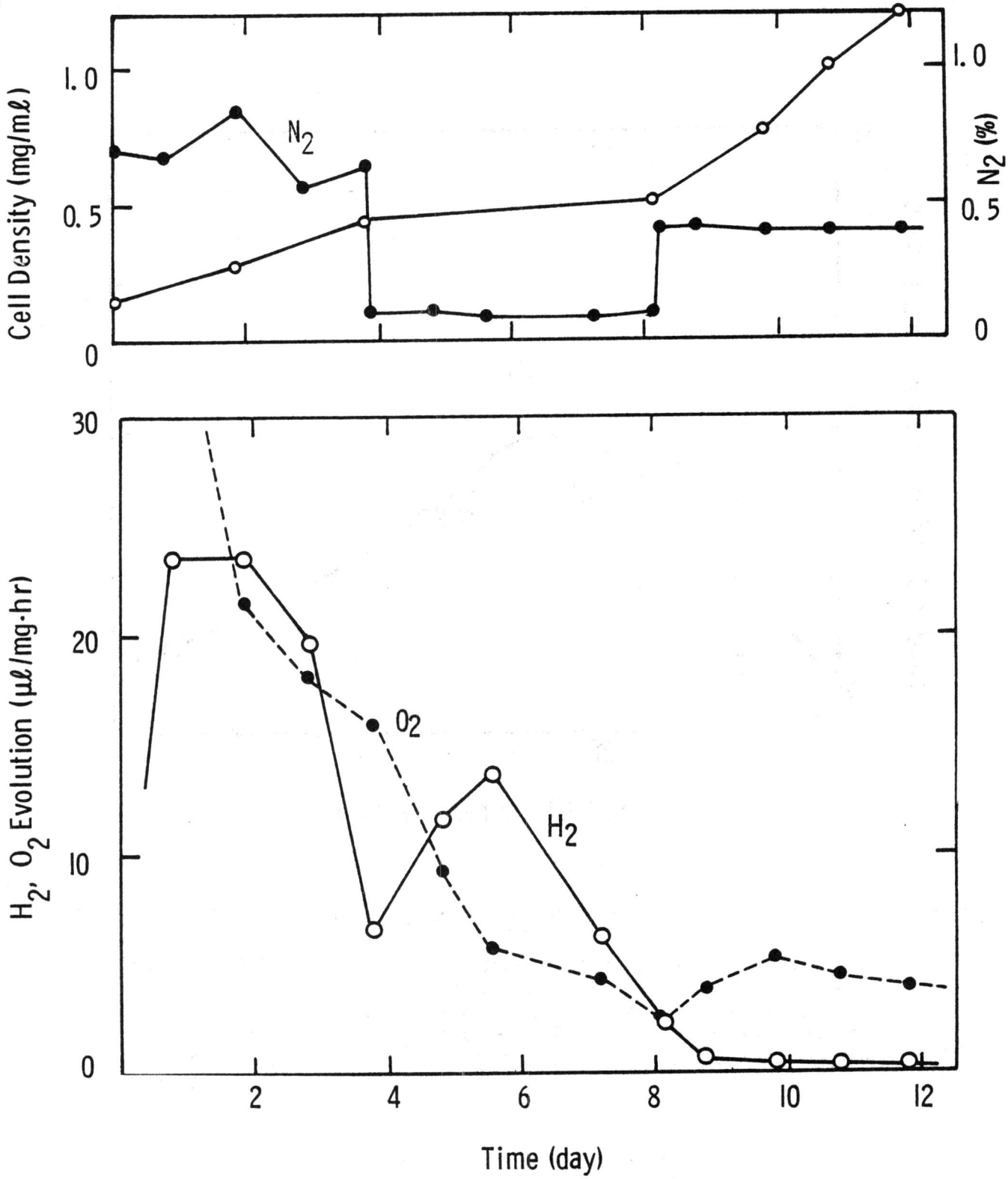

FIGURE 2. Effect of nitrogen supply on hydrogen evolution (bottom) and growth (top). The nitrogen content of the gas phase is indicated in the top figure (closed circles); algal cell density is also shown in the top figure (open circles). Hydrogen (open circles) and oxygen (closed circles) evolution are shown in the bottom figure.

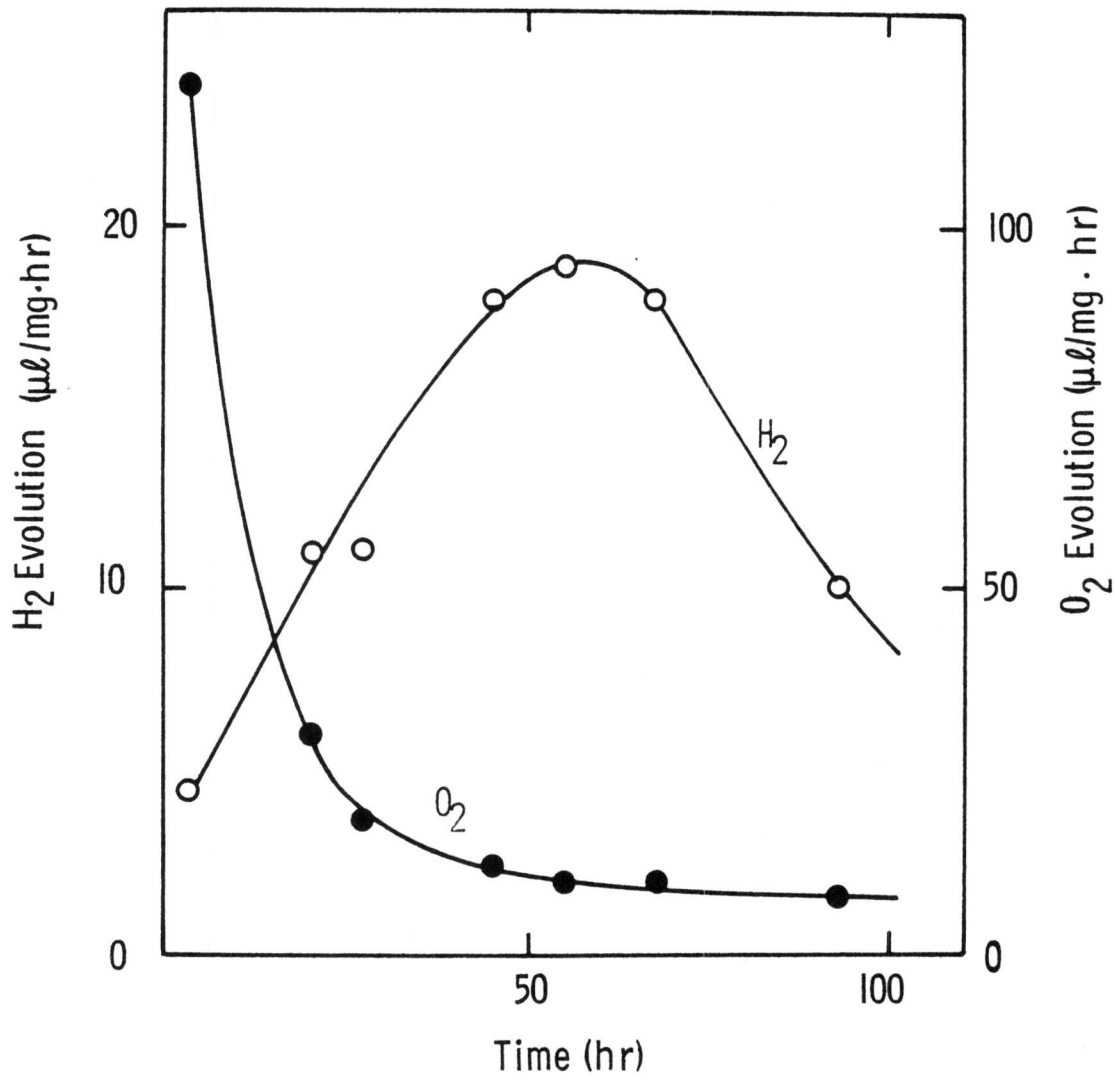

Figure 3. Typical pattern of hydrogen and oxygen evolution by nitrogen-limited cultures of M. laminosus.
Nitrogen limitation was initiated at time zero; the nitrogen content of the sparging gas was kept constant at 0.4% throughout this experiment. Algal cell density increased from 0.23 mg/ml at 20 hr to 0.36 mg/ml at 93 hr.

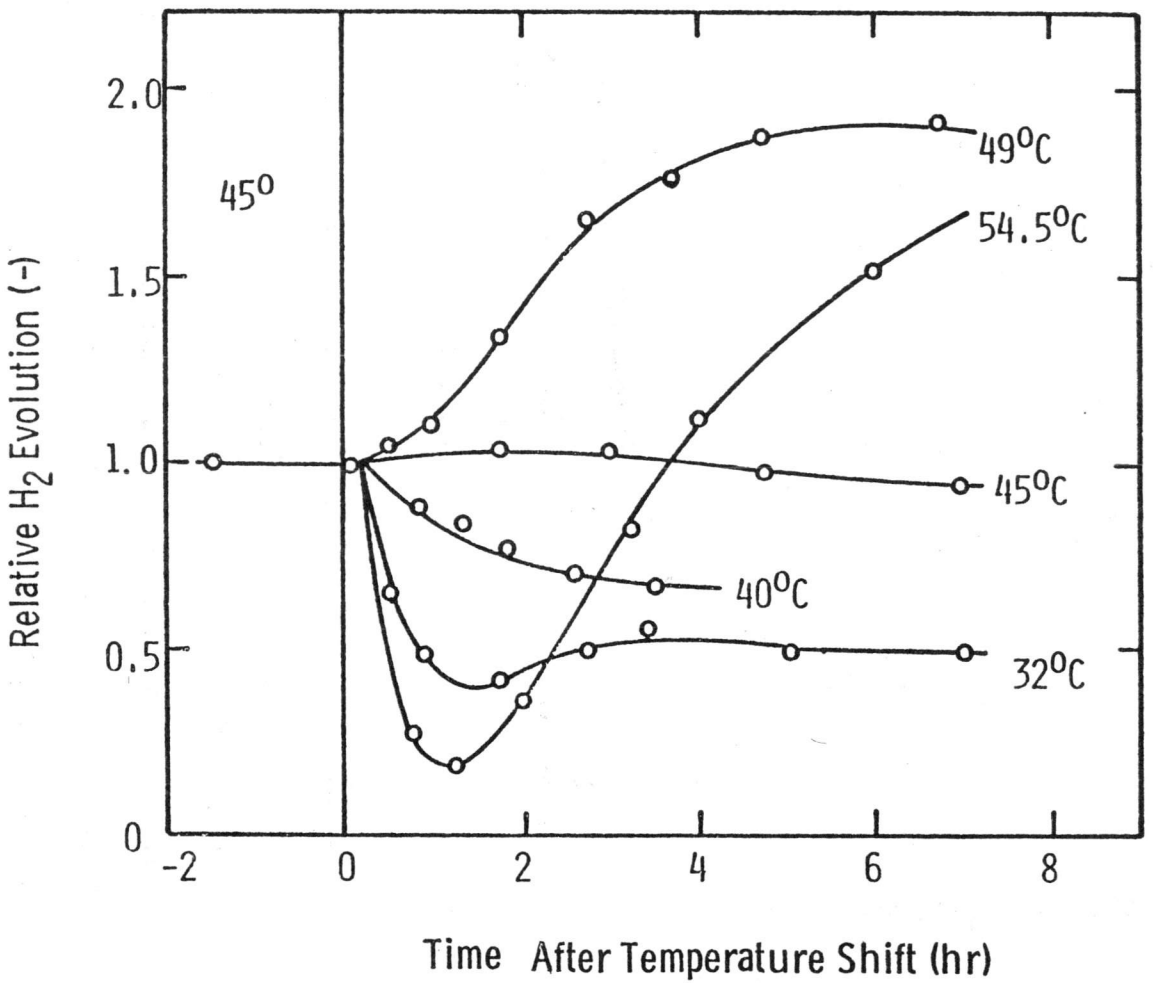

Figure 4. Short-term effect of temperature shift on hydrogen production. The temperature of hydrogen producing cultures was shifted from 45°C to the indicated temperatures at time zero. Algal cell density was 0.3 ~ 0.4 mg/ml.

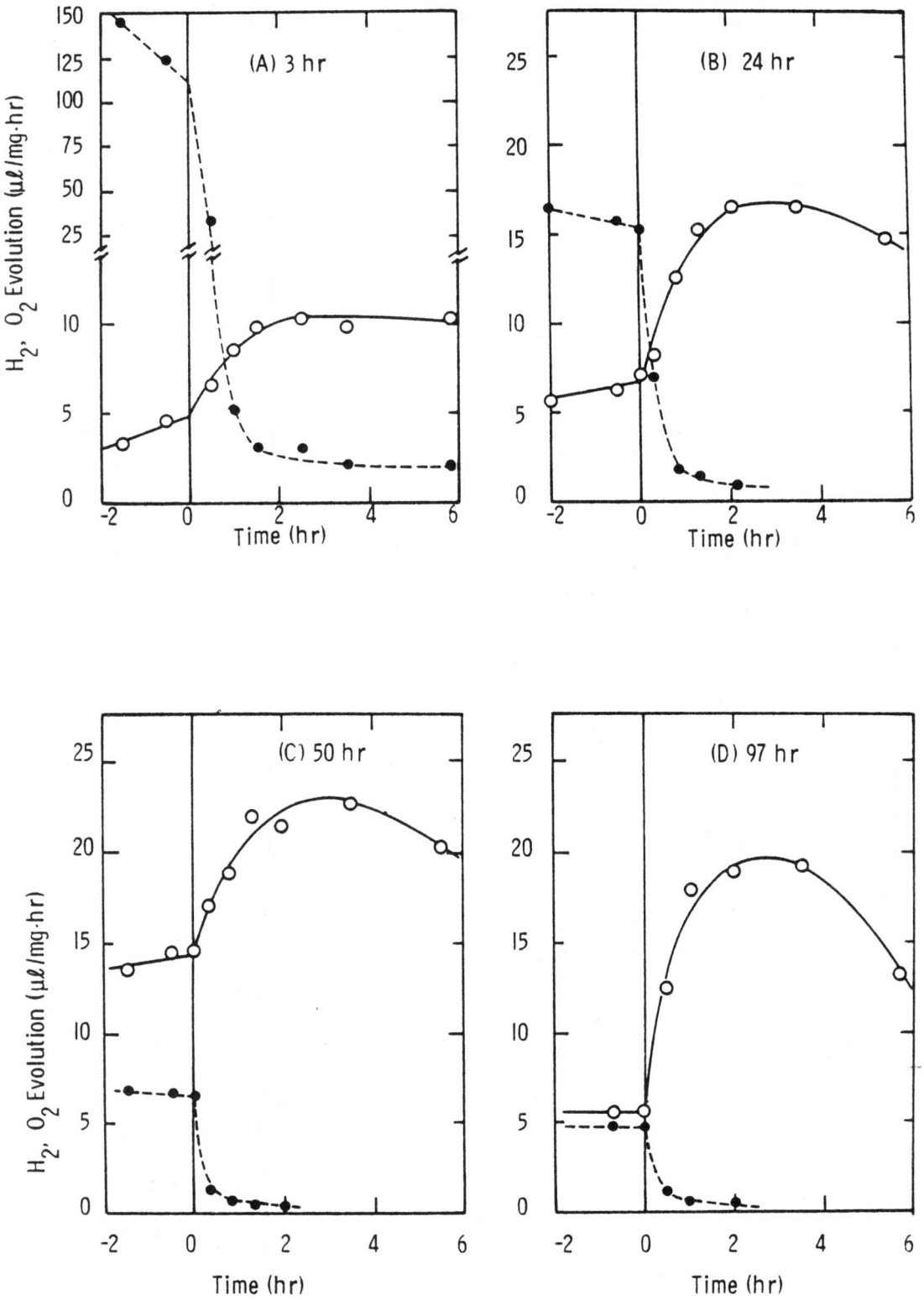

Figure 5. Effect of DCMU on hydrogen and oxygen evolution. Four cultures were injected with DCMU after different periods of nitrogen limitation: (A) 3 hr, (B) 24 hr, (c) 50 hr, and (D) 97 hr. Final concentration of DCMU was 2×10^{-5} M. Each figure shows hydrogen (—o—) and oxygen (—●—) evolution.

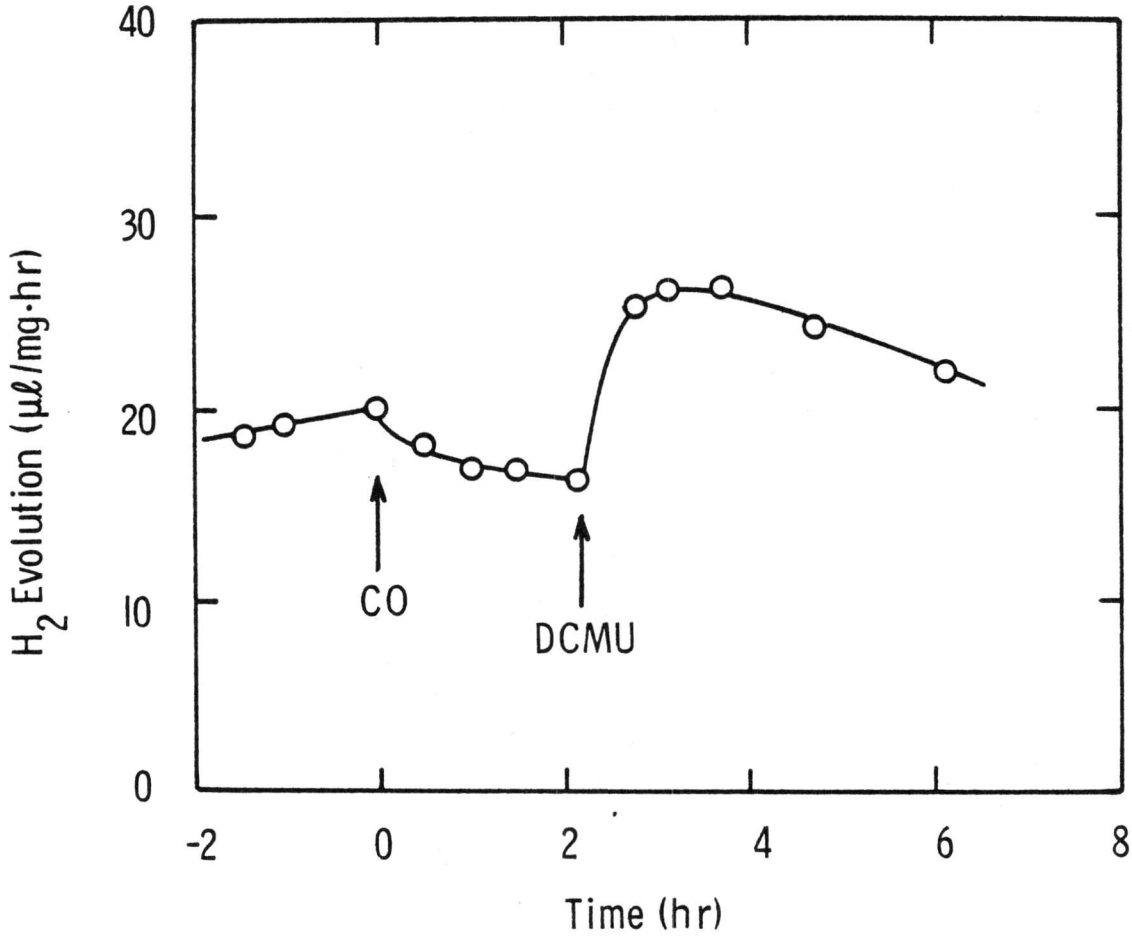

Figure 6. Effects of carbon monoxide and DCMU on hydrogen evolution. After two days of nitrogen limitation, carbon monoxide was included in the gas phase at a concentration of 11% (first arrow). Two hours later, DCMU was added to the culture (second arrow).

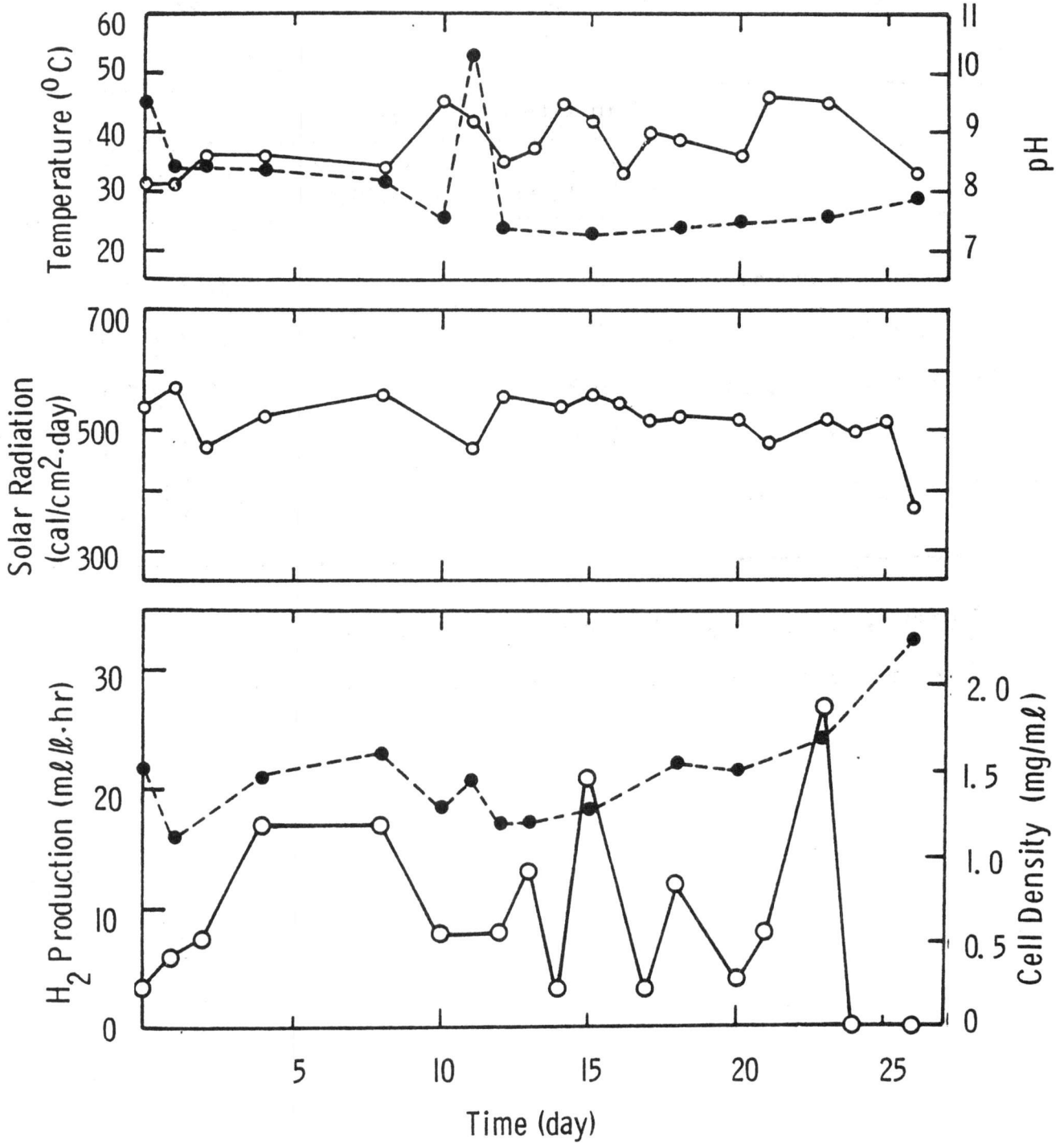

Figure 7. Hydrogen production by an outdoor culture. Mastigocladus laminosus was initially grown on air/carbon dioxide (99.7%, 0.3%). Hydrogen production was initiated by sparging the cultures with argon/nitrogen/carbon dioxide (98.7%, 1.0%, 0.3%). Bottom: hydrogen production (solid line) and algal cell density (dashed line); middle: daily insolation; top: culture temperature (solid line) and pH (dashed line).

Table 1. Acetylene reduction, hydrogen and oxygen evolution by nitrogen limited cultures of *M. laminosus*; the effect of DCMU

Cultures	*in situ* activities		C_2H_2 reduction (μl C_2H_4/mg·hr)
	H_2 evolution (μl H_2/mg·hr)	O_2 evolution (μl O_2/mg·hr)	
3-hr N_2-limitation	4.5	32.0	31.2 ± 3.8
" , + DCMU[a]	9.7	3.0	31.0 ± 0.6
50-hr N_2-limitation	9.3	3.4	49.8 ± 3.3
" , + DCMU	17.6	0.3	50.2 ± 1.6
47-hr N_2-limitation	--	--	57.4 ± 2.6 (50.8 ± 1.0)[b]

[a] +DCMU, samples assayed 1.5 hr after DCMU addition.

[b] Parenthesis indicates *in situ* acetylene reduction activity.

Table 2. Hydrogen consumption by a nitrogen-limited culture of *M. laminosus*; effect of DCMU

Treatment[a]	H_2 consumption ($\mu l/mg \cdot hr$)
none	13.6
DCMU, 2 hr	-14.1
DCMU, 2 hr Assayed with 5% O_2	7.5

[a] A N_2-limited culture (2 days) was assayed as a control, and 2 hr after DCMU addition, samples from the same culture were assayed with and without 5% O_2 in the gas phase. All assays were under light. Cell density was 0.38 mg/ml.

Table 3. Hydrogen production by outdoor cultures of M. laminosus

Culture	Cell density range (mg/ml)	Temp range (°C)	Duration of H_2 production (days)	Average H_2 production		Total H_2 production (ml H_2/l)
				(ml/l·hr)	(µl/mg·hr)	
RT 22	1.6-2.4	33-47	24	10.8	7.5	3100
RT 23	1.3-1.8	31-45	18	10.0	6.4	2160
RT 24	0.6-1.2	33-50	17	16.3	13.2	3330

VI. SOLAR ENERGY CONVERSION BY NITROGEN-LIMITED CULTURES OF *Anabaena cylindrica*

INTRODUCTION

Due to recent increase in costs of energy from conventional resources, a diversity of solar energy technologies are being developed. Biophotolysis is one of the approaches through which H_2 , a non-polluting fuel, could be produced by means of solar radiation. Among the different types of biophotolysis systems proposed (1), the most advanced system is based on the use of nitrogen-limited cultures of heterocystous blue-green algae (2,3,4,5,6,7).

In this system, the H_2 and O_2 evolving reactions are separated in the microscopic level in the heterocysts and vegetative cells of these filamentous algae. Thus, O_2 inhibition of H_2 evolving enzymes, the key problem in biophotolysis, is overcome. Using nitrogen-limited cultures of *Anabaena cylindrica*, H_2 evolution was demonstrated and sustained, both in the laboratory and outdoors for periods of up to 4 weeks at relatively low efficiencies (2,3). Recent studies (4) increased the conversion efficiency of light energy to free energy of H_2 to approximately 3% under laboratory conditions. This is about one-quarter of the maximal photosynthetic conversion efficiencies to algal biomass achieved during similar laboratory experiments with other algal cultures (8).

Some critical problems inherent to this system have been pointed out: The inefficiency of the nitrogen-fixing enzyme system on which the biophotolysis reaction depends, low photosynthetic activity in nitrogen-limited cultures and the induction of a H_2 consumption activity which decreases net H_2 production under some nitrogen-limited conditions (9,10,11). Here we report a study of these problems with nitrogen-limited cultures of *Anabaena cylindrica* operated outdoors during the winter of 1978.

RESULTS

Daily production pattern. Unlike the biophotolysis reaction carried out in the laboratory under continuous illumination, under outdoor conditions, the biophotolysis reaction gives a discontinuous production of H_2 because of the diurnal cycle of insolation. Fig. 2 shows a daily production pattern which was obtained from several measurements during a sunny day and used to calculate daily total H_2 production. This pattern was deformed depending on daily radiation. It should be noted that the H_2 production activities responded very rapidly to the start of sunshine in the morning, reaching a maximum within 30 min of direct sunlight shining on the converter. A similar, reverse pattern was observed at sunset. Clouds passing overhead had only minor effects.

Figure 2 also shows the effect of acetylene gas on H_2 production: inclusion of acetylene (11%) in the gas phase caused a sharp inhibition of H_2 production which was reversed upon elimination of acetylene. This demonstrates that nitrogenase was responsible for the majority of the H_2 evolution in the present biophotolysis system.

Effect of algal cell loading. Even in winter, solar radiation is much higher than laboratory illumination employed in previous studies (2,3,4,10). Algal cell loading was first optimized under natural insolation. Fig. 3 shows H_2 production in the outdoor converter as a function of cell density. Around two mg/mℓ of culture was the best loading. Although only a single experiment was carried out at a high cell concentration (above 3 mg/ℓ), inhibition of H_2 evolution at high cell densities was also noted in the laboratory. This inhibition would be expected from increased maintenance energy and light attenuation effects at high cell densities. Fig. 4 shows H_2 and O_2 production at the optimum loading; H_2 production for up to 5 weeks was demonstrated. Daily

MATERIALS AND METHODS

Organism and culture method. Anabaena cylindrica 629 was grown aerobically in a 45-liter-tank illuminated on both sides by six 40-watt fluorescent bulbs. Media and other cultivation methods were similar to our previous paper (4). When the culture had reached a density of about 0.35 mg/mℓ, the culture was transferred to outdoor converter columns, in most cases, after concentration by screening or sedimentation. Induction of heterocysts was initiated by sparging with N_2/CO_2/argon (0.2-0.4%, 0.5%, balance). H_2 production was observed within 1 to 4 days after the transfer, depending upon both cell density and solar radiation. Culture variables, except solar insolation, were kept constant during outdoor operation: temperature = 26.5 $\pm$ 1.5°C, pH = 7.5 $\pm$ 0.5, and gas flow rate = 5.0 $\pm$ 0.5 ℓ/hr. No effort was made to keep the outdoor cultures sterile, but microscopic observations were made to confirm the dominance of Anabaena cylindrica. All experiments were done during the winter season, from November 6 to December 16, 1978.

Converter. The outdoor converter was an array of one-liter glass columns (5 cm in diameter, with 0.8 liters of working volume) and was placed at a 35° angle to the horizontal facing south (Fig. 1). Daily total solar radiation data were collected with an Epply Model 848A pyranometer and a recorder.

Gas analysis. H_2 , O_2 , and N_2 in the effluent gas lines of the converter were measured simultaneously by gas chromatography, as previously described (12). Acetylene reduction and H_2 consumption activities were measured under an artificial illumination of 3.3×10^4 erg/cm^2·sec (12). Total H_2 production for each day was calculated by measuring H_2 production four times a day and integrating by triangular approximation.

total solar flux is also shown in the upper part of Fig. 4. The whole production phase may be divided into three parts, based on the H_2/O_2 ratio: 1) a high H_2 production period which appeared initially after the induction phase, 2) a phase in which H_2 and O_2 were produced equally for a rather long period, and 3) after 30 days, O_2 evolution became greater than H_2 production.

We interpret these three phases as follows: the initial phase of high H_2 and low O_2 production follows the adaptation of the culture to N_2 deficiency. This results in an increase in reductant stored in the cells and an almost total elimination of photosynthesis by phycocyanin breakdown, resulting in high H_2/O_2 ratios for several days. After this, photosynthesis recovers somewhat and H_2 production declines due to reductant limitations, resulting in a period of matched (though not stoichiometric) rates of H_2 production. Finally, in phase 3, H_2 production declines before O_2 production does due to causes not yet established. (Filament breakage and metabolite accumulation may be causes.)

A comparison between daily solar flux and H_2 and O_2 production shows that, as would be expected, O_2 production correlates better with decreases or increases of solar energy than H_2 production. A closer analysis shows a lag of one to two days between H_2 production and changes in solar flux. This is most clearly seen during the latter part of the experiment (day 17 and 25). Similar observations were made with the other cultures that had non-optimal cell densities.

Conversion efficiency. Daily H_2 production rates by cultures of different densities during the first high production period are correlated with daily solar radiation in Fig. 5. In a low-density culture (0.4 mg/mℓ) where nitrogenase activity was limiting, H_2 production was independent of solar radiation. In the optimally loaded (1.6 mg/mℓ) culture, however, H_2 production was proportional to solar radiation up to approximately 150 cal/cm^2 · day. A

maximum conversion efficiency would be attained during these low radiation days in the high-production period. The highest value of conversion efficiency obtained was 0.6% and the average efficiency over the 36-day experimental period was 0.2% for the culture shown in Fig. 4.

It must be recognized that the high initial rates of H_2 production which are not accompanied by stoichiometric amounts of O_2 production are, in part, due to previously accumulated reductant. Thus, at 1.6 g/ℓ of dry weight algal biomass, if 70% were in the form of a carbohydrate reductant decomposed to H_2 and CO_2 in the heterocysts, this could support H_2 production at 100 mℓ H_2 /ℓ/day for about 5 days. This is within the time range observed in these experiments. On the other hand, during the latter phases of the experiment, the higher O_2 production rates indicate that not all the photosynthetic reductant produced is transformed to H_2. Careful calorimetric measurements would be required during this phase of the experiments to determine the true solar conversion efficiency of the system.

Limiting factors in H_2 production. The higher conversion efficiencies observed in the laboratory under controlled conditions of continuous illumination led to an examination of possible factors limiting the outdoor biophotolysis system. Acetylene reduction, H_2 production, and H_2 consumption of three outdoor cultures at different phases were measured under artificial saturating light at the same time. The results are shown in Table 1 with the available in situ data included. In the high-rate H_2-producing culture in the first phase, specific acetylene reduction was much higher than H_2 production. However, specific activity of nitrogenase decreased as the cultures get older (phases 2 and 3), and H_2 production by old cultures consequently declined with age. H_2 consumption activity of those cultures was also measured in the dark with 1.2% O_2 and 1.0% H_2 in the gas phase. The results are shown in the last column

of Table 1. H_2 consumption was at most 10% of net H_2 production. Therefore, H_2 consumption activity in cultures of A. cylindrica was, unlike M. laminosus (10), not an important factor in low net H_2 production during the latter phase.

H_2 consumption might be a significant factor during the early phases of H_2 production since there was an almost two-fold difference in H_2 production and acetylene reduction (Table 1). However, H_2 consumption assays indicated only a minor H_2 consumption activity. In the latter phases of the experiment, a distinct shortening of filaments was observed in the cultures, indicating filament breakage which takes place preferentially at the heterocysts, as a factor in the decline of H_2 production.

Another factor implicated in the decline of H_2 production after the first phase or "burst" of H_2 production is reductant limitation. That reductant limitation could be a significant factor in reducing H_2 evolution is seen from the experiment shown in Fig. 6 which follows H_2 evolution after addition of DCMU (a potent photosystem II inhibitor). During the early stage of H_2 production, DCMU is rather ineffective in inhibiting H_2 production in the short term, while one day later the DCMU has significant short-term effects. Cultures from the latter phases of the experiment were even more susceptible to DCMU. This supports the suggestion that the initial high rates of H_2 production are due to stored reductant, while in later phases of H_2 production, reductant becomes limiting.

DISCUSSION

Biophotolysis by N_2-limited cultures of heterocystous blue-green algae is the only one in an advanced stage of development. Nevertheless, many critical bioengineering and physiological problems remain to be solved (1). The key limiting factor in this system is the rather low efficiency of solar energy conversion observed in the outdoor cultures. The presence of an uptake hydrogenase activity that lowers net H_2 production rates (9,10) under some conditions,

particularly with thermophilic blue-green algae (10) was not found to be important in the outdoor culture of A. cylindrica, as H_2 uptake activity was at most 10% of net H_2 evolution throughout prolonged periods of N_2 limitation. Thus, other factors must account for the low efficiency observed outdoors.

Through the optimization of N_2 supply (4) and algal cell loading in the converter, the conversion efficiency was improved in this work, but was still lower than the efficiency attained in the laboratory converters. In our most recent study (4), the average efficiency was 2.5% over 15 days of continuous artificial illumination. Since the same cultivation methods were employed in both laboratory and outdoor experiments and all operational variables except natural insolation were controlled, the differences in illumination are the key factors in the low conversion efficiency

The two key differences in the outdoor illumination was its diurnal and variable nature and its much higher, almost ten-fold, intensity. The rapid and smooth response to diurnal variations in solar insolation suggest that the cultures can cope with variable insolation fairly well. Thus, we identify the high natural insolation as the crucial factor. Indeed, blue-green algae are known to be, in general, better adapted to lower light environments than green algae (13). Although the problem of photooxidation would not be significant under the conditions of low O_2 tensions and pigmentation present in the cultures, photosynthesis may well be best adapted to low light intensities. Indeed, the optimal culture density exhibited maximal rates of H_2 production at 150 cal/cm^2/day, a rather low insolation. This corresponds also to the observation that, even in the laboratory, conversion efficiencies declined as light intensities were increased.

The data presented above also suggests additional factors for the relatively low overall rates of H_2 production (compared to O_2 evolution): decline in nitrogenase activity and reductant supplies which are probably due to a combination of filament breakage and breakdown of the vegetative cell-heterocyst system. It appears likely that significant increases in rates of H_2 production and solar conversion efficiency could be achieved with this biophotolysis system. This will require a continued study of the limiting factors in N_2-limited cultures.

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Table 1. Comparison between C_2H_2 reduction and H_2 production

Culture	Culture age (days)	Culture density (mg/ml)	C_2H_2 reduction (µl/mg · hr)	H_2 production[b] (µl/mg · hr)	H_2 consumption in the dark[c] (µl/mg · hr)
A4-2	8	1.47	18.4	9.0(7.5)[a]	0.6
A5-1	26	1.54	5.1(7.7)[a]	4.8(5.4)	0.3
A3-1	37	1.75	1.7	ND(1.8)	0.2

[a]Values in parenthesis represent acetylene reduction or hydrogen production activity measured under outdoor conditions.

[b]Hydrogen production under indoor conditions was measured for 30 min with 1.0% H_2 in argon atmosphere.

[c]Hydrogen consumption activity was measured in the dark with 1.2% O_2 and 1.0% H_2 in tne gas phase.

ND not determined.

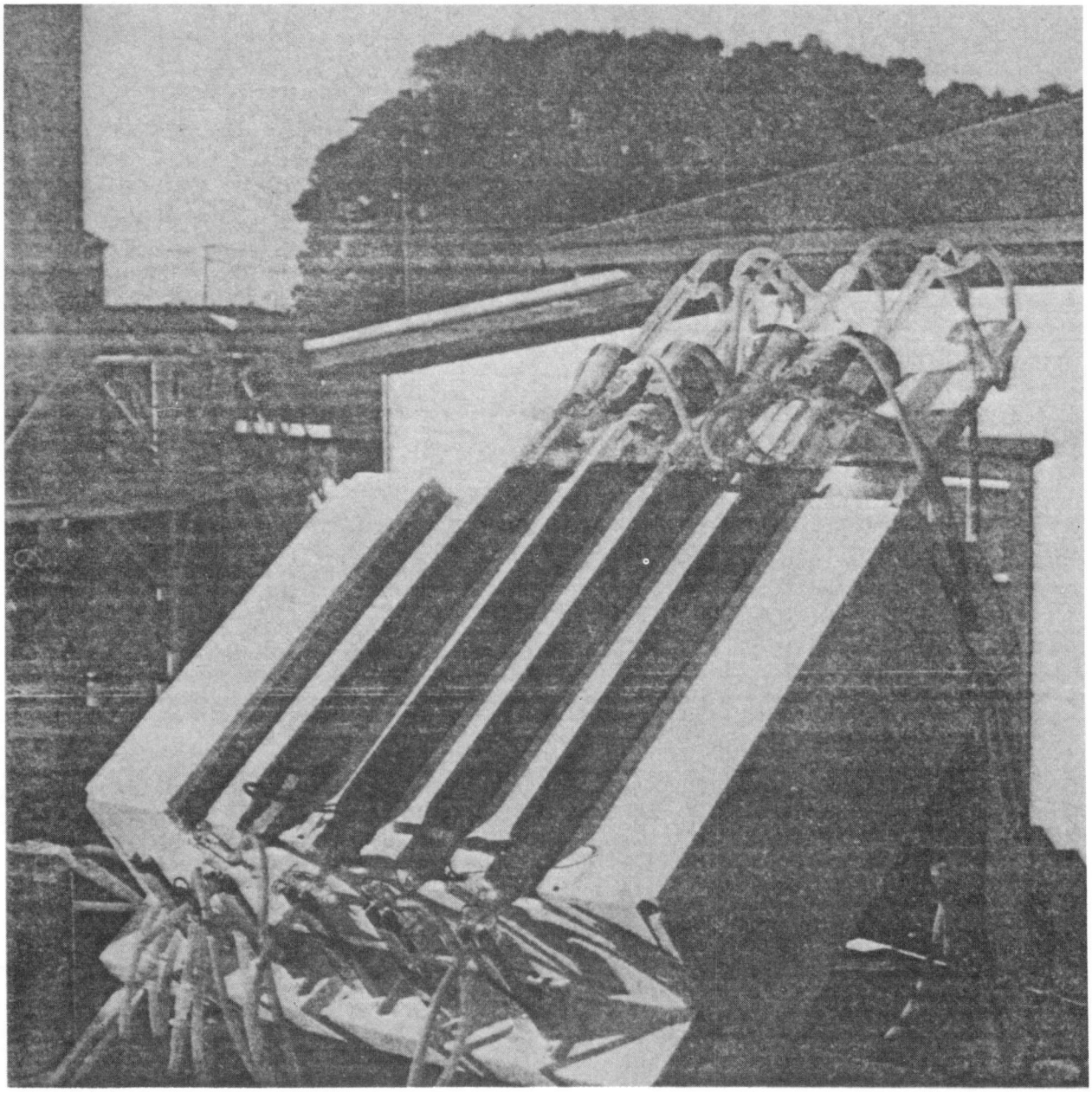


FIGURE 1. EXPERIMENTAL OUTDOOR BIOPHOTO-CONVERTER

The back half surface of the columns was enclosed by a dark box to simulate a close packed array of columns.

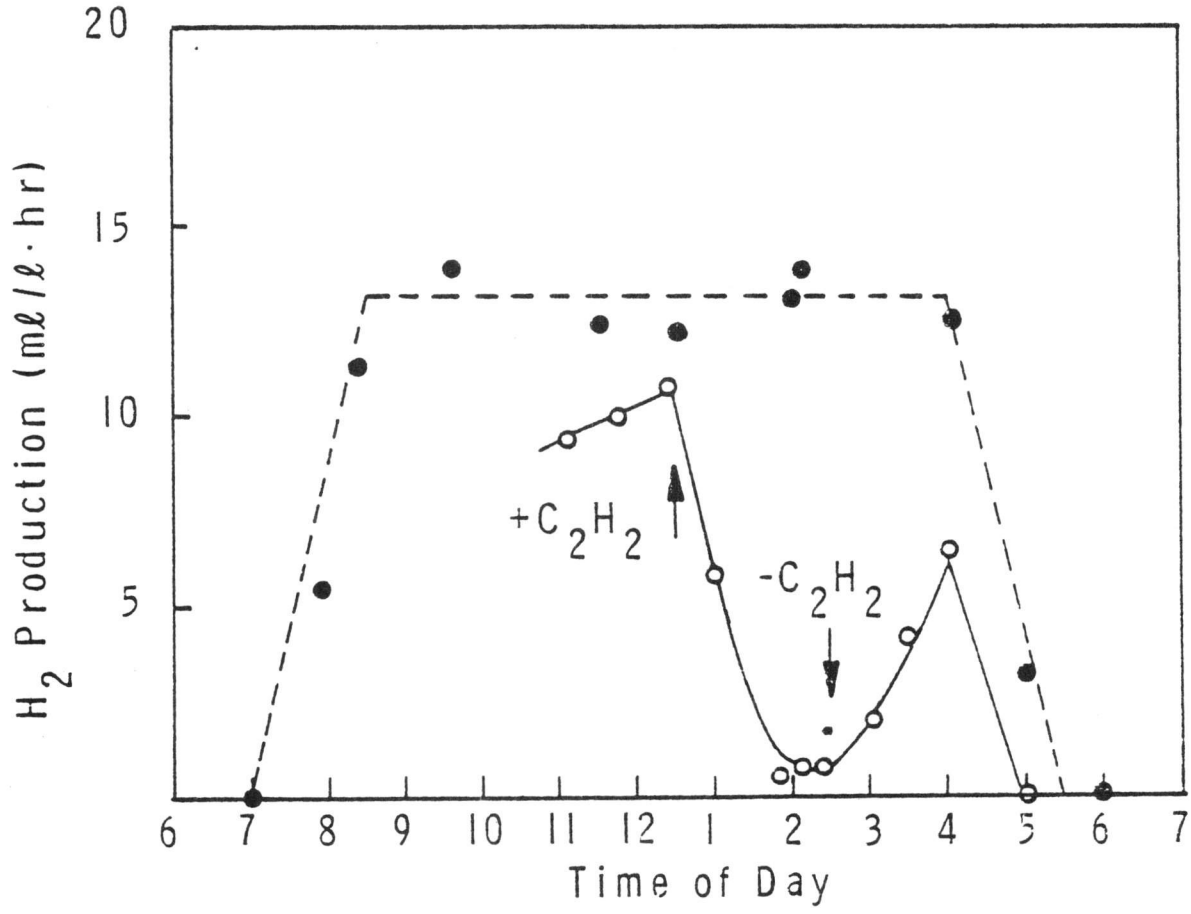


FIGURE 2. DAILY HYDROGEN PRODUCTION PATTERN AND HYDROGEN PRODUCTION IN THE PRESENCE OF ACETYLENE.

The curve representing daily hydrogen production (dashed line) was obtained from assays of a typical culture on November 9, 1978. This culture had a density of 0.9 mg/mℓ and was in the high hydrogen production phase. Hydrogen production in the presence of acetylene (solid line) was for a culture on December 14, 1978 that had a density of 1.5 mg/mℓ (optimal), but was in the declining hydrogen production phase (28 days after induction). Acetylene premixed with argon/carbon dioxide (11% acetylene, 0.5% carbon dioxide and balance argon) was used.

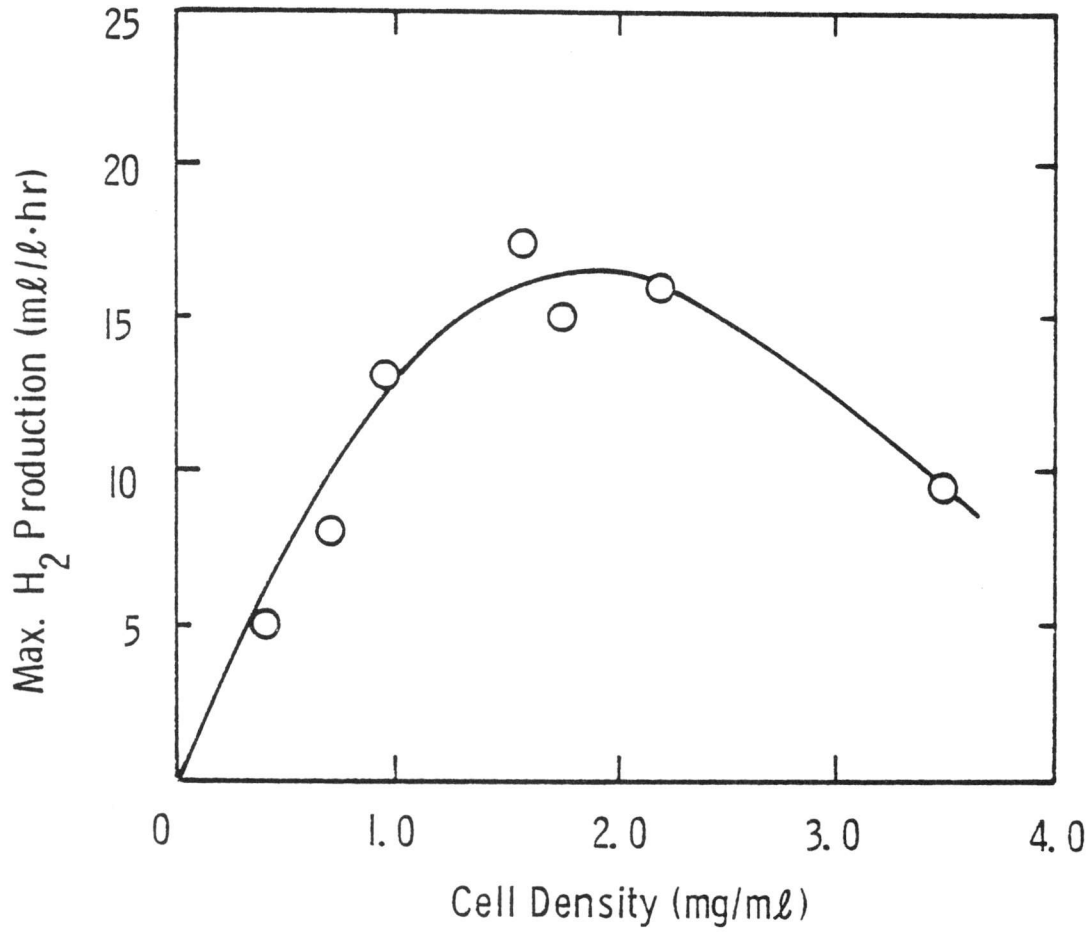
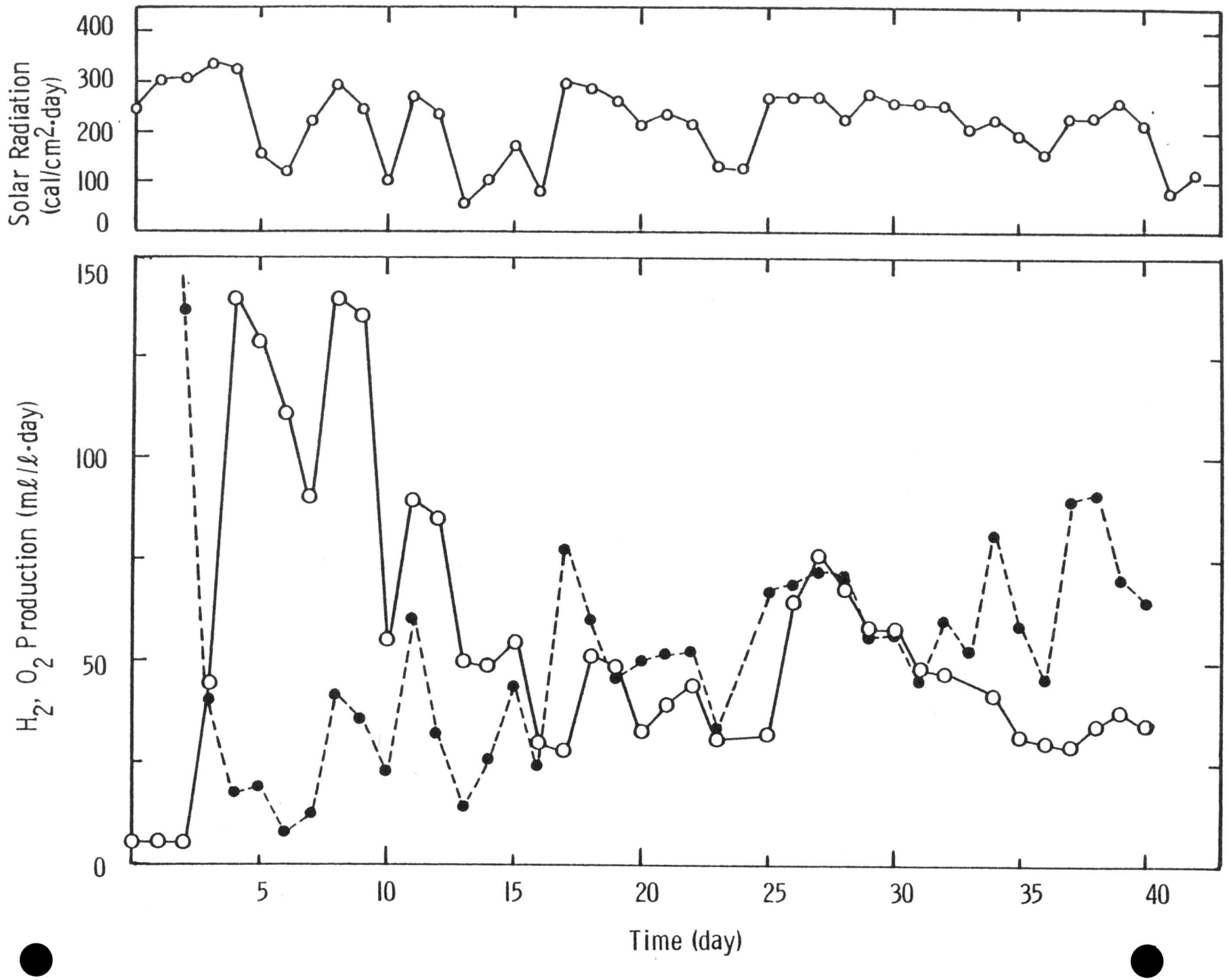


FIGURE 3. EFFECT OF ALGAL LOADING ON HYDROGEN PRODUCTION.

Cultures were run at the densities shown in the figure, and the maximum hydrogen production was measured at noon on a sunny day during the first ten days after induction.

FIGURE 4. HYDROGEN (open circles, solid line) AND OXYGEN (closed circles, dashed line) PRODUCTION IN AN OUTDOOR CONVERTER.

Daily solar radiation data are also shown in the upper part. Algal loading was kept constant at around 1.6 mg/mℓ throughout the experimental period. Data were taken during Nov. 6 - Dec. 16, 1978.


Solar Radiation (cal/cm²-day)
400
300
200
100
0
H₂, O₂ Production (mℓ/ℓ-day)
150
100
50
0
5
10
15
20
25
30
35
40
Time (day)

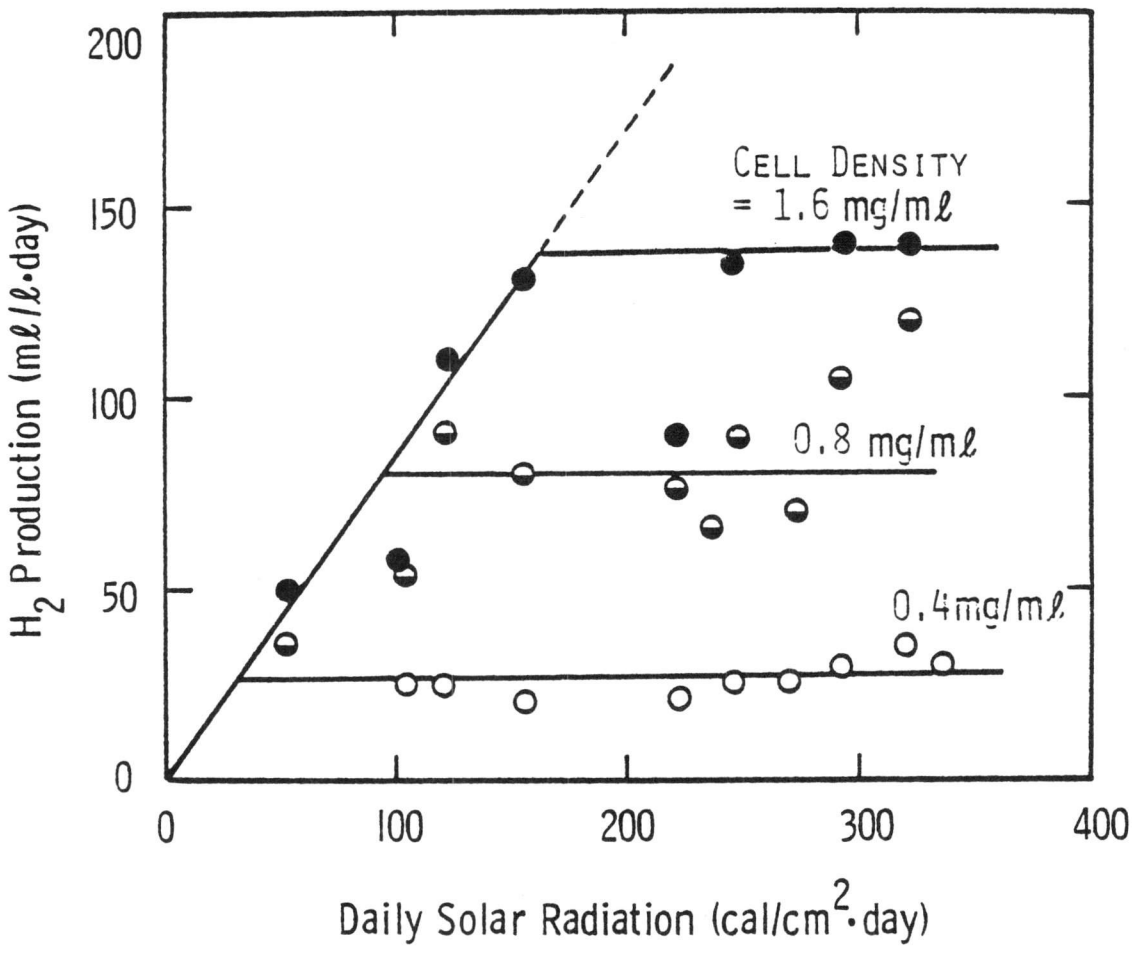


FIGURE 5. RELATIONSHIP BETWEEN HYDROGEN PRODUCTION AND DAILY SOLAR RADIATION.

Data were obtained during first ten days after induction when nitrogenase activity was highest.

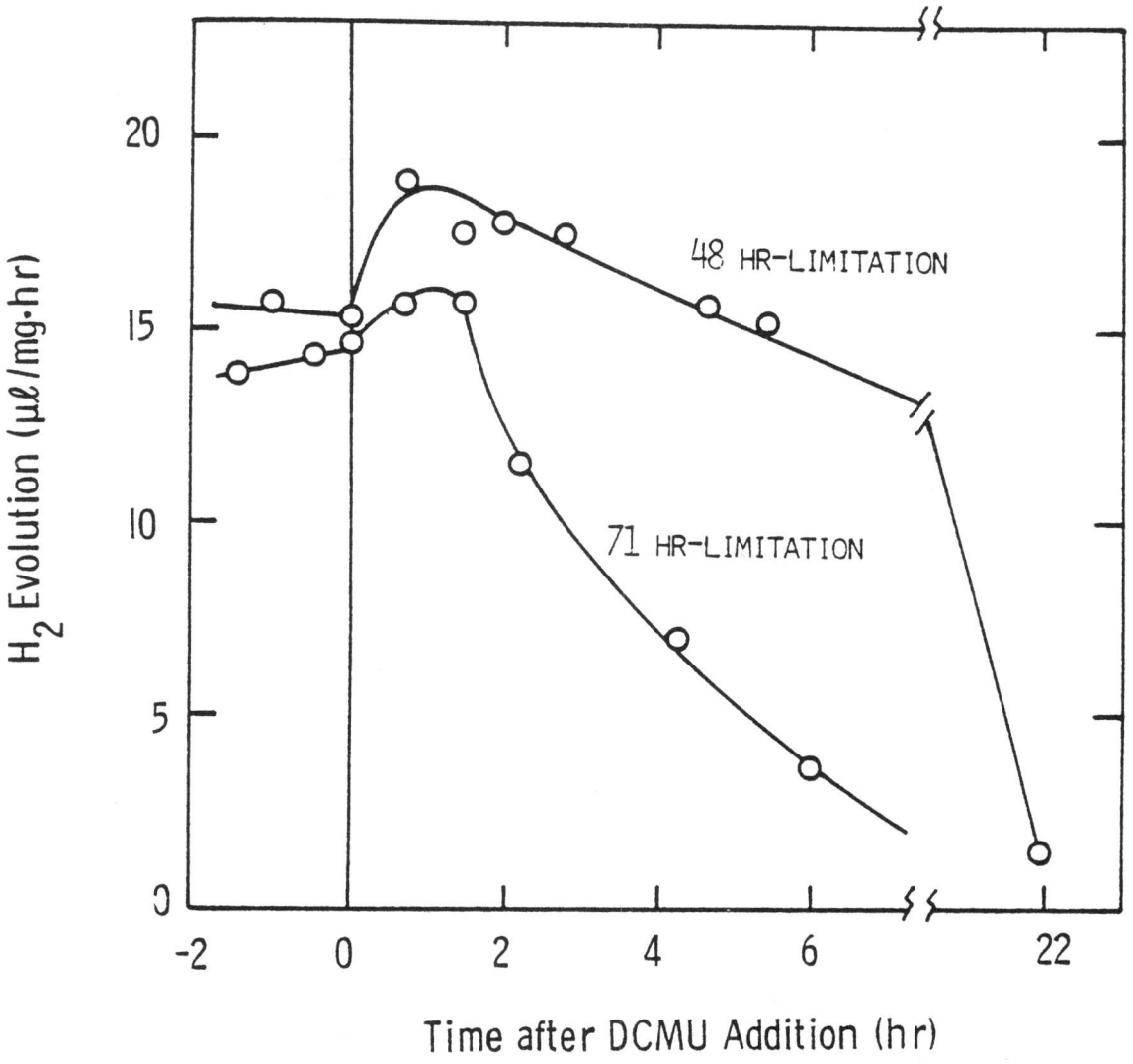


FIGURE 6. EFFECT OF DCMU ADDITION ON HYDROGEN EVOLUTION BY INDOOR CULTURES OF A. cylindrica.

DCMU was injected to two hydrogen-producing cultures under continuous artificial illumination (3.0×10^4 erg/cm^2·sec) after 48 hr and 71 hr.

VII. THE EFFECT OF MOLYBDENUM, VANADIUM, AND TUNGSTEN ON NITROGENASE SYNTHESIS AND ACTIVITY IN *Anabaena cylindrica*

INTRODUCTION

As with other nitrogen-fixing organisms, molybdenum is required for the growth of cyanobacteria on atmospheric nitrogen [1]. With the isolation and characterization of different nitrogenases, the requirement for molybdenum has been found to be due to the association of this metal with one of the two nitrogenase components [2,3]. The results of substituting vanadium or tungsten for molybdenum in the growth media has been different for different organisms [4-10]. In some cases there is an apparent requirement for molybdenum for the synthesis of nitrogenase components.

Vanadium partially relieves the effects of molybdenum starvation in some *Azotobacter* species [4], and it has been suggested that this vanadium effect is due to an increased effective utilization of trace amounts of molybdenum by molybdenum-deficient cultures [5,6]. In that organism it is likely that tungsten is incorporated into nitrogenase which, however, is inactive [7]. Thus, in *Azotobacter*, nitrogenase may be synthesized in the presence of all these metals. Partial purification and properties of a vanadium-containing nitrogenase have been reported [8, 9, 11]. This question has also been investigated by immunochemical quantitation of nitrogenase protein synthesized under various conditions. The absence or presence of Mo, V, or W has no effect on the synthesis of the non-Mo component of nitrogenase (Fe-protein, or component II) in *Azotobacter*, but serological analysis has shown that appreciable amounts of component I (which normally contains Mo) are only synthesized in the presence of molybdenum or tungsten and not vanadium [12]. Residual nitrogenase activity under molybdenum-deficient conditions, with or without vanadium, is probably due to trace amounts of molybdenum.

In Klebsiella pneumoniae, neither component is synthesized in the absence of molybdenum, as determined by both in vitro activity and serological assays, and the addition of tungsten fails to induce the synthesis of either component [13]. Molybdenum also appears to be required as an inducer of nitrogenase synthesis in Clostridium pasteurianum [14]. It is likely that addition of tungsten to cultures of that organism inhibits nitrogenase synthesis by competing with molybdenum for permeation into the cell [15]. On the other hand, tungsten has been reported to support a diminished, but significant evolution of hydrogen by nitrogenase in Rhodospirillum rubrum [16].

Vanadium has been reported to be either slightly stimulatory [1], or inhibitory [17] to residual nitrogenase activity in cultures of Anabaena cylindrica grown on media that lacks molybdenum. Replacement of molybdenum by tungsten in the growth medium permits the synthesis of active nitrogenase component II [18]; however, nitrogenase activity itself is very low [19, 20], suggesting that component I of nitrogenase may be synthesized, but is inactive. In the non-heterocystous cyanobacterium, Plectonema boryanum, both nitrogenase component proteins appear to be synthesized when the cultures are induced under molybdenum-deficient conditions (with or without tungsten) [10]. Component II activity was determined by in vitro complementation with bacterial component I, and component I activity could be restored by addition of molybdenum in the presence of chloramphenicol. Here we have investigated the effects of molybdenum starvation (with and without tungsten) on in vivo and in vitro nitrogenase activity, and the role of these metals in the regulation of nitrogenase biosynthesis in Anabaena cylindrica. Our results are consistent with the results obtained with Plectonema boryanum; namely that nitrogenase component proteins continue to be synthesized in the absence of combined nitrogen under conditions of molybdenum starvation.

MATERIALS AND METHODS

Organism and Culture Methods

Anabaena cylindrica 629 was grown in modified Allen and Arnon media [21] made up with distilled, deionized water and with molybdenum, tungsten, and vanadium omitted from the trace metal solution (nil media). When desired, these metals were added from stock solutions of sodium molybdate, sodium tungstate, or sodium meta vanadate. (All reagents used were of the highest grade commercially available.) Two M NH_4Cl was added to a final concentration of either 5 mM or 1 mM to provide fixed nitrogen; 5 mM NH_4Cl was used for maintenance of stock cultures on nil media; 1 mM NH_4Cl was used for growth of cultures in which derepression of heterocyst differentiation was desired. (This amount of nitrogen supported growth to about 0.245 g dry weight/L.) Nitrogen-starved cultures with active nitrogenase (grown on media containing molybdenum) were prepared as previously described [22]. Cultures were used when nitrogen starvation derepression of heterocyst differentiation was complete (36-48 hr). The cultures were grown as either non-axenic 47 L or axenic 2 L batch cultures, and *in vivo* experiments were conducted in either 2 L or 250 ml culture vessels. Lighting (2.0×10^4 ergs/cm^2/sec), growth determinations, and dry weight measurements were as previously described [21]. During nitrogen starvation and *in vivo* experiments, cultures were sparged with 99.7% argon, 0.3% carbon dioxide.

Assays

In vivo [21] and *in vitro* [22] nitrogenase activities were determined as previously described. Determination of component I protein was performed by a modification of the Laurell electroimmuno diffusion technique [23].

RESULTS

Cultures grown on nil media and subsequently nitrogen starved had very

low *in vivo* nitrogenase activity, typically about 3% of the activity of a control culture (Table 1). Cultures grown on media containing tungsten also showed a drastically decreased *in vivo* nitrogenase activity which usually was somewhat lower than cultures grown on nil media alone. This residual nitrogenase activity is probably due to the presence of trace amounts of molybdenum present in the media despite the precautions taken and is similar to or lower than the residual activity noted in previous studies [12,13,14].

The lower activity in the tungsten culture is probably due to an inhibition by tungsten of molybdenum assimilation [6,14,15]. As shown in Figure 1, tungsten inhibited the recovery of nil cultures upon the addition of molybdenum, with higher concentrations being increasingly inhibitory. Addition of 0.07 ppm Mo (0.73 μM) gave a ten-fold increase in nitrogenase activity in a control culture, 20 ppm W (0.11 mM) inhibited this recovery by 80%. The effect of various external molybdenum concentrations on nitrogenase recovery is shown in the form of a double recipricol plot (Fig. 2). The data gave a fairly good straight-line fit with an apparent Km for molybdenum of 2.7 μM. Using this data, the molybdenum contamination in the nil media can be estimated as approximately 0.23 μM (22 ppb).

Protein synthesis is required for maximal recovery of nitrogenase activity from molybdenum deficiency, although substantial stimulation was observed even in the presence of chloramphenicol (an effective inhibitor of protein synthesis in *Anabaena cylindrica* [24]). Addition of 4 ppm Mo to a molybdenum-deficient culture resulted in a twenty-fold stimulation which was about 50% inhibited in the presence of chloramphenicol (Table 1). Recovery of activity upon addition of molybdenum to a tungsten culture was lower, due

to the inhibitory effect of tungsten, and the effect of inhibition of protein synthesis was also less marked. Previously, it was shown that in Azotobacter vinelandii molybdenum incorporation into tungsten derepressed nitrogenase can occur in the absence of protein synthesis [12], and in Plectonema boryanum, nitrogenase activity of molybdenum-starved cultures is restored upon addition of Mo in the absence of protein synthesis [10]. Our results (Table 1) show that in Anabaena all the proteins required for formation of active component I are present in cells grown on nil media and tungsten-supplemented media.

Molybdenum stimulation of nitrogenase activity in the absence of protein synthesis also suggests the presence of nitrogenase component I apo enzyme in these cells. This was confirmed by quantitative immunoelectrophoresis (Table II), which showed that immunologically reactive material was present at the same or higher level than in a control culture. In vitro nitrogenase activities were as proportionately low as the in vivo activities. Thus, in Anabaena nitrogenase component I is synthesized in the absence of molybdenum, unlike the case with Klebsiella pneumoniae [12] or Clostridium pasteurianum [12] where molybdenum is required for synthesis, or Azotobacter vinelandii [11], where tungsten (or molybdenum) appears to be required.

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TABLE 1

RECOVERY OF *in vivo* NITROGENASE ACTIVITY UPON ADDITION OF Mo to -Mo CULTURES

Culture Media (trace metal)	Additions	Nitrogenase Activity (nm C_2H_2/mg dry wt per min) 0 h	5½ h
--	--	0.698	0.950
--	Mo Chloramphenicol	0.595	5.10
--	Mo	0.862	11.2
W (2 ppm)	--	0.546	1.13
W (2 ppm)	Mo Chloramphenicol	0.535	3.16
W (2 ppm)	Mo	0.558	4.45
Mo (0.1 ppm)	--	28.8	28.8
Mo (0.1 ppm)	Chloramphenicol	28.8	28.8

Where added, the final concentrations of Mo and chloramphenicol were 4 ppm and 100 mg/L respectively. Chloramphenicol additions were made 15 min prior to Mo additions. (Similar effects were seen when chloramphenical was added 30 and 45 min prior to Mo addition.)

TABLE 2

ACTIVITY AND NITROGENASE PROTEIN IN EXTRACTS
FROM CULTURES GROWN ON VARIOUS METALS

Culture Media (trace metal)	Specific Activity (mn C_2H_2/mg protein per min)	% Cross-Reacting Material
Mo	54.5	100
nil	1.1	107
W	0.41	112

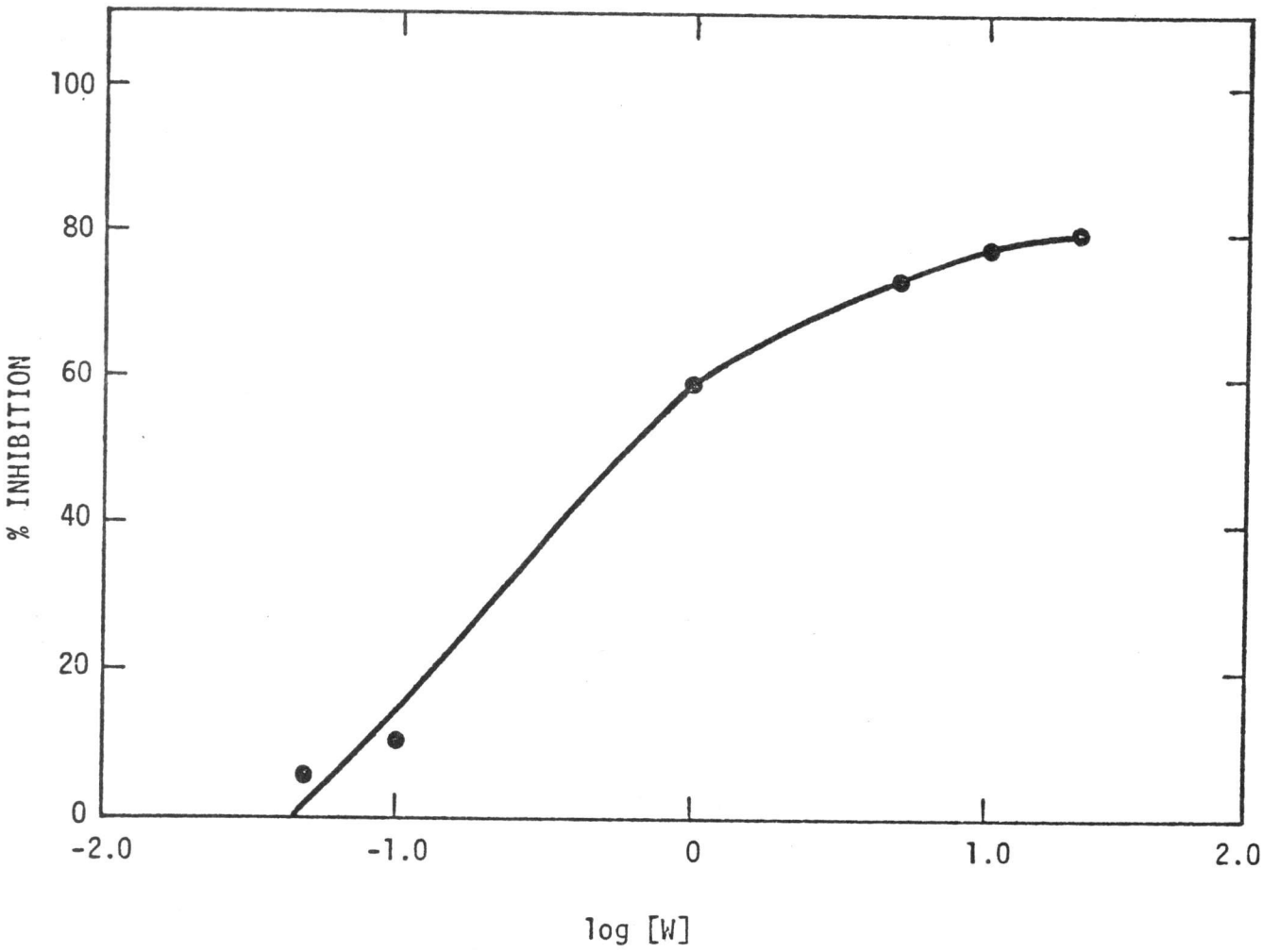

FIGURE 1. INHIBITION BY TUNGSTEN OF RECOVERY OF NITROGENASE ACTIVITY UPON ADDITION OF MOLYBDENUM.

Aliquots of a nil culture were incubated under illumination in 250 ml culture flasks. All flasks received 0.07 ppm Mo and W as indicated. After 5 h the cultures were assayed for nitrogenase activity. Results are expressed relative to a culture that received Mo only (0.698 nm/mg dry wt per h initially, increasing to 6.27 nm/mg dry wt per h).

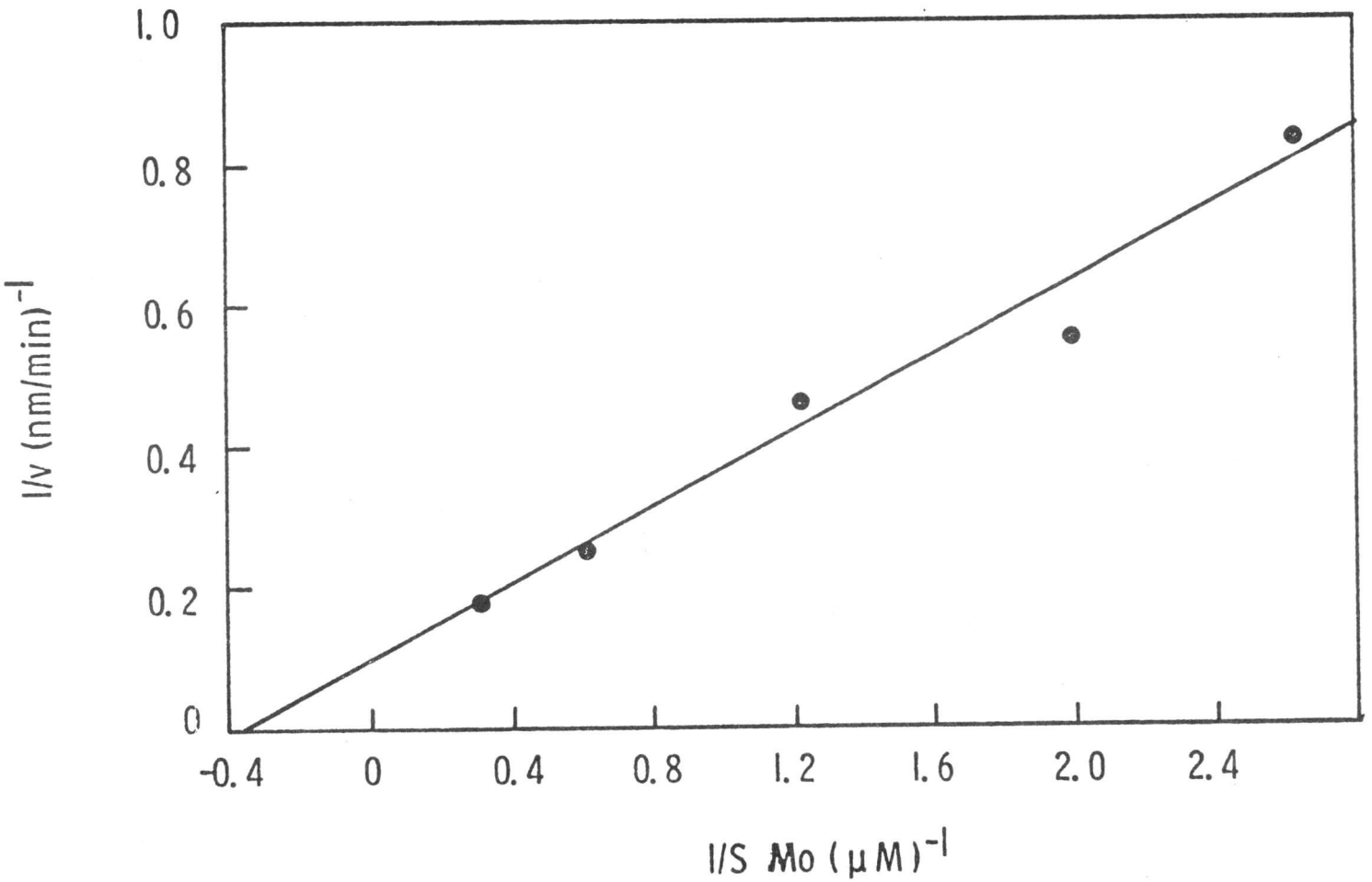

FIGURE 2. RECOVERY OF NITROGENASE ACTIVITY AT VARIOUS CONCENTRATIONS OF MOLYBDENUM. Portions of a 48-liter nil culture were incubated in the presence of the indicated concentrations of Mo for 16 hrs and then assayed for nitrogenase activity.

VIII. PREPARATION OF ANTIBODY PROBES AND THEIR CHARACTERIZATION

Efforts on the development of an electron-dense immunological probe for the electron microscopic localization of nitrogenases have centered on the preparation of an antibody conjugate that retains immunological activity after reaction with activated ferritin. (Previous preparations were made with glutaraldehyde-activated ferritin and were unreactive in an Ouchterlony double-diffusion analysis--see last year's Final Report.) Another conjugation scheme, using toluene 2,4 diisocyanate as crosslinker, was tried. This bifunctional reagent has the advantage of possessing crosslinking groups that are reactive at different pHs, thus preventing the formation of ferritin oligomers during the initial activation step. The procedure that was used is, in brief, as follows:

> 1.5 ml of purified ferritin (29.9 mg) was mixed with 1.5 ml of 0.3 m NaCl buffered with 0.1 M phosphate, pH 7.5. While stirring, (and with the solutions at room temperature), 0.075 ml of 2,4 tolune diisocyanate was added. The reaction was allowed to proceed for 30 mins and was stopped by centrifugation at 4°C (15,000 rpm for 10 mins). Since tolune 2,4 diisocyanate is insoluble at this temperature, most of the unreacted crosslinker is removed. The activated ferritin was reacted with 25 mg of partially purified IGG for 48 hrs, dialyzed for 48 hrs, and then applied to a column of agarose (1.5 m) to purify the product.

However, the preparations obtained with this procedure were variable. The first preparation showed a reaction on an Ouchterlony double-diffusion analysis but appeared inadequate for use in electron microscopy due to a large amount of non-specific staining. This might be due to a side reaction of the active isocyanate groups to acyl ureas. Loss of immunological activity upon storage was also observed with this preparation; it is not known whether this is due to further crosslinking and aggregation during storage.

Several new strategies have been developed that should lead to active

immunological probes. Ferritin can be linked to rabbit anti goat antibody. The use of a secondary (indirect) label should increase the sensitivity of this approach (since more than one ferritin is likely to be bound per nitrogenase localized) and should obviate some of the problems with preparations that may be monovalent.

One method that was used to prepare these conjugates was to isolate a specific antibody using affinity chromatography and then measure the extent of activity left after conjugation by quantifying how much of the conjugate was bound to the affinity column (1). Purified goat antibody was covalently bound to agarose (1.5 m) using a modification (2,3,4) of the original cyanogen bromide procedure (5). Purified rabbit anti goat IGG was obtained by eluting the column (after loading on a partially purified preparation[1], washing out unabsorbed material with 1.5 M NaCl in phosphate-buffered saline PBS) with either 3.5 M KSCN in PBS or 0.2 M glycine-HCl pH 2.3 (which proved to be more effective).

The purified IGG conjugate was purified by column chromatography (6). When rechromatographed on the affinity column (goat IGG-agarose), 41% of the original protein was bound, indicating that possibly 41% of the prepared conjugate might be immunologically active. However, the bound material was not removed using the previously used procedure, and the conjugate failed to give a reaction when tested using Ouchterlong double diffusion.

It was not clear from these results whether or not such a conjugate would be suitable for electron microscopy, so a more direct and highly sensitive method of determining the antigen-binding activity of ferriten-antibody conjugates was developed. The principle of this method is essentially that of normal rocket immuno electrophoresis. However, since both antigen (goat IGG)

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[1] purified from serum obtained from Antibodies, Inc., Davis., Calif.

and antibody (rabbit IGG antigoat IGG) are both isoelectric at the same pH, one or both must be modified to obtain differential electromobility. Since it was decided to quantify the conjugate, the procedure was developed so that the antibody was electrophoresised into an antigen-containing gel ("reverse" rocket immunoelectrophoresis). The isoelectric point of the goat IGG was changed from pH 8.6 to pH 5.0 by carbamylation (7) and checked by agar electrophoresis, using the same buffer used for the reverse rocket electrophoresis, 0.05 M acetate buffer, pH 5.0.

The results of a reverse rocket-immune electrophoresis (pH 5.0) where various amounts of rabbit anti-goat IGG were electrophoresed into an agar gel containing 0.7 µg of carbamylated goat IGG/cm^2 are shown in Figure 1. This system proved suitably sensitive, with a linear relationship between precipitate peak height and µg's rabbit antigoat IGG applied (4.2 mm/µg rabbit IGG). Shown in Figure 2 is a trial run of this system with an old conjugate preparation. Unconjugated IGG has a net positive charge at this pH and migrates towards the negative electrode. However, ferritin is a very acidic protein and is negatively charged at this pH. Therefore, free ferritin or ferritin-antibody conjugates (Fig. 2) migrate toward the positive electrode. Thus, not only does this system allow the quantification of the antigen-combining activity of conjugate preparations, it also can detect the presence of potentially interferring unconjugated rabbit IGG. As shown in Figure 2, free rabbit antigoat is removed by the conjugate purification procedures. This procedure should allow the preparation and characterization of immunologically active ferritin-antibody conjugates in the near future.

ROCKET IMMUNOELECTROPHORESIS ASSAY

The initial development of a quantitative immunological assay (using rocket immunoelectrophoresis) for nitrogenase was detailed in last year's

Final Report. Use of the procedure as developed then led to several problems, notably distorted morphology of the rocket peaks, and in some cases, the failure to obtain any reaction. Some of these problems have now been traced to electro-endoosmosis, which has been corrected by using lower voltages. In addition, we have shown that incorporation of 4% polyethylene glycol (PEG) into the gels has led to an enhancement of the precipitin lines, allowing higher sensitivity and contrast over the stained background. (For details of the new rocket immunophoresis procedure, see the appendix to this chapter.) Addition of 4% PEG is reported (8) to increase sensitivity five-fold by excluding the antibody-antigen reaction from 80% of the gel through steric hinderance. This, in effect, concentrates both the antigen and the antibody proportionately. We have also found that identical samples and IGG concentrations produce different peak heights when different brands of agarose are used. (Presently, Biorad agarose is used.)

Several plates were run using a well-characterized antibody-antigen system to test the physical system that we were using for electrophoresis. We have experienced many problems with faint precipitates, unusual rocket morphology and blank plates with the anti-nitrogenase antibody and we did not know whether this was due to a technical problem involving the electrophoresis materials and equipment or due to the nature of the nitrogenase antibody complex and the lability of nitrogenase during extraction and storage. Using BSA against 1% anti ^{BS} A we found a clearly resolved peak with a distinct peripheral precipitation line showing the typical rocket shape reported in serum immunoelectrophoresis studies. We concluded that our system was acceptable for immunoelectrophoresis studies.

A typical example of some of the results obtained with this new procedure and the anti-nitrogenase antibody is shown in Figure 3. All samples showed two or three distinct rockets. The major cross-reacting species was

was present in large excess and, hence, was not resolved on this plate (seen as the outermost line extending off the plate). These minor fractions may be proteolytic breakdown products of nitrogenase and/or O_2-damaged nitrogenase.

Crossed immunoelectrophoresis was carried out to study the relationship between O_2 exposure and cross-reacting protein changes in a sample of partially purified nitrogenase. Two 15-µl samples were run against 1.5% anti-mo Fe-nitrogenase serum. One of the samples had been stored anaerobically while the other sample had pure O_2 (g) blown over it for 15 mins prior to electrophoresis. The sample exposed to O_2 showed five rockets and the anaerobically stored samples showed three rockets. When the areas of cross-reacting proteins were compared, it was found that the total area of the five rockets from the O_2-treated sample was only 52% of the total area of the anaerobic sample. Therefore, there must be more than five degradation products when a sample is exposed to O_2, some of which lose their cross-reacting properties. Both plates had diffuse bands of protein precipitates at approximately the same level. These bands did not show the rocket shape typical of antibody-antigen reaction in this system and may be due to precipitation of extraneous cellular proteins.

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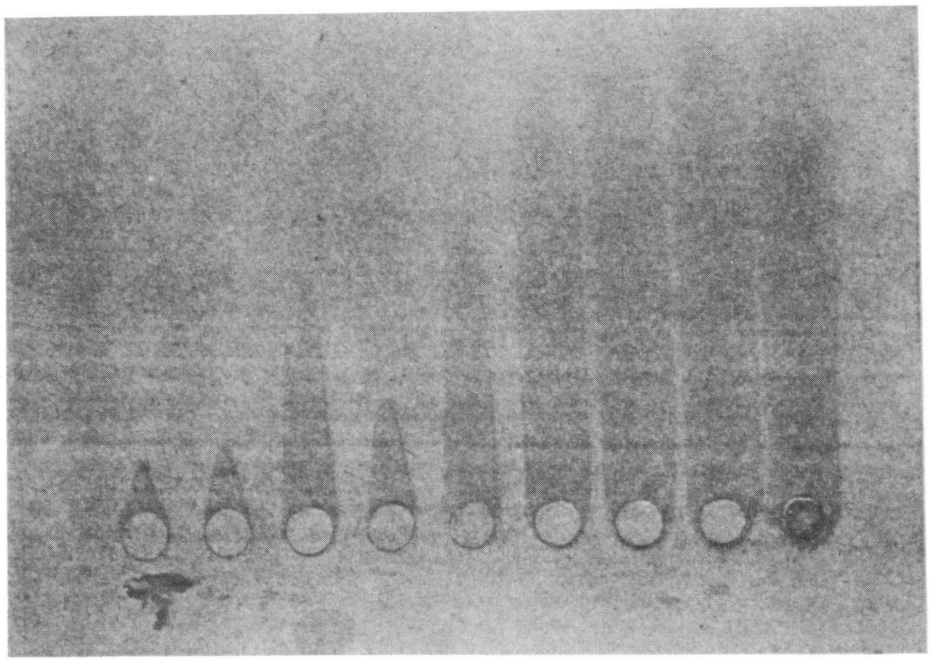


FIGURE 1. REVERSE ROCKET ANALYSIS OF IMMUNOLOGICAL ACTIVITY OF NATIVE RABBIT ANTIGOAT ANTIBODY. Gel was 1.5 mm thick, 1% Baker agarase in 0.05 M acetate buffer. The gel contained 4.8 µgm/ml carbamylated goat IGG. Rabbit IGG samples, forming visible rockets, were from 4.75-0.1 µgms.

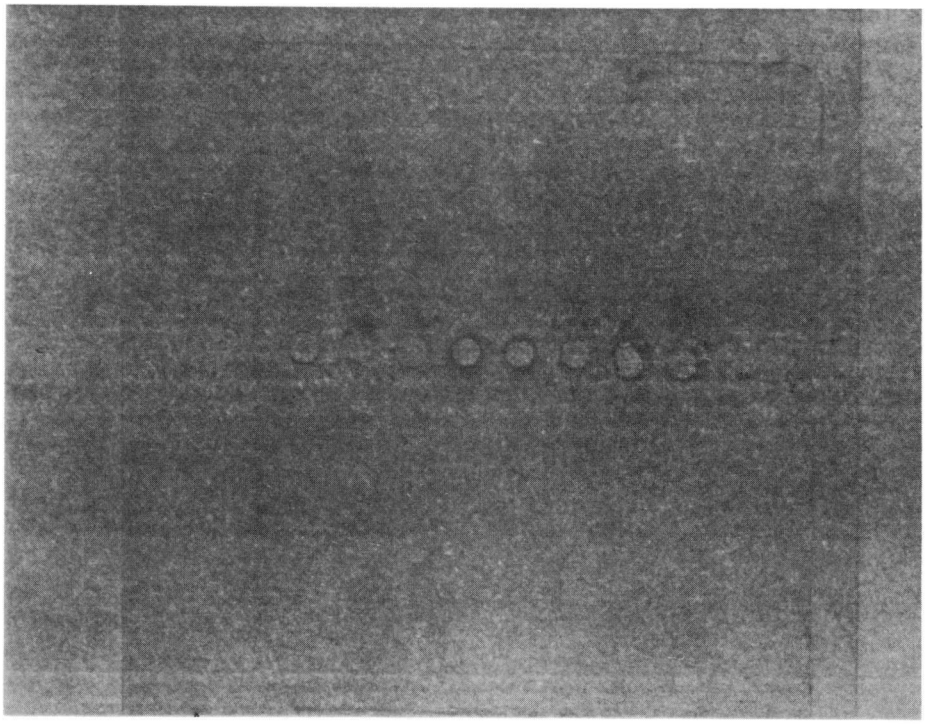
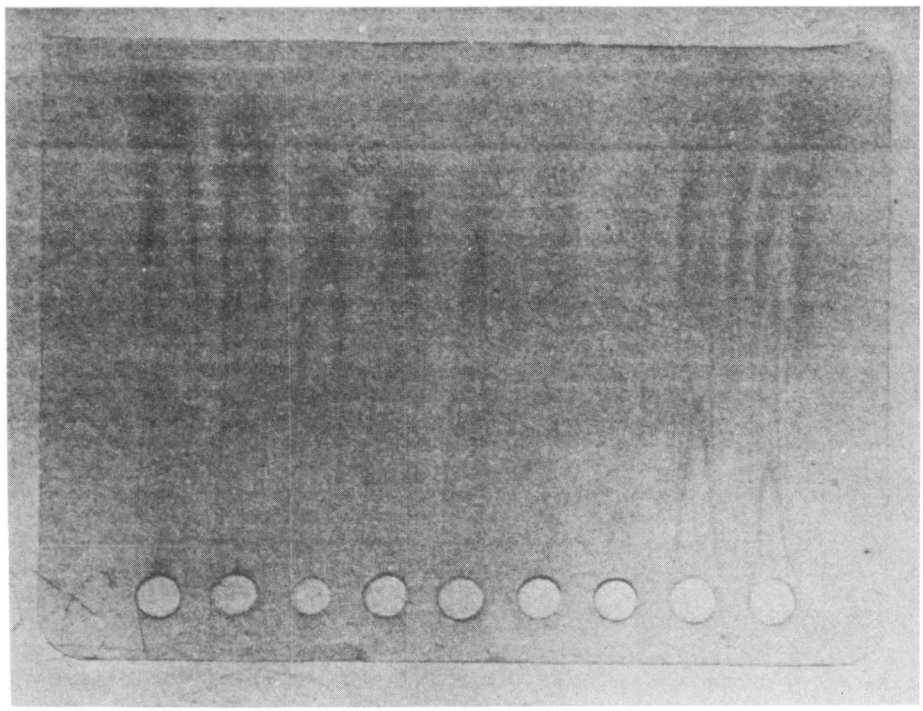


FIGURE 2. REVERSE ROCKET OF FERRITIN-ANTIBODY CONJUGATES. The gel composition was as in Figure 1. Wells 1-3 contained native rabit antigoat, well 4, an antibody-ferritin preparation, well 5,6, 7, 8 were side fractions from preparative column chromatography. Wells 6,8 showed native rabbit antigoat activity.



1 2 3 4 5 6 7 8 9

Figure 3. Typical results of immunoelectrophoretic "rocket" assay of nitrogenase fractions.

Walls contained the following nitrogenase samples:

1 15 µℓ extract normally grown, nitrogen-starved culture
2 10 µℓ " " " " " "
3 15 µℓ extract tungstem-grown, nitrogen-starved culture
4 15 µℓ extract nil grown, nitrogen-starved culture
5 10 µℓ " " " " " "
6 15 µℓ 52.8 pm acetylene reduced/min/µℓ
7 10 µℓ " " : " " "
8 15 µℓ 626 pm acetylene reduced/min/µℓ
9 10 µℓ " " " " " "

APPENDIX

PROCEDURE FOR IMMUNOELECTROPHORESIS

Reagents and materials for electrophoresis

1. 1% BioRad Agarose, 4% 6000 MW polyethylene glycol in 25 mM BioRad Barbital Buffer I, pH 8.6. BioRad Agarose minimizes gel dessication due to electroendosmosis at voltages under 50 V. Addition of 4% polyethylene glycol (J.T. Baker Chemical Co, max wt 6000) enhances the sensitivity of the antigen-antibody reaction five-fold and results in increased visibility of precipitation lines (8). Buffer is chosen at the isoelectric point of antibody to minimize migration in the electric field.
2. 25 mM BioRad barbital Buffer I, pH 8.6 for wells.
3. Veronal, tris, glycine buffer, pH 8.8 for electrode vessels. This buffer was found to maximize pH stability in the buffer vessels

   30.14 g BioRad Barbital Buffer I

   112.40 g glycine

   90.40 g tris

   4 L H_2O
4. Telfa non-adhesive strips previously soaked in Ba/tris/glycine buffer are used to connect buffer vessels to gel.

Preliminary Procedures

1. Melt 10 ml aliquots of buffered agarose at 100°C.
2. Place test tubes of melted agarose in dry bath at 55°C.
3. Level electrophoresis cell in refrigerator.
4. Switch anode and cathode to maintain buffering capacity of vessel buffer.
5. Heat flask of distilled H_2O for rinsing syringes.
6. Melt antibody and antigen at 4°C.

Procedures

1. Secure U-frame between plates with clamps on three sides.
2. When agarose $\leq$ 55°C, add antibody, invert tube gently to mix without incorporating bubbles, withdraw mixture with syringe, place syringe aperature on inside edge of U-frame and quickly inject mixture. This should be done quickly so that the gel does not solidify during the procedure. Normal melting temperature of the agarose is 60°C but the lower temperature minimizes denaturation of the antibody and can be used successfully if the gel does not remain at 55°C for too long.
3. Make wells in solidified plate with gel puncher. These should be 1-1.5 cm from each plate because current is least stable at the gel edges.
4. Place plates on level cell platform with wells at cathode (-) side.
5. Place wicks on edge of gel so that they extend 3/4 cm onto gel.
6. Inject diluted samples with Hamilton microsyringe placed vertically into center of well.
7. At voltages $\geq$50, place parafilm over gel to minimize dessication from electroendosmotic migration of gel. The film should not adhere to the gel in the region of the wells.
8. Immediately turn on power supply to avoid precipitation from antigen diffusion into the gel.
9. Turn off power when electrophoresis is complete (usually 20 hrs for 20V) and allow plates to incugate for > 48 hrs. Incubation may be continued for several weeks if convenient.
10. Wash plates in 1 M NaCl, 50 mM K H_2PO_4/K_2HPO_4 pH 7.6, 0.2% NaN_3 for 15 min overnight. If plates have not been precoated, the gel will loosen from the plate during this step. Extreme care must be taken while handling loose gels. The gel is best replaced in its original position on the plate while still immersed.

11. The gel is then pressed. Place 10 x 10 filter paper on gel and saturate with distilled H_2O. Air bubbles may be removed by gently stroking wet filter paper with the flat of your thumb. Cover filter paper with 2 cm of blotting paper and a glass plate. Place a 750 g weight on the top plate and press for 10 mins. Greater weights or longer pressing times will trap unprecipitated proteins in the gel and obscure the background of the final stained plate.
12. Repeat wash and press steps three times diluting saline 1:1 twice and washing finally in distilled water.
13. Plates may then be dried under a blower. Cool air gives better results but takes longer than hot air. The best drying technique for keeping the gel intact and avoiding air bubbles is to simply press the gel overnight or longer.
14. Stain the plate with 0.2% Coomassie blue in 5:5:1 methanol/water/acetic acid for 10 mins. Destain with 5:5:1 methanol/water/acetic acid until background is light enough to see rockets. Plates are best preserved if repressed at this point.

CROSS ROCKET

1. Pour first plate with no IGG using U-frame; punch with 2,4 mm wells; inject sample and place in cells.

   Place 1 drop of bromothylmol blue at edge of each well and run electrophoresis at 10 V/cm until tracking dye approaches next well. Cut off excess agar on top. Place plate in U-frame and pour IGG + 1% Agarose and 4% PEG to top of frame.

   Place plate in cell and run as for 1 dimensional rocket.

IX. OXYGEN PROTECTION MECHANISM IN THE HETEROCYSTS

ABSTRACT

Possible mechanisms of O_2 protection of nitrogenase in the heterocysts of A. cylindrica were studied in vivo. O_2 sensitivity of nitrogenase was negatively correlated with the O_2 tension of the media in which the algae were induced to produce heterocysts. O_2 sensitivity was also correlated with the apparent Km for acetylene. In an argon-induced culture, the in vivo Km for acetylene was about two times the Km in vitro. In contrast, aerobically induced cultures had an apparent Km about five times greater than the in vitro value. The Km was intermediate in a microaerobically (N_2/CO_2) induced culture. We interprete these results to indicate that the postulated gas diffusion barrier of the heterocyst envelope is poorly developed under low O_2 tensions. The role of respiratory activity in protecting nitrogenase from any O_2 diffusing into the heterocyst was studied using the cytochrome inhibitor, sodium azide. At concentrations (30 mM) sufficient to completely inhibit dark nitrogenase activity (a process dependent on oxidative phosphorylation for its ATP supply), azide was found to severely inhibit short-term acetylene reduction in the presence of O_2 but not in its absence. Inhibitors of carbon catabolism were investigated to identify the metabolic pathways responsible for providing substrate for nitrogenase activity and respiratory protection. 2-4 dinitrofluorobenzene, an inhibitor of glucose-6-phosphate dehydrogenase, completely inhibited acetylene reduction in the light and dark, with or without O_2. Photosynthetic ^{14}C incorporation was not affected, indicating its effect was not generally toxic. Malonic acid, a competitive inhibitor of a Kreb's cycle enzyme, succinate dehydrogenase, had no effect on acetylene reduction in the presence or absence of O_2 in log phase cultures. However, in reductant-limited cultures, malonate stimulated nitrogenase activity to the same or greater degree as H_2. We conclude that the O_2 protection mechanism is dependent on both an O_2 diffusion barrier and an active respiration in the heterocysts.

INTRODUCTION

The photosynthetic production of O_2 presents a unique problem to the functioning of nitrogenase in the blue-green algae. In common with bacterial nitrogenases, all cyanobacterial nitrogenases studied to date are irreversibly inactivated by O_2 (1,2,3). Despite this, a wide variety of species, representing the major groups of cyanobacteria, are capable of growth on atmospheric N_2. Species of the Nostoc line (Nostocaceae) have evolved the most elaborate systems to allow the simultaneous operation of these basically incompatible processes: the heterocyst.

There are probably several mechanisms by which the heterocyst is able to maintain an anaerobic environment required for activity of O_2-labile nitrogenase. The loss of O_2-evolving photosystem II components is well documented (4,5,6). The presence of four unique glycolipids in the laminated layer of the heterocyst envelope has been suggested to provide a passive barrier to the diffusion of O_2 (7). This is supported by the recent isolation of mutants deficient in these envelope glycolipids which have little or low nitrogenase activity when assayed aerobically (8). The findings that the *in vivo* Km of nitrogenase for acetylene is five-fold higher than the *in vitro* Km in *A. cylindrica* but only slightly higher in the non-heterocystous alga, *Plectoneoma boryanum*, further support this notion (9). It is unlikely, however, that O_2 is completely excluded from the heterocyst since dark nitrogenase activity is O_2-dependent (10,11,12). Furthermore, an impermeable gas diffusion barrier would also exclude N_2 from the site of N_2 fixation.

An O_2-scavenging system is another means by which the heterocyst could lower the intercellular O_2 tension. Enhanced rates of O_2 uptake (13) in isolated heterocysts suggest the operation of a respiratory protection mechanism,

perhaps analogous to that proposed for Azotobacter (14,15). The O_2 sensitivity of nitrogenase activity in reductant-limited, but not exponentially growing cultures (16), supports this view. In Azotobacter the site or sites that protect nitrogenase from O_2 inactivation in vivo are sensitive to azide and 2,4-dinitrophenol, suggesting a role for the cytochrome system (17). Large increases in peroxidase (18) uptake hydrogenase (53), and ascorbic acid oxidase (19) activities following differentiation of heterocysts suggest other mechanisms for O_2 reduction.

The key questions regarding these O_2-protection mechanisms (diffusion barrier and respiratory protection) are their relative role in protecting the nitrogenase enzyme and activity, their exact nature, and the effects of various environmental parameters in the development and effectiveness of these O_2-protection mechanisms. In the development of biophotolysis with heterocystous blue-green algae, relatively high O_2 tensions will be desirable and, thus, the O_2-protection mechanism becomes an important problem.

MATERIALS AND METHODS

Anabaena cylindrica (629) was grown axenically as previously described (20) in 2-L batch cultures using modified Allen and Arnon media sparged with air/CO_2 (99.7%/0.3%). Heterocyst-free cultures were obtained by successive transfer in medium supplemented with 5 mM NH_4NO_3 and induced as described earlier (20). Log-phase cultures were obtained by dividing a low-density 2-L culture into several 250 mL cylindrical bottles and incubating at 28° to 29°C under lowered light intensities (provided by fluorescent white lights) with the desired gas phase for at least 12 hrs before initiating an experiment. The light intensity was doubled to 2×10^4 erg/cm^{-2} sec^{-2} when the cultures reached a density of 40 to 50 Klett units. The doubling time under these conditions ranged between 13 and 15 hrs. Reductant-limited cultures were obtained by two methods. 0.5 mL of a DCMU [3-(3,4-dichlorophenyl)-1,-dimethylurea]

solution in 100% ethanol was added to a final concentration of 10^{-5} M to each 250 mL bottle of exponentially growing cultures sparged with N_2/CO_2. Cell density stayed constant and the cultures remained green when sparged with anaerobic gases. Reductant limitation was also induced by covering a log-phase aerobically cultured 250 mL bottle of algae with aluminum foil and incubating in the dark until nitrogenase activity in the light decreased by approximately 50% (usually in 5 hrs).

Acetylene reduction assays were performed as previously described (20) in argon-filled 5-6 mL Fernbach flasks which contained all the gases indicated in the text; 17% acetylene was used with the exception of the Km measurements. Assays were incubated at 30°C with shaking and saturating light (6×10^4 erg cm^{-2} sec^{-1}) from below for 30 min unless otherwise indicated. Assays were terminated by injection of 0.2 mL of 25% trichoroacetic acid. Ethylene production was measured using a series 1200 Varian aerograph gas chromatograph equipped with a 5 ft Poropak R stainless steel column and a flame ionization detector.

Photosynthetic carbon fixation was measured by injecting 50 µL of 10 µC $NaH^{14}CO_3$ into argon-flushed 6 mL Fernbach flasks containing 2 mL of algae. The samples were incubated as described earlier for acetylene reduction. After 15 min of incubation, 1 mL of algae was removed, filtered on a 2.5 cm millipore filter (pore size 0.45 µ) and washed with acidified distilled water (pH 4.0) to remove external bicarbonate. The filters were dissolved in 10 mL of Beckman "Filter-Solv" for liquid scintillation counting.

O_2 tension of the culture media was measured using a galvanic dissolved O_2 probe with a D.O. analyzer Model DO-40 (New Brunswick Scientific, N.J.). The probe was inserted into the neck of the 250 mL magnetically stirred culture bottle immediately after removing the gas sparger, and D.O. was read within 30 sec. The reaeration rate of N_2-sparged media was 0.105 mg O_2/L/min.

RESULTS

O_2 Sensitivity of Nitrogenase Activity During Heterocyst Differentiation

To assess the physiological state of differentiating cultures with respect to the development of O_2 protection mechanisms, acetylene reduction was followed during the course of heterocyst development under a variety of conditions. Figure 1 shows the time course of apparent nitrogenase induction and heterocyst differentiation in a culture induced under an argon/CO_2 atmosphere. The time course of both processes were similar in cultures induced under N_2/CO_2; however, nitrogenase activity increased faster than under argon, exhibiting an autocatalytic induction pattern. Furtheremore, under N_2 growth resumed following the appearance of nitrogenase activity, and heterocyst frequency reached a peak (approx. 6%) 24 hr following transfer (data not shown). In both cases, the rise in nitrogenase activity coincided with the appearance of mature heterocysts and continued to increase as proheterocysts matured.

The effect of O_2 tension on nitrogenase activity during induction under an argon/CO_2 or N_2/CO_2 atmosphere has been reported previously (21). It was found that early in the induction process (12 hr) O_2 (20%) inhibited nitrogenase activity by 55% while a few hours later (19 to 25 hr) O_2 sensitivity was reduced to about 25% (Figures 10 & 12, Ref. 21). There was a somewhat greater O_2 resistance in the N_2/CO_2-induced cultures than in the argon-induced cultures. H_2 was not stimulatory at any time during the induction process, suggesting high reductant pools. Also, dark activities were relatively high and DCMU did not inhibit. Thus, it can be surmised that the O_2 sensitivity during the early phase of induction may be due to a different diffusion barrier rather than ineffective respiratory protection.

Further comparisons of the O_2 sensitivity of nitrogenase activity in cultures induced under different gas phases are shown in Figure 2. Each

culture was assayed 24 hr following induction. Under all culturing conditions, proheterocysts matured into heterocysts. Large differences, however, were found in response to O_2 tension. Air-induced cultures were very resistant to O_2. Inhibition of nitrogenase activity was minimal even at 50% O_2. The cultures sparged with anaerobic gases during the induction period were much more sensitive to O_2.

The most O_2-sensitive cultures were induced under an atmosphere of argon/CO_2. A marked decline in phycocyanin pigments is apparent under all culturing conditions within 12 hr of N_2 removal. Associated with this loss is a dramatic decline in photosynthetic capacity (Fig. 1). Under an atmosphere of air or N_2, phycobilin proteins and photosynthesis are restored in the vegetative cells as heterocysts mature and growth resumes (22,23). Under an argon atmosphere, however, nitrogen starvation is complete and photosynthetic capacity continues to decline. This situation is apparently reflected in the O_2 tensions found in the culture media (Table 1). Although there was some variation in sparging rate in these experiments, O_2 tensions were significantly higher in the N_2/CO_2-sparged cultures than in the argon/CO_2 cultures. This presumably represents photosynthetic O_2 production which would be much greater under N_2. Since DCMU at 10^{-5} M inhibits $^{14}CO_2$ incorporation by 95%, the low levels of O_2 present in the DCMU culture probably reflects reaeration during measurement and should be considered background levels. In an algal-free control bottle, oxygenation of N_2-sparged media occurred at a rate of 0.105 mg O_2/L/min. The O_2 sensitivity of nitrogenase activity in cultures induced under microaerophilic conditions correlates well with the O_2 tension in the culture media (Fig. 3). Thus, we conclude that the development of O_2 protection mechanisms are regulated by the presence of O_2.

Development of the Heterocyst Gas Diffusion Barrier

Since we could detect no evidence for reductant limitations during induction, differential development of the heterocyst gas diffusion barrier could account for the O_2 sensitivity of nitrogenase induced under microaerophilic conditions. The observation made earlier in this laboratory (9) that the apparent Km for acetylene reduction by nitrogenase was about five times greater *in vitro* than *in vivo* was interpreted as evidence for the existence of a passive gas diffusion barrier in intact cells. Since the affinity of the enzyme for acetylene presumably is the same *in vivo* and *in vitro*, this suggests that the acetylene concentration inside the heterocyst is much lower and, thus, its entrance into the heterocyst is impeded by the heterocyst envelope. Table 2 shows differences in the Km for acetylene found in cultures induced under different gas phases. In an argon-induced culture (Fig. 4a), the apparent Km for acetylene was about two times that reported for nitrogenase *in vitro* (9). In contrast, the apparent Km was five times greater in an air-induced culture (Fig. 4) than the Km *in vitro*. The Km for the N_2/CO_2-induced culture (Fig. 4b) was intermediate. It must, however, be pointed out that this data is preliminary. The difficulties in *in situ* O_2 measurement and Km determinations suggest that additional data will be required to strengthen the interpretation.

Respiratory Protection Mechanisms of the Heterocyst

Figure 5 shows that O_2 sensitivity of nitrogenase activity increases after addition of DCMU, resulting in a reductant-limited culture. Reductant limitation was induced by treating a log-phase N_2/CO_2-grown culture with DCMU for 4 hrs. Within this period, nitrogenase activity had dropped by 20% but was restored to the original level by the inclusion of 15% H_2 in the assay, indicating reductant limitations to enzyme activity (24).

The role of the cytochrome system in scavenging O_2 from the site of N_2

fixation was studied in vivo by the use of the cytochrome inhibitor sodium azide. Under anaerobic conditions, azide had no inhibitory effect on nitrogenase activity until concentrations of more than 30 mM were reached (Fig. 6). Dark acetylene reduction was almost completely inhibited at this concentration. In the dark, nitrogenase activity is apparently dependent on oxidative phosphorylation for ATP since O_2 is required (10,11,12). At 20 mM azide inhibition of this process is complete. In the light, under anaerobic conditions, azide-sensitive reactions are not involved in substrate supply to nitrogenase. Atmospheric levels of O_2 which had only a small inhibitory effect on nitrogenase activity under these conditions (see Fig. 2), inhibited nitrogenase activity by almost 70% when 20 mM sodium azide was also included. Azide does not affect nitrogenase directly at this conc. as seen from the anaerobic control. Thus, it appears that cytochrome activity is an important factor in O_2 protection of nitrogenase The inhibitory effects of high azide concentrations under anaerobic conditions may be attributed to inhibition of reductant or ATP generation in heterocysts or competitive inhibition of nitrogenase.

To identify the metabolic pathways responsible for providing substrate for nitrogenase activity and O_2 protection, selective inhibitors of carbon catabolism were also studied for their effect on short-term acetylene reduction. 2-4 dinitrofluorobenzene, a selective inhibitor of glucose-6-phosphate dehydrogenase (25), the first enzyme in the oxidative pentose phosphate pathway, nearly abolished acetylene reduction in the light and in the dark (Table 3). The presence of O_2 made little difference. Photosynthetic $^{14}CO_2$ incorporation was not influenced (Table 4), suggesting that the effect of this inhibitor was specific. These results indicate that the oxidative pentose phosphate pathway is the sole source of reductant for nitrogenase both in the light and in the dark under these conditions. The suggestion that organic substances can supply reductant to nitrogenase by donating electrons to photosystem I (26,27) is not supported

by these results. The results, however, do not allow any conclusion about the role of this pathway in O_2 protection.

Malonic acid, a competitive inhibitor of the Kreb's cycle enzyme, succinate dehydrogenase, had no effect on acetylene reduction rates in exponentially growing cultures (Table 5). Under conditions of reductant limitations, however, malonate actually stimulated nitrogenase activity to the same or greater degree as H_2. Figure 7 shows that maximum stimulation of acetylene reduction occurs at 5 mM malonate. The presence of 20% O_2 had little effect on either H_2 or malonate stimulation of nitrogenase activity.

Neither H_2 nor malonate stimulated the low residual nitrogenase activity in the dark. It is likely, however, that dark activities are limited more by ATP than reductant. Malonate is also known to inhibit phosphoenol pyruvate carboxylase, an enzyme involved in C_4 photosynthesis in blue-green algae (28) and some higher plants. No malonate effect, however, was observed in photosynthetic $^{14}CO_2$ incorporation (Table 5). It is interesting to note that azide stimulated $^{14}CO_2$ uptake, probably by inhibiting its subsequent oxidation. Further experiments are planned to elucidate the exact mechanisms of these inhibitors.

DISCUSSION AND PLANNED WORK

The protection from O_2 afforded nitrogenase by its localization in the heterocyst apparently involves at least two mechanisms--a diffusion barrier and respiratory protection. During induction under microaerophilic conditions, nitrogenase activity (measured under argon) begins to rise with the appearance of newly developed heterocysts 9 to 10 hr after transfer to N_2-free media. O_2 sensitivity, however, is much greater in these early stages of heterocyst maturation than later when most proheterocysts have developed into heterocysts

(21). This indicates that under these conditions, at least, nitrogenase synthesis precedes the full development of O_2 protection mechanisms in the developing heterocysts. The O_2-lability of nitrogenase and its dependence on substrate supply for activity, however, make it impossible to determine by *in vivo* assays of enzyme activity when during the differentiation process, nitrogenase synthesis is initiated. This question is of interest since it has recently been shown that nitrogenase synthesis in a number of organisms is repressed not only by the presence of ammonia but also by O_2. In organisms such as *Plectonema* (29) and *Klebsiella* (30,31) that have no apparent means of protecting nitrogenase from O_2 inactivation, extremely low O_2 tensions are necessary before nitrogenase synthesis is derepressed. In the obligate aerobe, *Azotobacter*, nitrogenase synthesis occurs in the presence of exogenous O_2, but respiratory activity is required. When respiration was blocked by azide, synthesis of this enzyme was inhibited (17), indicating that intercellular O_2 tension is a regulatory factor in this organism also.

Although under aerobic conditions nitrogenase activity can only be detected *in vivo* with the appearance of mature heterocysts, it may be that its synthesis is initiated at an earlier stage in heterocyst differentiation. Electrophoretic analysis of the proteins in differentiated and vegetative cells during the time course of heterocyst differentiation under aerobic conditions has suggested that the components of nitrogenase may appear in all cells during the early proheterocyst stage (32). Nitrogenase is supposedly shut off in the vegetative cells beyond this point but continues in those cells destined to become heterocysts. Since activity is not detected until the appearance of mature heterocysts, nitrogenase may be inactivated until O_2 protection mechanisms are developed. Alternatively, an inactive, O_2-resistant precursor may be synthesized in these early stages or perhaps activity is not expressed because substrate supply is not yet developed. These questions will be addressed

using immunoelectrophoretic techniques now being developed in this laboratory (Chapter VIII). Time course experiments during heterocyst induction in A. cylindrica should allow us to pinpoint the stage at which the Fe-Mo component first appears. The use of either an ouchterlony double diffusion test or immunoelectrophoretic methods should also allow us to differentiate between the normal Fe-Mo component and an inactive precursor. Some partial non-identity with the normal Fe-Mo component is found in crude extracts of tungsten-grown cultures of A. cylindrica where in vivo activity is nearly zero (33). As discussed in the following chapter, O_2 exposure of nitrogenase results in the formation of several molecular forms of the Fe-Mo component which can be identified by their different electrophoretic mobility. Thus, the 2-dimensional "rocket" assay should permit detection of O_2-inactivated enzymes.

Whether low O_2 tension is a prerequisite for nitrogenase synthesis in A. cylindrica will be difficult to ascertain, even during induction under aerobic conditions since intercellular O_2 tension certainly must be the key factor. To avoid the complications of simultaneous development of O_2-protection mechanisms in the developing heterocysts, we will attempt to correlate the appearance of the Fe-Mo component with heterocyst differentiation in mutants of A. variabilis which are deficient in the heterocyst glycolipids and are unable to fix N_2 under aerobic conditions.

Another possibility for the extreme O_2 sensitivity of nitrogenase activity in cultures induced under microaerophilic conditions is that the vegetative cells are capable of synthesizing nitrogenase under these conditions. Several workers (34-38) have reported the occurrence of nitrogenase activity in the vegetative cells of N_2-starved, argon-induced cultures of Anabaena. Our results provide little support for this idea. Although atmospheric levels of O_2 inhibited activity by more than 50% in argon-induced cultures (Fig. 2), 10% O_2

which completely inhibits nitrogenase activity in the non-heterocystous algae, Plectonema ( ), was only slightly (20%) inhibitory. Moreover, under the same culturing conditions, we have been unable to detect nitrogenase activity in N_2-starved Plectonema cultures (21). Only when light intensity was reduced ten-fold or when DCMU was added to inhibit photosynthetic O_2 production was nitrogenase induced in this alga. When DCMU is added to differentiating cultures of A. cylindrica 12 hrs after transfer to N_2-free media, neither heterocyst development or the rate of increase of nitrogenase activity is influenced over the next 12 hrs. Apparently sufficient carbon reserves are present after 12 hrs of N_2 starvation to support continued cellular differentiation and adequate substrate supplies to nitrogenase without additional carbon fixation. Differentiation, however, is arrested at the proheterocyst stage if DCMU is added immediately after initiating induction. In heterotrophic organisms, exogenous glucose alleviates the inhibitory effect of DCMU on the differentiation process. Heterocyst induction in the presence of DCMU then should provide the strict anaerobic conditions necessary for induction of nitrogenase synthesis in the vegetative cells of A. cylindrica if, in fact, undifferentiated cells are capable of doing so. Figure 2 shows that nitrogenase activity in DCMU-treated cultures was substantially more sensitive to O_2 than argon-induced cells. However, this may reflect less efficient O_2 protection by the heterocyst, both in terms of development of the gas diffusion barrier and to lowered reductant pools. Furthermore, if there were two pools of nitrogenase, then a biphasic response to O_2 would be expected. Due to the indirect nature of this approach, we are now conducting experiments to address the question of whether vegetative cells can synthesize nitrogenase under strict anaerobic conditions by directly labelling nitrogenase in situ with a labelled antibody (see Chapter XI).

The O_2 sensitivity of nitrogenase activity in fully differentiated

(i.e. after 24 hrs of induction, when most proheterocysts had matured), anaerobically induced cultures apparently reflect the development of an O_2 protection mechanism distinct from the protection afforded nitrogenase by the availability of carbohydrate pools. Since resistance to O_2 inhibition correlated well with the O_2 concentration of the media during induction (Fig. 3), we conclude that O_2 tension is an important regulatory factor in the development of the O_2-protection mechanism.

Ambient O_2 tension during differentiation strongly influences the development of the postulated gas diffusion barrier of the heterocyst envelope. The apparent Km for acetylene in an argon-induced culture was about twice that reported for nitrogenase *in vitro* (see Table 2). In contrast, the apparent Km was five times greater in an air-induced culture and an intermediate value was found in N_2/CO_2-induced algae. Since the affinity of the enzyme for acetylene presumably is the same *in vivo* and *in vitro*, we interprete this variation in apparent Km for acetylene as an indication of the intercellular level of acetylene and, thus, as an indirect measure of the resistance of the heterocyst envelope to gas diffusion. We cannot, however, exclude the possibility that O_2 tension also influences the development of respiratory protection mechanisms. In *Azotobacter* the cytochrome content correlates well with the O_2 tension of the media in continuous cultures (39). An analogous situation may exist in the heterocyst and, thus, even in the presence of high carbohydrate pools, the cytochrome content may be insufficient to reduce all the O_2 which enters the cell. At any rate, the correlation of O_2 resistance with *in situ* O_2 concentrations (Fig. 3) indicates that O_2 tension is an important factor in the development of the O_2-protection mechanism.

That respiration may act as an O_2 protection mechanism is suggested from reports in the literature of high rates of O_2-uptake (13) and elevated levels of glucose-6-phosphate dehydrogenase (40) in isolated heterocysts. Reduced carbon compounds produced on the vegetative cells are transported into the heterocyst to provide substrates for nitrogenase activity. A portion of this could be used to scavenge any O_2 that diffuses into the heterocyst. The addition of DCMU to A. cylindrica cultured under an atmosphere of N_2/CO_2 results in a steady loss of nitrogenase activity over the course of several hours (24). In short-term (15-30 min) experiments, however, DCMU has only a slight inhibitory effect on nitrogenase activity, indicating that the enzyme is not directly dependent on photosystem II activity for reducing power (41, 42). However, with prolonged incubation in the absence of carbon fixation, the heterocysts become depleted of reductant. Under these conditions, nitrogenase can be restored to nearly normal levels by the inclusion of H_2 in the gas phase during the assay period (24). This has been attributed to the presence of an uptake hydrogenase, which is localized in the heterocyst (43), which can couple to the electron transport pathway of nitrogenase, at least under conditions of reductant limitation (24).

Figure 8 shows that O_2 sensitivity of nitrogenase activity increases substantially in N_2/CO_2-grown cultures treated with DCMU for four hrs. Enzyme activity was apparently limited by reductant supply since activity (which had dropped by 20% in this period) was restored when assayed in the presence of H_2. These observations corroborate the findings of O_2 sensitivity of nitrogenase activity in aging (light-limited) A. cylindrica cultures (16).

The strong inhibitory effect of sodium azide on nitrogenase activity in the presence of O_2 indicates that cytochrome activity is an important factor in O_2 protection of nitrogenase. However, since no inhibition was observed at azide concentrations which abolish dark nitrogenase activity, oxidative

phosphorylation is unimportant in providing ATP to nitrogenase in the light. This supports the earlier conclusion that cyclic photophosporylation can supply all the necessary ATP for N_2 fixation (44-46). Inhibition of the oxidative pentose phosphate pathway by the selective inhibitor of glucose-6-phosphate dehydrogenase, dinitrofluorobenzene, nearly abolished acetylene reduction in the light and in the dark. This supports evidence (40,47,49) that in the heterocyst, the route of electron transfer from reduced carbon compounds to ferredoxin occurs via glucose-6-phosphate dehydrogenase. Whether this pathway also provides electrons for O_2 reduction or if another route of electron transfer occurs is not clear. Kreb's cycle activity is apparently not an important source of reductant for either process. Malonate, a competitive inhibitor of succinate dehydrogenase, had no effect on either nitrogenase activity or its O_2 sensitivity in log-phase cultures. However, under reductant limitations, this inhibitor actually stimulated nitrogenase activity. Its effect apparently does not involve inhibition of phosphoenolpyruvate carboxylase (51) since ^{14}C incorporation is not influenced by its presence (Table 4). Experiments are now in progress to identify the mechanism of the malonate effect. If malonate acts by inhibiting succinate dehydrogenase, than if should be possible to reverse the stimulation for nitrogenase activity by adding sufficient succinate to overcome competitive inhibition by malonate. The Kreb's cycle in blue-green algae is incomplete and thought to act in a biosynthetic role, providing carbon skeletons of amino acid synthesis, rather than carbon catabolism as in heterotrophic organisms (52). Thus, it is conceivable that inhibition of the kreb's cycle may result in a diversion of carbon compounds from amino acid synthesis to reductant generation. If so, it should be possible to simulate the malonate effect by exogenous sugars or Kreb's cycle intermediates.

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TABLE 1. O_2 tension in culture media during heterocyst induction under different gas phases. O_2 tension was measured 25 hrs after transferring undifferentiated cultures of A. cylindrica to N_2-free media with a New Brunswick

Gas Phase	Inhibitors	mg O_2/L
Air/CO_2	--	8.85
N_2/CO_2	--	1.17
Argon/CO_2	--	0.79
Argon/CO_2	1 x 10^{-5}M DCMU	0.498

Table 2. Km for acetylene reduction by A. cylindrica cultures 24 hrs after induction under various gas phases.

Gas Phase	Km in Atmospheres of Acetylene ($\times 10^{-3}$)
Air/CO_2	22
N_2/CO_2	12.9
$Argon/CO_2$	8.4
(in vitro)*	4.25

* See last year's Final Report.

Table 3. Effect of 5 mM 2, 4-dinitrofluorobenzene (DNFB) on nitrogenase activity in log-phase, aerobic cultures of A. cylindrica.

Assay Conditions	nME/min/mg	% Control
Light		
Argon	9.79	100
Argon + DNFB	0.24	2.5
Argon + 20% O_2	8.45	100
Argon + 20% O_2 + DNFB	0.27	3.2%
Dark		
Argon + 20% O_2	4.6	100
Argon + 20% O_2 + DNFB	.16	3.6

Table 4. Effect of inhibitors of carbon catabolism and respiration on $^{14}CO_2$ incorporation.

Inhibitor	cpm/min mL	% Control
--	34.8	100
2,4-dinitrofluorobenzidene (10 mM)	35.6	102
Malonate (10 mM)	33.8	97
Malonate (]0 mM)	36.8	105
Malonate (50 mM)	33.5	96
Na Azide (20 mM)	45.8	132

TABLE 5

EFFECT OF MALONATE ON SHORT-TERM ACETYLENE REDUCTION ASSAYS IN LOG-PHASE AND REDUCTANT-LIMITED A. cylindrica. Sodium malonate and sodium azide were added to a final concentration of 10 mM. Hydrogen and oxygen were 15 and 20%, respectively, of gas phase.

Culture Conditions	Assay Mixture	% of Control (under argon)
Exponential growth under N_2	light + malonate	97
	light + H_2	101
Induced for 24 hr under N_2 DCMU (10^{-5}m) added 14.5 hrs prior to assay*	light + H_2	228
	light + malonate	417
Air-grown, incubated in dark for 5 hrs+	light + H_2	145
	light + malonate	152
	light + H_2 + O_2 20%	149
	light + malonate + O_2	142
	light + NaN_3	96
	light + NaN_3 + malonate	101
	dark + O_2	14
	dark + O_2 + malonate	15
	dark + O_2 + H_2	14

*Activity dropped to 20% of control culture after 14.5 hrs of DCMU treatment.

+Activity dropped to 50% of control after 5 hrs in dark.

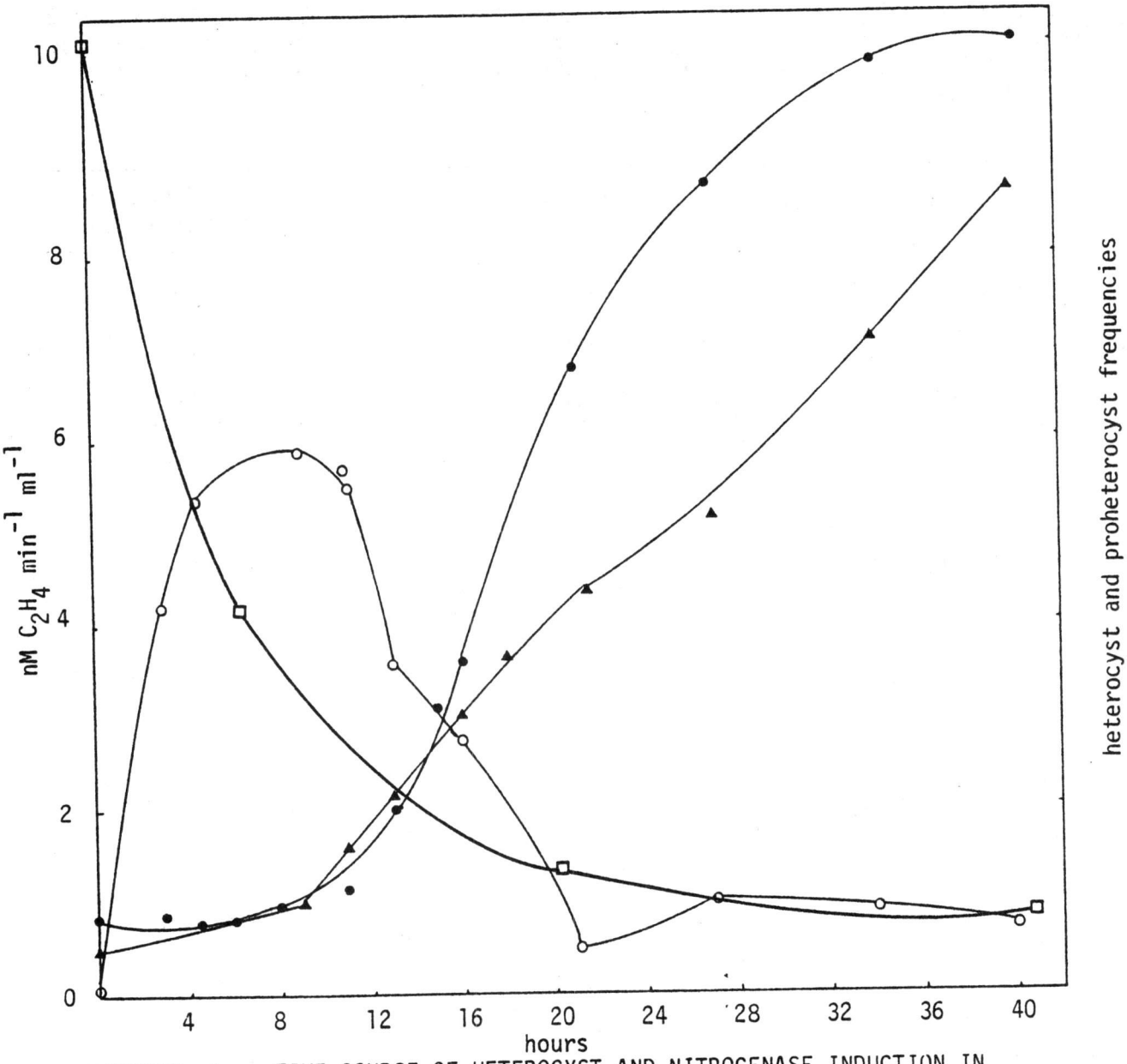

FIGURE 1. TIME COURSE OF HETEROCYST AND NITROGENASE INDUCTION IN A. cylindrica FOLLOWING TRANSFER TO NITROGEN-FREE MEDIA.

Cultures were incubated in the light under an atmosphere of argon/CO_2. Nitrogenase activity in n moles C_2H_4 min^{-1} ml^{-1} ( ); heterocyst frequency (number of heterocysts/100 cells) ( ); proheterocysts frequency (number of proheterocysts/100 cells) ( ). Proheterocyst and heterocyst frequencies are the results of two different experiments. -o- relative $^{14}CO_2$ incorporation cpm ml^{-1}.

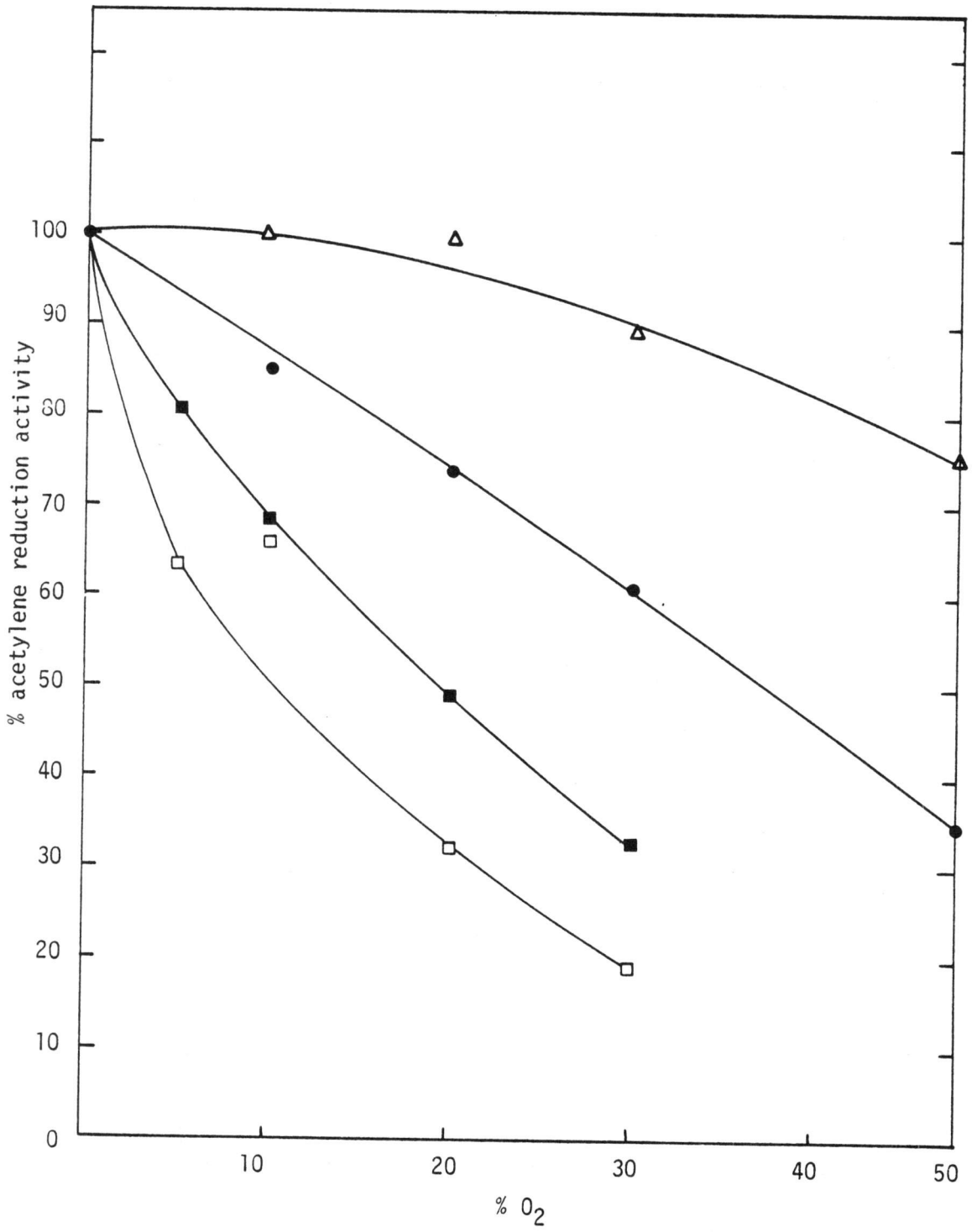

Figure 2. O_2 sensitivity of nitrogenase activity in A. cylindrica 24 hrs after initiating heterocyst differentiation. During the induction period, cultures were incubated under an atmosphere of air/CO_2 (Δ); N_2/CO_2 (●), argon/CO_2 (■) and argon/CO_2 plus 1 x 10^{-5} M DCMU which was added 12 hrs prior to the assay. The specific activities of the four cultures measured under argon were 36.2, 37.8, 43.4 , and 41.8 nME/min/mg dry wt, respectively.

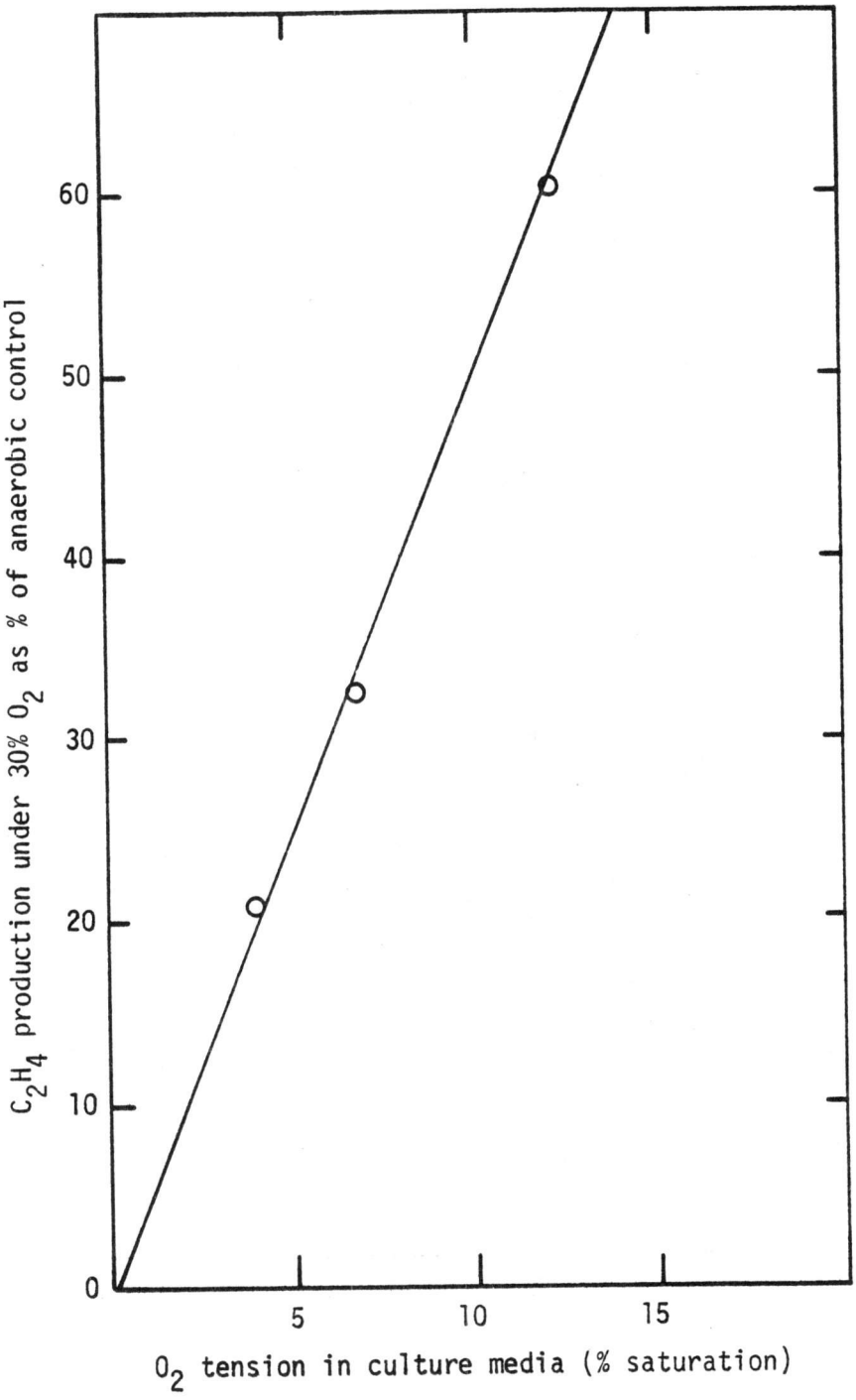

FIGURE 3. RELATIONSHIP BETWEEN O_2 SENSITIVITY OF NITROGENASE ACTIVITY IN A. cylindrica INDUCED UNDER MICROAEROPHILIC CONDITIONS AND O_2 TENSION OF MEDIA. Nitrogenase activity and O_2 tensions were measured 24 hr after initiating induction.

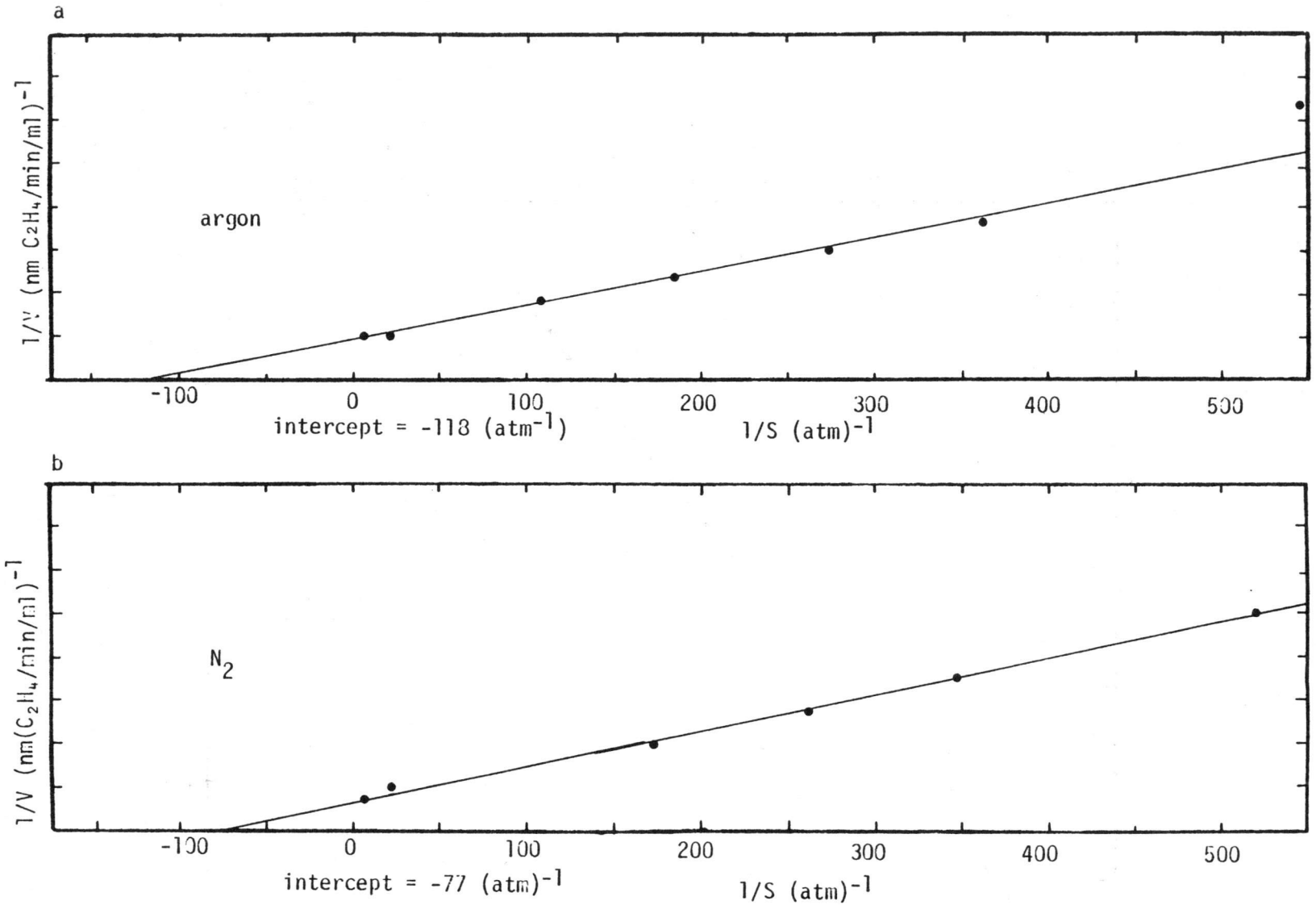

Figure 4. Apparent in vivo Km for acetylene reduction by A. cylindrica induced under air/CO_2 (c); argon/CO_2 (a) and N_2/CO_2 (b). 24-hrs following transfer to N_2-free media, 2 ml aloquots of algae were assayed for 15 mins under argon with varying concentrations of acetylene. Each point is the average of duplicate assays. The reported Km's were determined by linear regression analysis.

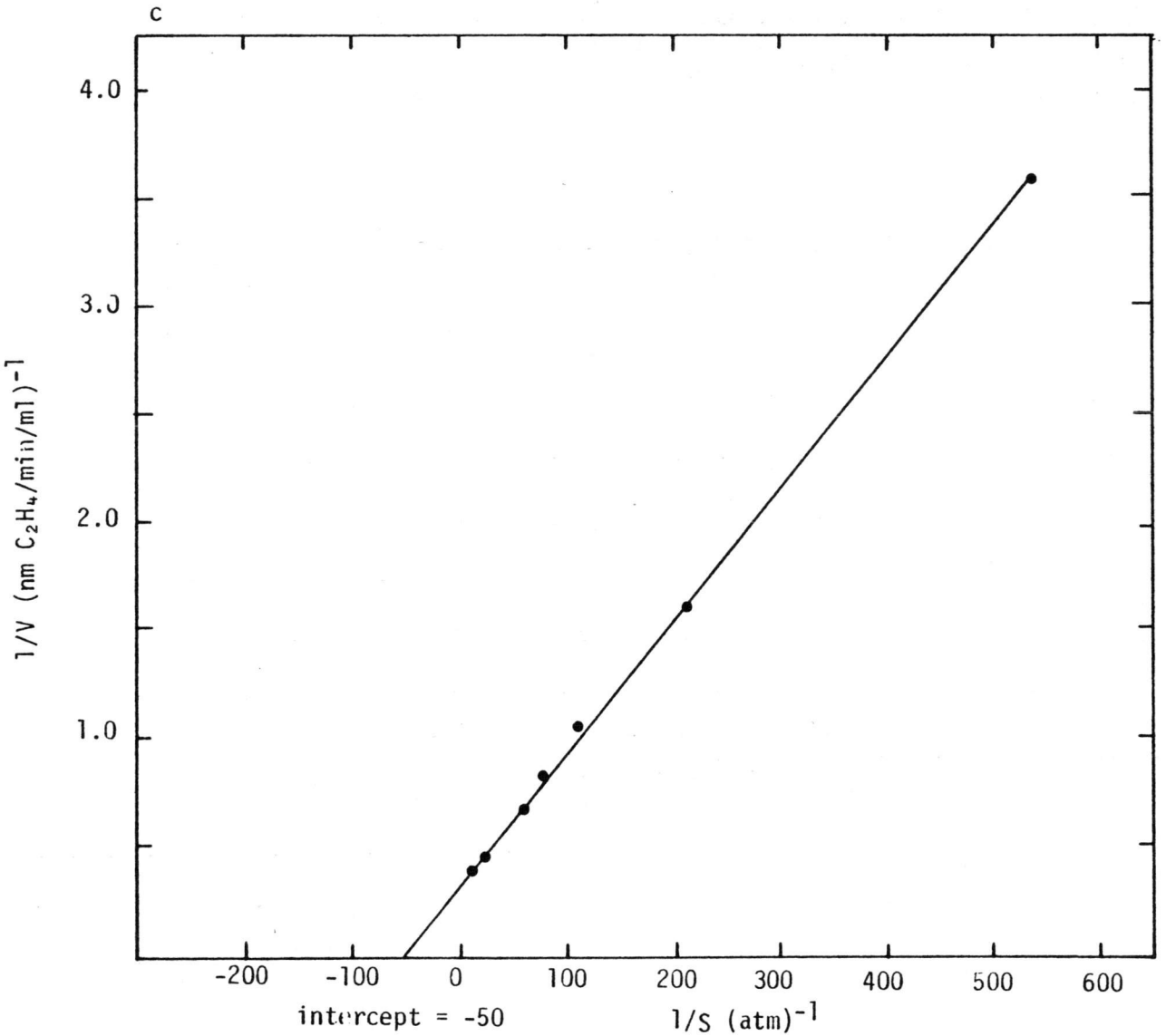
c
4.0
3.0
2.0
1.0
1/V (nm C_2H_4/min/ml)$^{-1}$
-200
-100
0
100
200
300
400
500
600
intercept = -50
1/S (atm)$^{-1}$

FIGURE 4

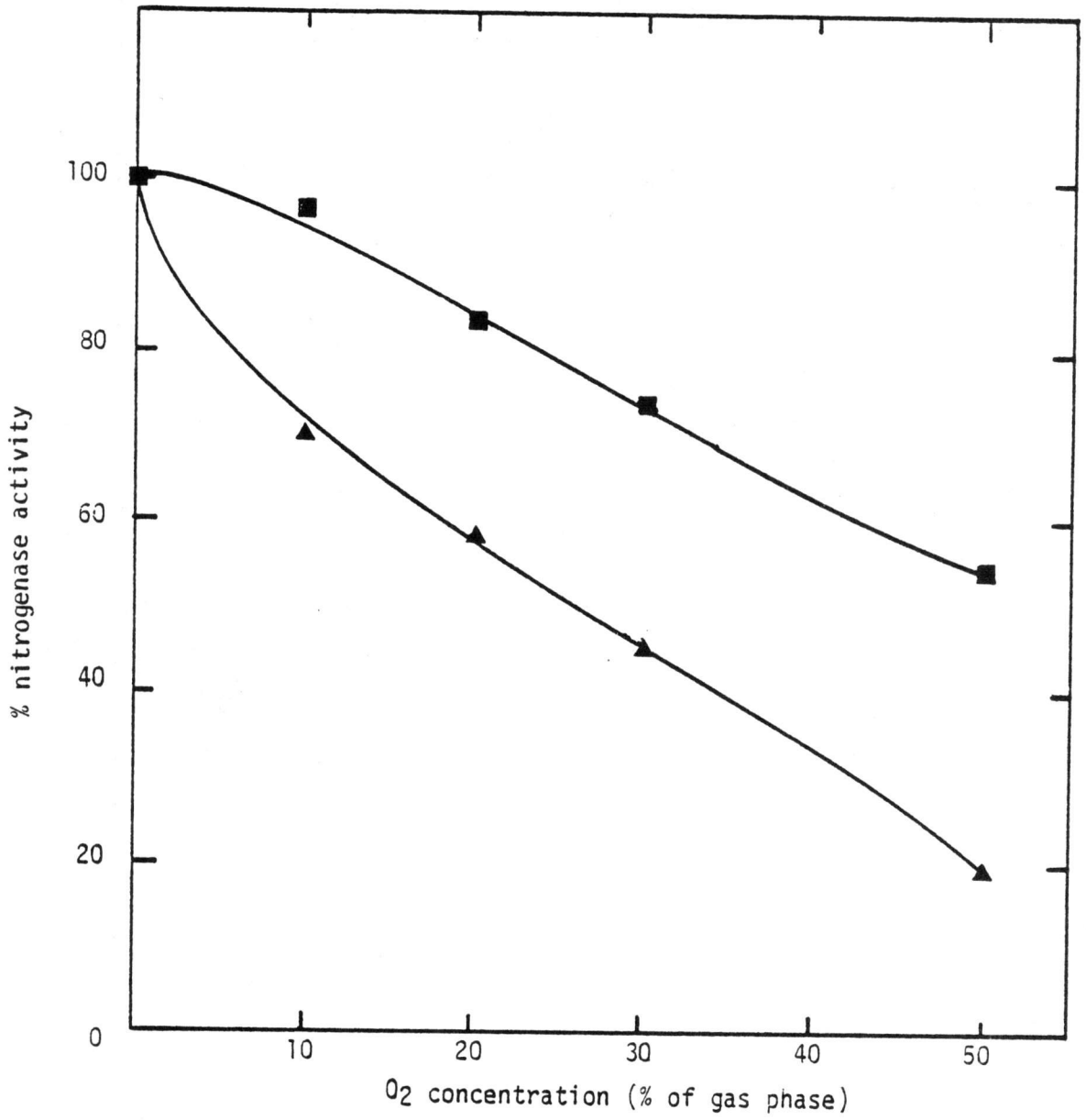

Figure 5. Effect of O_2 tension in log-phase (■) and DCMU-treated (▲) culture of A. cylindrica. N_2/CO_2-grown cultures were assayed for 30 mins, 4 hrs following DCMU treatment. Nitrogenase activity (under argon) was 10.58 nME/min/mg (dry wt) in the log-phase culture and 8.37 nME/min/mg (dry wt) in the DCMU-treated culture. 15% H_2 stimulated activity to 10.8 nM/min/mg in the DCMU-treated culture but had no effect on the log-phase culture.

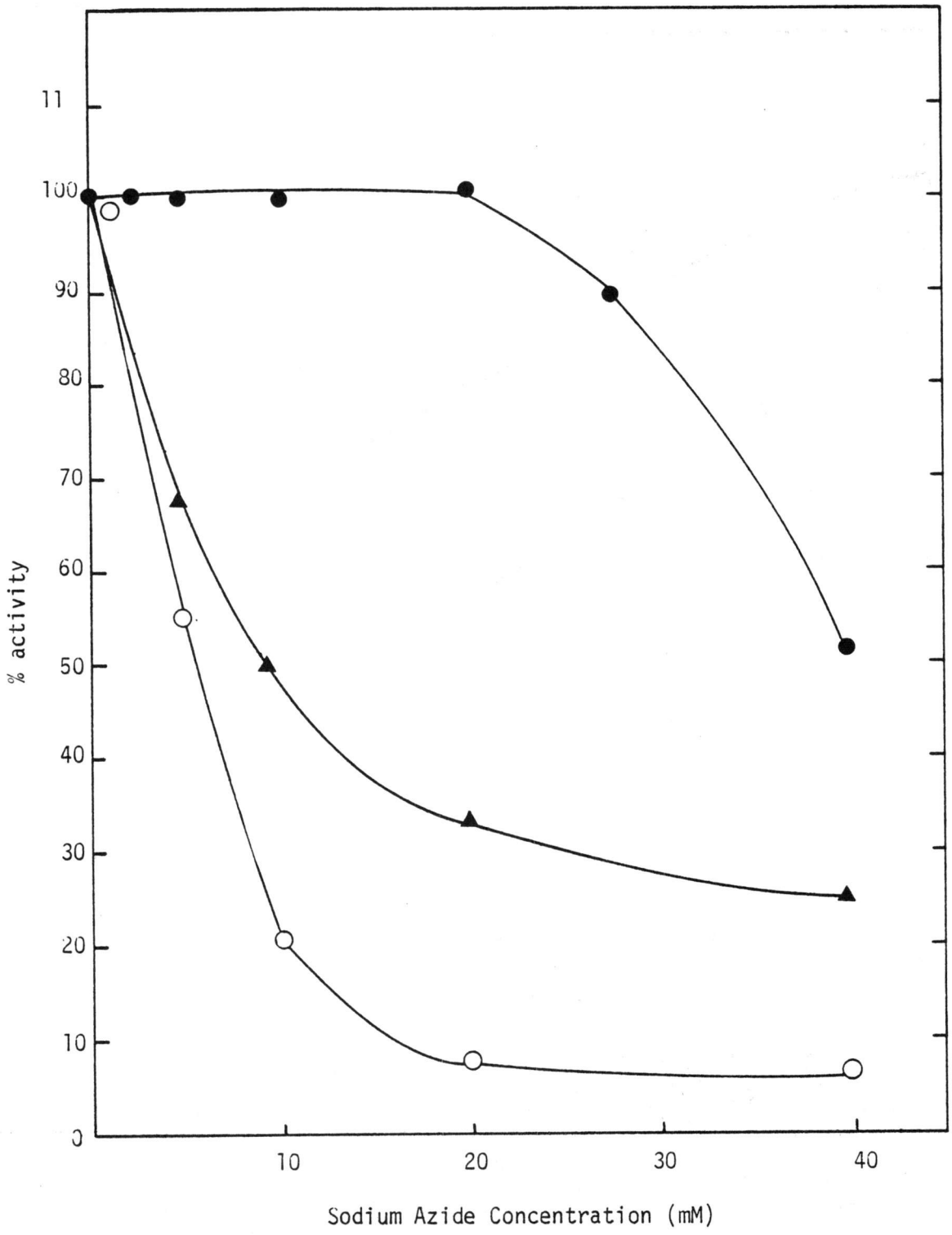

Figure 6. Effect of sodium azide on nitrogenase activity in N_2-grown log phase A. cylindrica. Cultures were assayed in the light under argon (●), and under argon + 20% O (▲) and in the dark (o) with 20% O_2.

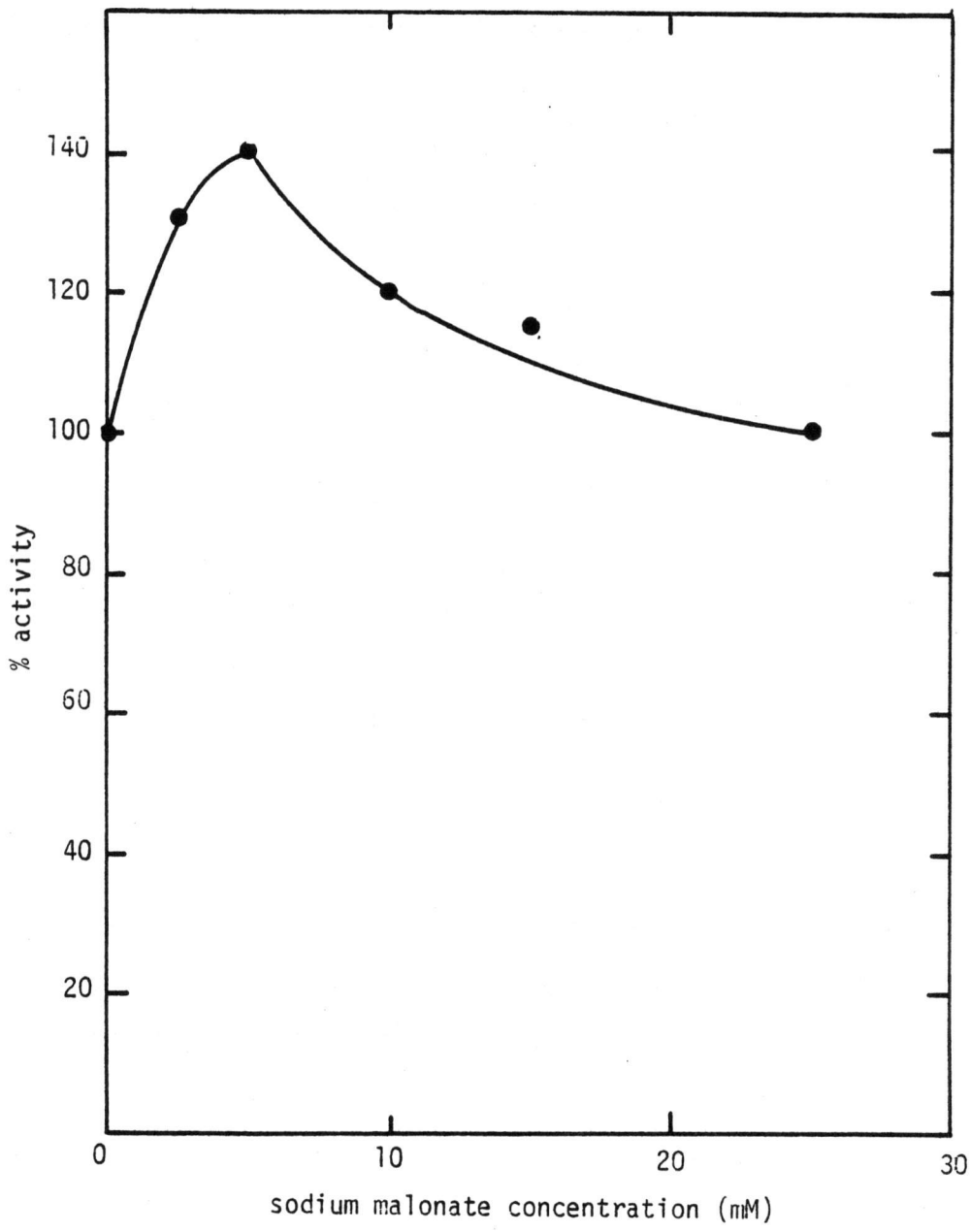

Figure 7. Malonate stimulation of nitrogenase activity in reductant-limited A. cylindrica cultures. Log phase N_2/CO_2 -grown cultures were pre-incubated in the presence of 1×10^{-5} M DCMU for 5 hrs.

X. NITROGENASE REGULATION: INACTIVATION AND TURNOVER

In an earlier communication (1), we reported a rapid loss of *in vivo* nitrogenase activity following inhibition of protein synthesis in exponentially growing, aerobic cultures of *Anabaena cylindrica*. This suggested that nitrogenase is continuously inactivated during aerobic growth and that the steady-state levels of enzyme activity found during growth must reflect a balance between nitrogenase synthesis and decay. The decline in nitrogenase activity appeared to be O_2 dependent since enzyme activity in filaments incubated under an argon/CO_2 atmosphere was quite stable in the absence of further protein synthesis. These observations, coupled with the fact that nitrogenase is extremely O_2 labile, suggests that O_2 may play an important role in the regulation of active nitrogenase concentration.

There is now appreciable evidence that the intracellular level of a specific protein is dictated not only by its rate of synthesis, but also by its rate of breakdown (2,3). Two distinct processes--enzyme inactivation and protein turnover--can effect protein breakdown in a cell. Procedurally, however, it is often difficult to distinguish between them. Inactivation, defined as the irreversible loss *in vivo* of catalytic activity of an enzyme (4), may or may not precede protein turnover which refers strictly to the hydrolysis of intracellular proteins to their component amino acids (2). To better define the nature of this apparent loss of enzyme, we have examined the effect of O_2 tension on the decay rate of nitrogenase activity *in vivo* and on the amount of the Fe-Mo component *in vitro* using immunological techniques.

MATERIALS AND METHODS

Axenic cultures of *Anabaena cylindrica* 629 were grown in 2.5-L glass

bottles on modified (2×10^{-3} M K_2HPO_4 and 5×10^{-3} $NaHCO_3$) Allen and Arnon Media. At least 12 hr before initiating an experiment, the culture was divided into several 250 mL bottles and incubated at 28-29°C as described in the previous chapter. Acetylene reduction and $^{14}CO_2$-fixation assays were conducted under argon for 15 and 30 min, respectively. Protein was determined using the Bio-Rad Protein Assay. The absorbance of the dye-protein complex was measured at 595 nM. Crossed immunoelectrophoresis was carried out on crude extracts of an argon-induced A. cylindrica culture by the procedure outlined earlier (1) except that molded rather than cast plates were used. Four percent polyethylene glycol was included in the agar to enhance sensitivity (5). One-hundred percent O_2 was blown across the surface of a freshly prepared cell-free extract for 0, 1, 3 and 5 min. After each exposure, approximately 200 of extract was removed and placed in argon-filled, serum-stoppered, 1 mL test tubes. The tubes were evacuated and filled with argon several times to remove all O_2 and then stored under pressure until assayed. Immediately after preparation 1 µl aliquots of extract were immunoelectrophoresed. The samples were stored at 4°C for 24 hr and then assayed again.

The area of the immunoprecipitate was estimated by tracing the outline of the precipitate on paper, cutting out the trace, and weighing it. The weight of the trace of anaerobically prepared samples immunoelectrophoresed at 0 hr and at 24 hr were identical and assigned the value of 100%.

RESULTS

Effect of Antibotics on Nitrogenase Activity

Figures 1 and 2 show the loss of in vivo nitrogenase activity in two different experiments where various inhibitors of protein synthesis were added to aerobically cultured, log-phase A. cylindrica. In each case, the half-life of nitrogenase loss was similar and ranged between 4.6 to 6.6 hr. A four-hr lag, however, was observed with both rifampcin and puromycin (Fig. 1). This may be due to the larger (MW > 500) size of these molecules and,

thus to the difficulty in penetration of the heterocyst. It is possible that the inhibitors were first taken up by the vegetative cells and then translocated. In the experiment shown in Figure 1, stock solutions of both rifampcin and puromycin were prepared by dissolving the inhibitor in small quantities of DMSO. DMSO increases cell permeability but at these concentrations had little effect on either growth or nitrogenase activity. Without DMSO, puromycin was only partially effective in inhibiting nitrogenase activity (Figure 2).

Table 1 shows that the concentrations of chloramphenicol and rifampcin used in these experiments were sufficient to inhibit further protein synthesis. Photosynthetic $^{14}CO_2$ incorporation was only slightly inhibited after 12 hr of incubation in the absence of protein synthesis. Thus, the photosynthetic apparatus appears to be turned over very slowly. Since reductant generation appears to be unaffected by antibiotic treatment, it is unlikely that substrate limitations to nitrogenase occurs with these antibiotics. This is supported by the lack of H_2 stimulation of nitrogenase activity, an indication of reductant limitations, 12 hours following treatment with these antibiotics (data not shown).

Effect of Oxygen Tension on in vivo Loss of Nitrogenase Activity

In contrast to the sharp decline of nitrogenase activity in aerobic cultures, the enzyme was quite stable after chloramphenicol treatment of cultures induced under an argon/CO_2 atmosphere (Figure 3). This is likely due to the low O_2 tensions on the culture media (see Table 3 in previous chapter). The relative stability of nitrogenase under these conditions suggests that O_2 inactivates nitrogenase on aerobically cultured algae. If this is the case, one would expect the half-life of nitrogenase activity to correlate with ambient O_2 tensions. Preliminary experiments (data not

shown) have shown that acetylene reduction was inhibited by 8 hr of chloramphenicol treatment only slightly more under aerobic culturing conditions than under N_2/CO_2. Inhibition was significantly greater when an N_2/CO_2 grown culture was switched to air/CO_2 immediately before adding chloramphenicol. The O_2 tension of the media, however, may not accurately reflect the inter-heterocyst O_2 tensions. As discussed in the previous section, the heterocyst gas diffusion barrier is poorly developed under microaerophilic conditions. Thus, photosynthetically produced O_2 may find its way into the heterocyst more readily in N_2/CO_2-grown cultures than in aerobic cultures. An ineffective gas diffusion barrier appears to be responsible for the more rapid loss of acetylene reduction activity when chloramphenicol treatment was followed by switching the N_2/CO_2-grown culture to air.

Table 2 shows an experiment in which air-grown A. cylindrica cultures were treated with chloramphenicol and then sparged with different concentrations of O_2. Since each culture was grown aerobically prior to the change in gas phase, presumably the development of the oxygen protection mechanism was similar in each culture at the start of the experiment. The loss of nitrogenase activity with chloramphenicol treatment was strongly influenced by the O_2 tension of the sparging gas; however, acetylene reduction and growth (data not shown) in the control cultures (no chloramphenicol) were not significantly affected. These observations suggest that to maintain steady-state levels of nitrogenase when incubated under increased O_2 tensions the rate of enzyme synthesis must increase to compensate for the greater loss of active enzyme.

In Vitro Loss of the Fe-Mo Component of Nitrogenase: Effect of Oxygen

Freshly prepared crude cell-free extracts of nitrogen-starved A. cylindrica were exposed to pure O_2 for varying amounts of time. The concentration

of the Fe-Mo component of nitrogenase was then measured immediately after O_2 exposure and again after 24 hours of incubation using 2-dimensional immunoelectrophoresis. Figure 4 shows that O_2 exposure resulted in moderate (up to 30%) increases in precipitate area of material immunoelectrophoresed immediately after O_2 treatment. This increase in peak area is due to the appearance of multiple immunoprecipitates. As discussed in last year's Final Report (1), exposure to air results in the appearance of several molecular forms of the antigen which differ in electrophoretic mobility from the component of anaerobic preparations. A similar situation has been reported for O_2-inactivated nitrogenase from Azotobacter (6). The multiple precipitates then arise from the formation of distinct immunocomplexes when the separated forms of the antigen are electrophoresed into the antibody gel.

The amount of cross-reactive material decreased dramatically upon incubation of the O_2-inactivated extracts. Within 24 hours at 4°C, more than 50% of the antigenic material was lost. O_2 exposure was required for the decline in cross-reactive material since in the anaerobic samples the area of the immunoprecipitates were identical when assayed immediately or 24 hours later (Figure 4).

DISCUSSION AND FUTURE WORK

If one assumes that steady-state levels of enzyme are regulated simply by a balance between enzyme synthesis and degradation as suggested first by Bone a decade ago (12), then the rate of either process can be measured by inhibiting the other. This approach, however, presents several difficulties related to the possibility of both direct and non-specific inhibitory effects of antibiotics on culture metabolism. This is particularly a problem when in vivo assays are used to indicate enzyme concentration since the inhibitor may disrupt, for example, substrate supply to the enzyme, thus

masking any specific effects an antibiotic may have on it synthesis. Furthermore, in in vivo studies, it is necessary to demonstrate that the drug can effectively penetrate the cell (in this case, the thick-walled heterocyst or translocate from the vegetative cells) and effect inhibition rapidly and completely. We have approached these problems by using a variety of inhibitors of protein synthesis, each with a different mechanism of action and comparing their effects on the loss of nitrogenase activity.

Chloramphenicol is widely used as an inhibitor of protein synthesis in prokaryotes. The drug exerts it inhibitory effect in E. coli by preventing the attachment of m-RNA to ribosomal sites (8). Evidence that this antibiotic is effective in blue-green algae has come from the demonstration that radioactive chloramphenicol binds to the ribosomes of Oscillatoria and chloroplasts but not cytoplasmic ribosomes (9). However, Rodriquez-Lopez et al. (10) found that chloramphenicol inhibited ^{14}C-phenylalanine incorporation in a cell-free system using A. nidulans ribosomes by only 30%. In contrast, similar concentrations of chloramphenicol completely inhibited protein synthesis in Anabaena cylindrica in vivo (11). Puromycin also effects inhibition of protein synthesis at the translational level but at a later stage in the process. This drug acts as an analogue of amino acyl-t-RNA's and inhibits protein synthesis by causing premature termination of the growing polypaptide chains (12). There is evidence that puromycin binds to the amino acyl-t-RNA binding site on the ribosome and then reacts with peptidyl t-RNA forming the corresponding peptidyl-puromycin (13). In contrast to these two drugs, rifampcin does not inhibit protein synthesis directly but rather acts by preventing synthesis of cellular RNA by its specific inhibition of DNA-dependent RNA polymerase (14). Once chain initiation is accomplished, however, RNA synthesis is resistant to rifampcin. Furthermore, protein synthesis continues unabated on preformed m-RNA until it decays (15). Purified preparations of RNA

polymerase from Anacystis nidulans are apparently not as sensitive to rifampcin as the bacterial enzymes. However, 130 μM rifampcin inhibited ^{3}H-uridine incorporation in reconsitituted systems of this alga by 88% (10). Cancanavine, in contrast to the other antibiotics, does not prevent protein synthesis per se, but rather this amino acid analogue is incorporated into proteins, rendering them enzymatically inactive.

Each of these inhibitors of protein synthesis resulted in similar rates of exponential decay of nitrogenase activity (Fig. 2). As discussed earlier, the lag (which is of variable length in different experiments) observed with rifampcin and puromycin apparently reflects slower penetration to the heterocyst. However, since inhibition of nitrogenase activity, once effected, occurred at the same rate as the smaller, presumably more permeable antibiotics, their inhibitory action was probably complete.

The photosynthetic apparatus apparently is turned over very slowly in this alga since photosynthetic capacity was inhibited by less than 10% after 12 hours of antibiotic treatment. A slow turnover rate for cellular protein in Anabaena was reported earlier (16). These workers estimated the half-life of total protein in aerobically grown Anabaena 7120 to be 150 to 170 hours. The small inhibitory effect of these drugs on photosynthetic capacity, coupled with the lack of H_2 stimulation of nitrogenase activity, further suggest that metabolic integrity is maintained in the absence of protein synthesis. Thus, it is unlikely that interference with substrate supply is responsible for the loss of nitrogenase activity but rather these results indicate that the enzyme is unstable under these conditions. However, we cannot as yet exclude the possibility that ATP generation or other cellular processes are adversely affected. An effect on ATP supply seems unlikely since cyclic photophosphorylation is considered to be the major

source of ATP for nitrogenase (19) and photosynthetic capacity was not influenced. Immunoelectrophoretic techniques now being developed will allow a more direct means of determining enzyme concentration and, thus, permit more definitive answers in this area.

In common with bacterial nitrogenases, cyanophycean nitrogenases are irreversibly inactivated by O_2. This, coupled with the negative correlation between stability of nitrogenase activity and O_2 tension (see Table 2 and Figure 3), strongly suggests that the decay of activity observed with antibiotic treatment is due to O_2-effected inactivation of the enzyme rather than protein turnover directly. This supposition is supported by the findings that the Fe-Mo component of anaerobically prepared cell-free extracts is not degraded by intracellular proteases. In contrast, more than 50% of the antigenic material in extracts exposed to O_2 was lost after 24 hours of incubation. Thus, it appears that this alga possesses an active protease which is specific to O_2-inactivated, but not native, nitrogenase. Experiments are now in progress to identify the class of protease responsible for nitrogenase degradation by its susceptibility to active site inhibitors. This information should allow us to firmly establish that O_2 inactivation rather than proteolysis is involved in the loss of enzyme activity by growing the algae in the presence of the specific protease inhibitor. Preliminary experiments (data not shown) indicate that PMSF, a specific serine protease inhibitor, will inhibit heterocyst induction (presumably by preventing turnover during nitrogen starvation) but has no effect on growth on ammonia. Similarly, exponential growth of several bacteria is unaffected by concentrations of other protease inhibitors that block protein degradation during metabolic shift-down (18, 19). The cellular location of this protease is also of interest. If it is specific to nitrogenase, then it is likely to be found only in the heterocyst and as such represent another example of a protein induced during differentiation.

The rather rapid apparent half-life of nitrogenase *in vivo* in aerobic cultures *A. cylindrica* indicates that large inputs of cellular energy are required to maintain the steady-state levels of enzyme. This seemingly represents a wasteful drain on the cellular economy of this organism. There is no obvious adaptive advantage to rapid nitrogenase inactivation as is the case with other examples of enzyme degradation (21-23). Thus, this phenomenon may be an inevitable consequence of carrying out simultaneously the basically incompatible processes of O_2-evolving photosynthesis and nitrogen fixation. Although heterocysts possess at least two mechanisms to protect this enzyme from O_2 (see previous chapter), it is unlikely that O_2 is completely excluded from the site of nitrogen fixation. Since nitrogenase is extremely O_2 labile, even a small amount of O_2 penetrating the heterocyst would result in nitrogenase inactivation. Furthermore, the results shown in Table 2 suggest that the algae are capable of altering the rate of nitrogenase synthesis to compensate for the rate of inactivation. Whether nitrogen starvation due to loss of active enzyme is responsible for the apparent increased rates of enzyme synthesis when the O_2 tension of the media is increased or if more subtle regulatory mechanisms are involved in this phenomenon deserves further consideration.

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TABLE 1. EFFECT OF RIFAMPICIN AND CHLORAMPHENICOL ON PROTEIN SYNTHESIS AND PHOTOSYNTHETIC CAPACITY IN A. cylindrica. Protein content and $^{14}CO_2$ incorporation were measured 12 hr following the addition of antibiotics to log phase, aerobic cultures.

Sampling Time	Treatment	Protein (µg/mL)	% of hour 0	$^{14}CO_4$ Incorporation	
				cpm/mg dry wt/min	% of control
Hour 0	none	.09	100	--	--
Hour 12	none	.168	187	400.5	100
Hour 12	10 µg/ml rifampicin	.114	126		
Hour 12	20 µg/ml rifampicin	.083	92.3	376	94
Hour 12	30 µg/ml rifampicin	.092	102		
Hour 12	10 µg/ml chloramphenicol	.113	125		
Hour 12	25 µg/ml chloramphenicol	.088	97	377	94.3
Hour 12	75 µg/ml chloramphenicol	.096	106		

TABLE 2. EFFECT OF OXYGEN TENSION IN THE CULTURE MEDIA ON THE LOSS OF NITROGENASE ACTIVITY 9.5 HR FOLLOWING CHLORAMPHENICOL TREATMENT OF A. cylindrica. At hour 0, 50 μg/ml of chloramphenicol was added to actively growing aerobic cultures (acetylene reduction activity = 0.7 nM C_2H_4 min^{-1} ml^{-1}) and the gas phase changed to N_2CO_2 (99.7:0.3); $N_2/O_2/CO_2$ (~50:50:0.3) or left with air/CO_2 (0.3% CO_2) sparging.

Gas Phase	Chloramphenicol	Nitrogenase Activity After 9.5 hr of Treatment (nM C_2H_4 min^{-1} ml^{-1})	Activity as % of hr 0 value
N_2CO_2	-	1.4	200
	+	0.385	55
Air/CO_2	-	1.25	180
	+	0.21	30
$N_2/O_2/CO_2$	-	1.3	186
	+	0.085	12.1

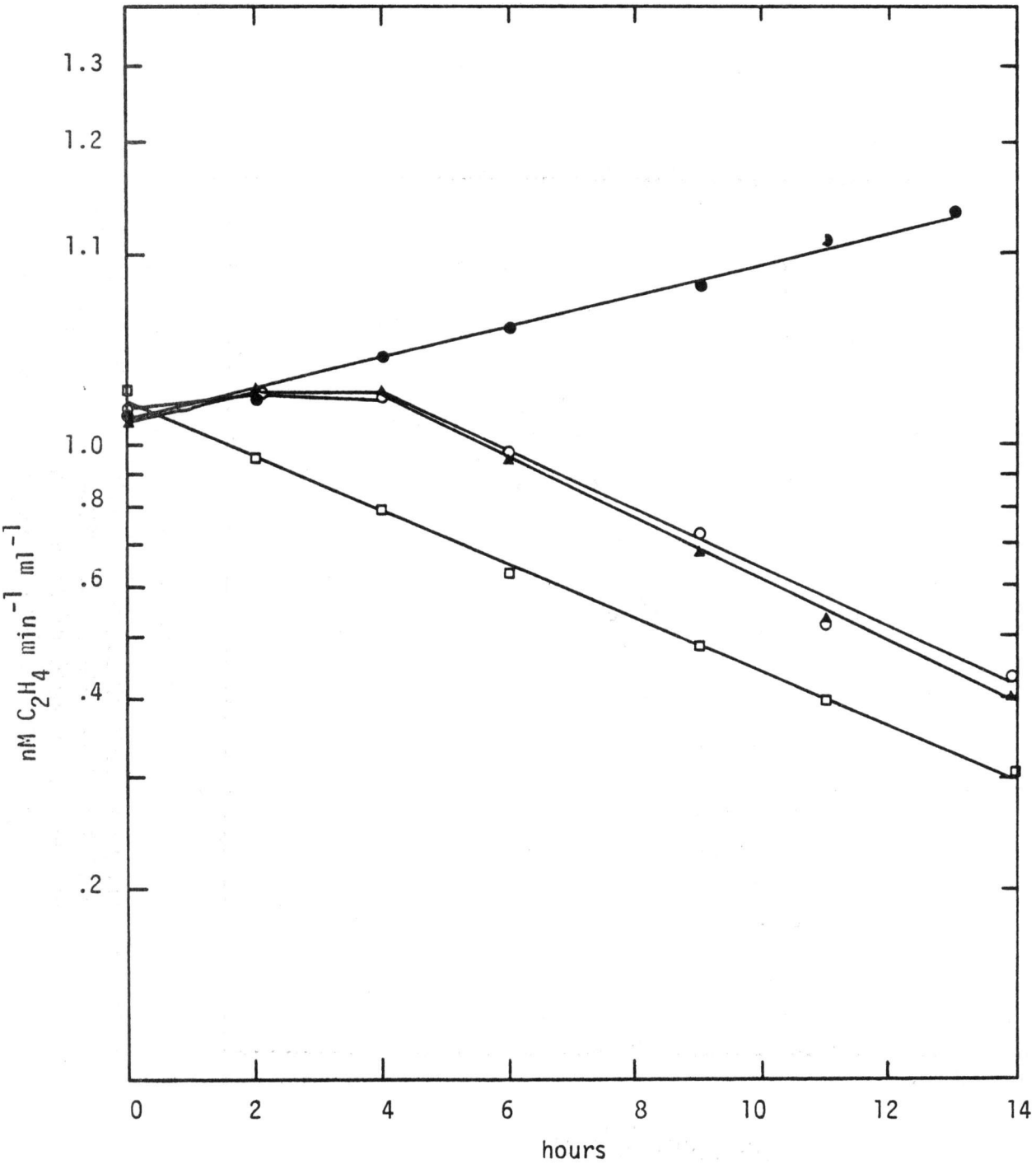

FIGURE 1. EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON ACETYLENE REDUCTION ACTIVITY IN LOG-PHASE, AEROBICALLY CULTURED A. cylindrica. At hour 0, 50 µg/ml chloramphenicol (□), 40 µg/ml puromycin (○) and 20 µg/ml rifampcin (▲) were added to parallel cultures. Control series (●). Puromycin and rifampcin were dissolved in DMSO, chloramphenicol was dissolved in 100% ethanol.

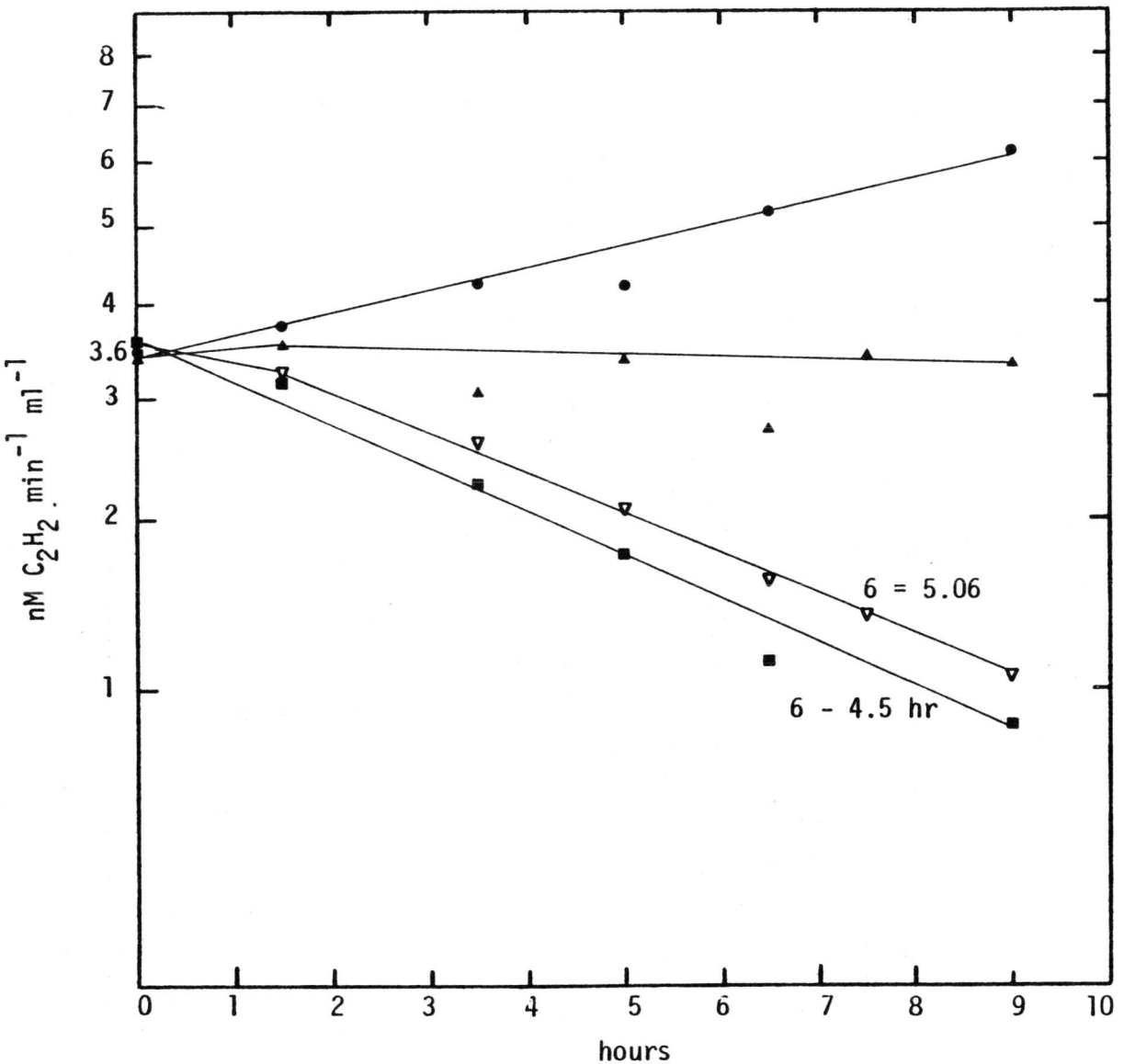

FIGURE 2. EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON NITROGENASE ACTIVITY IN LOG-PHASE, AIR-GROWN A. cylindrica. At hour 0, 50 µg/ml of chloraphenicol (■), 25 µg/ml cancanavine, 40 µg/ml of puromycin (▲) were added. Control (●).

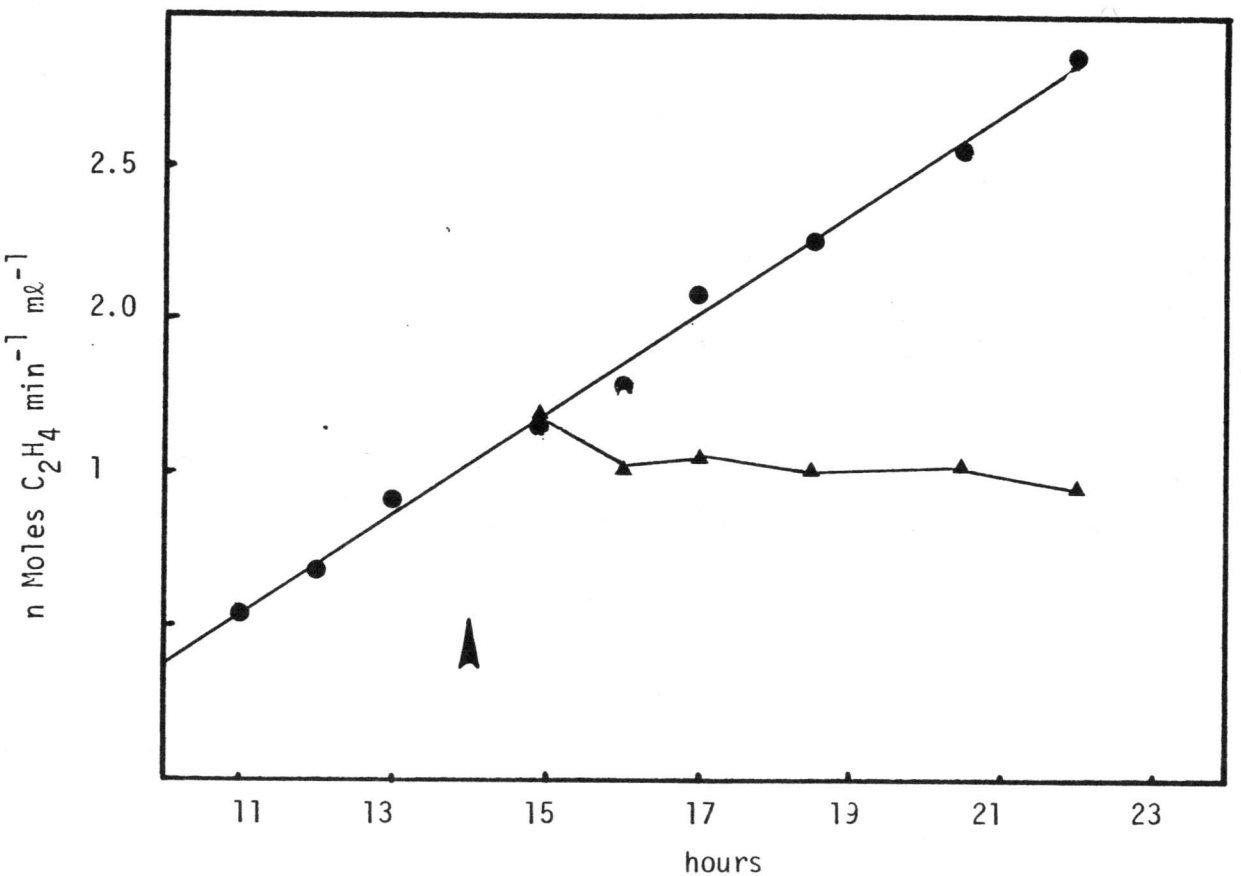

FIGURE 3. EFFECT OF CHLORAMPHENICOL ON NITROGENASE INDUCTION IN A. cylindrica INCUBATED UNDER ARGON/CO_2. Fifteen hours after transfer to nitrogen-free media, 50 µg/ml chloramphenicol (▲) was added to parallel culture. Control (●).

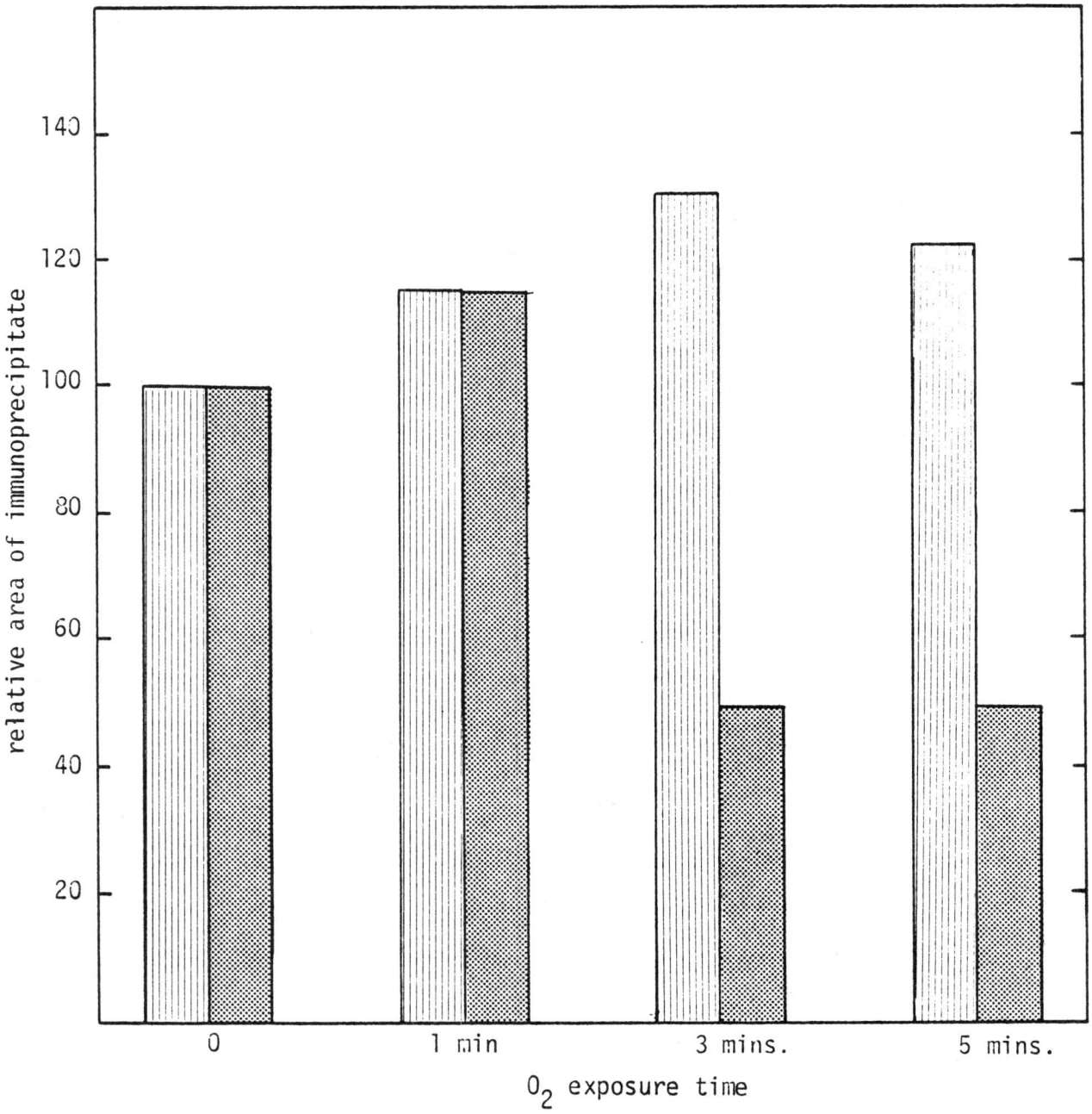

FIGURE 4. EFFECT OF OXYGEN EXPOSURE OF CRUDE EXTRACTS OF A. cylindrica ON THE AREA OF IMMUNOPRECIPITATE FORMED BY TWO-DIMENSIONAL IMMUNOELECTROPHORESIS. 1 µl samples were arranged immediately following O_2 exposure (III) and after 24 hrs of incubation at 4°C (■).

XI. LOCALIZATION OF NITROGENASE AND OXIDASES IN FROZEN SECTIONS OF *Anabaena cylindrica*

INTRODUCTION

The recent developments in cryomicrotomy provide powerful new techniques for cytological localization of enzymes at both the light and electron microscopic level. Ultrathin frozen sections were first obtained in the laboratory of Bernhard more than a decade ago [1,2]. Since then, several procedures for cryomicrotomy have been published [3,4,5]. The basic procedure involves light glutaraldehyde fixation to immobilize soluble components, infusion with cryoprotetants and gelatin encapsulation. The tissue is then frozen and sectioned at very low temperatures. The rationale behind this technique is simple. The conventional sectioning procedures, involving harsh chemical fixation, dehydration, and embedding in highly reactive epoxy resins, produce remarkable electron micrographs, but the process also destroys enzymatic activity, antigenicity and extracts most soluble and numerous macromolecular components. Thus, cytochemical and immunocytochemical reactions were generally not possible.

Previously, enzyme localization by cytochemical and particularly immunological means had been largely limited to extracellular components. The large size of immunological markers, especially ferritin, impedes penetration into intact cells. Similarly, diffusion of reagents for cytochemical enzyme reactions may likely be influenced by cellular structures. Thus, comparisons of enzyme activity in different cellular or subcellular locations, which would require that the reactants have equal access, is difficult to achieve in intact tissue. A further problem with conventional enzyme and immunocytochemical techniques involves the difficulty in washing unreacted substrate from the tissue. These shortcomings can be avoided by carrying

out the reactions directly on thin sections of the cellular material. The major drawback to this approach lies in the difficulty of obtaining ultrathin sections of tissue which still retain enzyme activity or antigenic components.

The preservation of biological activity in the hydrated state in frozen ultrathin sections is generally quite good. However, the necessity of immobilizing soluble components requires fixation by cross-linking agents which can greatly modify the cellular proteins. Gluteraldehyde is widely used in ultrastructural studies; however, this reagent can seriously reduce immunological labelling of antigens either by modifying the antigenic components or rendering them inaccessible by extensive cross linking (6). Aldehyde fixation may also inactivate or alter the substrate specificity of cellular enzymes (7). For these reasons, greatly reduced aldehyde concentrations and fixation periods have been used in recent cytochemical studies. Consequently, conservation of ultrastructure is not always possible. Improved procedures, however, have been reported (8,9) and morphological detail, approaching that of conventional techniques, has been obtained in frozen ultrathin sections suitable for cytochemical studies.

Here we report our progress in cytochemical studies using frozen sections of Anabaena cylindrica. Although ultrathin frozen sections, suitable for ferritin-labelled antibody reactions, have been obtained, the poor immunological activity of ferritin conjugates, coupled with technical difficulties encountered in the staining procedure, have led us to explore other antibody markers. Antibody probes, using fluorescent dyes conjugated directly to antibody, were introduced almost 30 years ago as a means of visualizing antibody-antigen reactions (10). Peroxidase-labelled antibodies also allow identification of immunological reactions and has an advantage over fluorescent markers in that the system is applicable at both the light and electron

microscopic level. The location of the antigen is visualized by reacting the peroxidase labelled antibody-antigen complex with diaminobenzidine (DAB). DAB is oxidized by peroxidase forming an insoluble osmospilic polymer. Our use of a peroxidase-labelled antibody preparation to localize nitrogenase on thick (1μ) sections led to the observation of endogenous peroxidase activity that was localized in the heterocyst (see below). This precluded the use of this system for nitrogenase localization studies, but this oxidase activity is of interest since it may be an important factor in heterocyst function. DAB is known to react with a number of cellular oxidases including catalase, peroxidase, cytochromes (including cytochrome oxidase) and other heme proteins (11). The O_2-consuming nature of these reactions, the location in the heterocyst, and the reports of large increases in peroxidase (12) and ascorbic acid oxidase (13) activity during heterocyst differentiation, suggest that the enzyme (or enzymes) responsible for DAB oxidation may be involved either in substrate production or O_2 protection of nitrogenase.

MATERIALS AND METHODS

Fixation and Eucapsulation

Aerobic nitrogen-fixing cultures of A. cylindrica 629 were prepared for immuno-flourescent, immunoferritin and 3,3-diaminobenzidine (DAB) staining of ultrathin and thick (0.5 μ) sections using a light glutaraldehyde fixation followed by freeze-encapsulation. For immunoferritin and fluorescence studies, 1 L of log-phase culture was incubated in an equal volume of 0.5% glutaraldehyde in cold 0.1M Na_2PO_4 buffer pH 7.5 (final concentration 0.25% fixative in 0.05 M buffer) for 15 min at 4°C. The 2 L were aliquoted to 250 ml bottles and harvested by a 15-min centrifugation at room temperature. Cells were twice resuspended and centrifuged as above in 1 L 4°C 0.05 M Na_2PO_4 buffer. The subsequent resuspensions in 400 ml, 400 ml, and 100 ml buffer were

centrifuged for 10,000 rpm at 4°C for 10 min. The final 2 pellets were re-suspended in approximately 1 ml cold buffer. For DAB studies, fixation was as above, except that one 1 L/20°C centrifugation was replaced by an additional 400 ml/4°C centrifugation. Prior to freezing, cells were infused with an equal volume of a 2 M sucrose/1.5% gelatin mixture in buffer for at least an hour at 4°C. Gelatin does not serve as a cryoprotectant but its addition was found to greatly enhance sectioning ability. Fast freezing was accomplished by immersing the tissue held to a copper stud in liquid nitrogen-cooled freon. Storage of the prepared tissue was in liquid nitrogen.

Cryomicrotomy

Ultrathin (70-100 μM) and thick (0.5 μ) sections were obtained using a Sorvall-Blum MT-2 ultramicrotome equipped with a Sorvall Model Cryokit. The most satisfactory temperature for obtaining ultrathin sections ranged between -70° and -90°C. -50°C was sufficient for thick sections. Ultrathin sections were retrieved from the dry knife edge by adherance to a small droplet of 2 M sucrose and 1.5% geletin; the geletin serves to reduce surface tension minimizing ultrastructural damage to lightly fixed cells ( ). Sections were transferred to Formvar-coated, 200 mesh nickel grids by touching the surface of the droplet on which the sections floated to the surface of the grid. The sucrose was washed from the sections by floating the grids for 5 to 15 min on successively lower concentrations of sucrose (1M, 0.5M, 0.25M, and 0.1M) and 1.5% gelatin in PSB. The grids were then washed on 0.1M tris buffer prior to ferritin staining. Thick (.5 μ) frozen sections were retrieved with an eyelash and transferred to agar-coated (1%) glass slides. Immediately after transferring the sections, the slides are placed in a large volume of 0.1 M tris buffer (pH 7.5) to prevent the sections from drying out and to wash off the sucrose.

Cytochemical Staining

Ferritin-labelled antibody. In the present study we employed an indirect immunological procedure in which the ferritin was conjugated to rabbit anti-goat IgG by Kashida's two-step glutaraldehyde method as described in Chapter VIII, rather than directly to the anti-nitrogenase serum as described in last year's report (14). The ferritin conjugate was reacted with ultrathin sections that were first treated with the ammonium-sulfate fraction of anti-nitrogenase goat serum.

Freshly cut, ultrathin sections on formvar-coated grids were floated for 5 to 15 min on droplets of either 4% BSA, 0.5% gelatin, or 1% cytochrome or combinations of these solutions to decrease non-specific ferritin-staining. The grids were washed by floating on large volumes of 0.1M tris (pH 7.5) buffer and then transferred to small droplets of the primary antibody diluted 1:5 or 1:10 with tris buffer. Sections were incubated at room temperature for 20 to 30 min in covered petri dishes with moistened filter paper on the top surface to prevent desication. The grids were then washed with buffer and floated on 1:5 or 1:10 dilutions of the ferritin-conjugate (0.63 mg protein/ml) for 30 min to one hr. As a control, the primary antibody treatment was either left out or followed by incubation with rabbit anti-goat IgG prior to the ferritin conjugate application. Unreacted ferritin was washed off by swirling the grids for 1 to 2 min in several changes of buffer and was followed by a final wash in distilled water. The grids were air-dried and observed with a Zeiss electron microscope at an accelerating voltage of 60 KV.

Fluoroscein-labelled antibody. Glutaraldehyde-fixed, 1 micron-thick frozen sections were incubated with the staining reagents added directly to the surface of the slide, covering the sections. Care was taken to leave a slight film of liquid on the sections at all times. Incubation was conducted at room temperature in covered petri dishes containing moistened filter paper

to prevent dessication. To prevent non-specific staining, the sections were incubated with a 1:20 dilution of normal goat serum for 15 to 20 min. The sections were washed by dipping the slide into several changes of tris buffer (0.05 M, pH 7.5), excess liquid was drained and the sections were incubated for 15 min with a 1:5 dilution of whole anti-nitrogenase goat serum. After washing the slide in buffer, a 1:5 dilution of fluorescent-labelled rabbit antigoat gamma globulin purchased from Antibodies Incorporated, Davis, California (protein concentration was 5.5 mg/ml, F/P ratio was 2.75) was added to cover the sections. After 1 hr of incubation in the dark (fluoroscein is light sensitive), the excess antibody was washed off and the sections were immediately viewed in a Zeiss phase contact, fluorescent microscope at 640 x magnification. A UV light source (Zeiss) and barrier filters 40 and 50 and an excitor filter B512 were used to visualize the fluoroscein.

Diaminobenzidine (DAB) reaction. Glass slides with sections attached were left immersed in buffer (0.05 M tris pH 7.5) prior to DAB incubation. DAB (0.5 mg/ml) was made fresh each hr in 0.05 tris buffer, filtered through No. 1 Whatman filter paper, stirring constantly, and adjusted to the desired pH (7.5 or 8.5) with HCl. Hydrogen peroxide (30% V/V) was added to the DAB solution immediately prior to incubation at 0.03 ml/100 ml solution (0.01% or 3.4 mM). Controls were incubated in buffer only (pH 7.5 and pH 8.5) or in the DAB solution without the added H_2O_2 substrate. Incubation was accomplished in magnetically stirred, 100 ml beakers shielded from excess direct light. The slides were washed by magnetic stirring in buffer for at least 10 min; all were subjected to a final wash in distilled water. Permanent mounts were made by adhering coverships with piccolyte. Slides were viewed and pictures taken on a Zeiss photomicroscope at 640x.

RESULTS AND DISCUSSION

Ferritin Conjugated Antibody Staining

Figure 1 shows the ultrastructural detail we have been able to obtain using cryomicrotomy to prepare ultrathin sections of Anabaena. Even without osmium or uranylacetate post staining, some detail of membrane structure is readily seen. The use of gelatin is the encapsulating media greatly facilitates sectioning of the material and has resulted in thinner, more intact sections than previously obtained (see last year's final report). Furthermore, the severe ice-crystal damage found in our earlier attempts has been avoided by freezing the encapsulated algal material in molten freon prior to emersion in liquid nitrogen. This avoids the insulating effect of bubble formation that occurs when freezing material directly in liquid nitrogen (15).

Figures 1 to 3 show the distribution of ferritin on ultrathin sections of heterocysts at two magnifications. In both cases, the ferritin appeared to be more concentrated on the heterocyst than on the formvar support grid. However, the reaction was light and, to some extent, represents non-specific staining. Furtheremore, in some heterocysts in the same experiment, there was no evidence of a specific ferritin antibody-antigen reaction (see Fig. 1). Short (30 min) incubation periods were used to react the sections with both primary and secondary antibody reagents in these experiments. Prolonged treatment with the antibody preparations is likely to improve the intensity of the reaction. However, when former-coated grids were incubated for 1 hr with the ferritin conjugate used in these experiments, a large amount of non-specific ferritin staining occurred. Pretreatment of the grids with combinations of BSA, gelatin, and cytochrome c solutions improved but did not alleviate this problem. Furtheremore, extensive washing of the protein-treated, ferritin-stained formvar grids with detergents (0.1% triton), high salts (1.5 M NaCl) or glycine (0.01 M) dissolved in the buffer was unsuccessful

in removing ferritin. Non-specific staining, however, has varied considerably from one ferritin preparation to another and; thus, may not be an insurmountable problem. Further work is in progress.

Fluorescent-labelled Antibody Staining

Preliminary experiments with a fluorescein-labelled, indirect antibody staining method showed a preferential labelling of the heterocysts in aerobically grown A. cylindrica. The heterocysts appeared as a bright fluorescent green in a dark background. There was some fluorescence associated with small particles and folds in the agar coating of the slides. However, vegetative cells and the background in general were unstained. Although the fluorescent heterocysts were strikingly bright against the dark background when viewed through the microscope, even 3-min exposures of Polaroid film (ASA 75) were insufficient to obtain an image. A more light-sensitive film will be necessary to record these observations.

Diaminobenzidone Oxidation

At the light microscope level, the only discernible site of the black DAB oxidation product was localized in the polar plug area of the heterocysts, even after incubation times of up to 50 min. All vegetative cells and the central regions of the heterocysts remained greenish in both controls and DAB media, indicating the reaction did not involve the entire cell. Lauritis et al. (16) observed that whole cell preparations incubated in DAB turned brown, indicating DAB oxidation. The observed DAB oxidation product in this study, however, was black and readily visible in discrete areas. The dark appearance of some cells (both vegetative and heterocyst) in some photos (Fig. 4 and 6a) is a function of the thickness of the section, and are actually green when viewed in the microscope.

A reaction product was visible as early as 5 min in all sections

incubated in a DAB-containing medium (Fig. 5). The product deposition was not enhanced by increasing pH (data not shown) in DAB-only media, but was stimulated by introducing the H_2O_2 substrate (Fig. 6). While the reaction becomes darker under these conditions, it still maintains its discretness. Sections through different planes of the heterocyst present a different aspect. Some sections of longer incubation times exhibit a dark refractile reaction product in the polar plug regions. The reason for the refractile appearance is not clear.

The reaction sites in all sections are clearly associated with what Lang and Fay (17) call the "pore channel" (Fig. 5b) and the "honeycomb network" (Fig. 6). The highly contorted membranous structure seems to be differentiated in the area immediately adjacent to the pore channel during heterocyst development and undergoes a breakdown in older heterocysts. There does not seem to be a reaction in the pore channel itself (at least not extending to the neighboring vegetative cell), but is often very close and sometimes extends into it. This tubular membrane structure is unique to developing heterocysts. A similar membrane proliferation occurs in Azotobacter vinelandii only under nitrogen-fixing conditions (18).

In preliminary studies a number of inhibitors had no apparent effect on the intensity or site of DAB deposition when H_2O_2 was included in the reaction media. Amino triazole (0.02 M), a known inhibitor of catalase and sodium azide (0.02M), which inhibits the activity of heme-containing enzymes, both had no effect. This may be due to the fact that the sections were immersed in a DAB/H_2O_2/inhibitor solution without prior incubation in the inhibitor alone, and the reaction could have taken place before the inhibitors took effect.

The effect of several oxidase inhibitors on nitrogenase activity in air-grown A. cylindrica is shown in Table 1. Aminotriazole, an inhibitor of catalase, had no effect on acetylene reduction, with or without the presence of O_2 in the gas phase. Phenythiourea, an inhibitor of polyphenoloxidase (19), was strongly inhibitory to nitrogenase activity. Its inhibitory effect was only somewhat more inhibitory in air than under argon; however, the algae were cultured under air and thus some O_2 was transferred to the assay flask. Sodium sulfide, an inhibitor of peroxidase activity, was also inhibitory in the presence of air. The specificity of these inhibitors on the respective oxidases is unclear at this time. Future work will require study of the effect on other physiological processes. Furtheremore, the relation of the presumed oxidase function of the DAB oxidizing enzyme system to O_2 protection of nitrogenase will require more extensive work.

CONCLUSIONS

The work described above suggests that the electron microscopic localization of nitrogenase work is near to achieving its goals. The indirect ferritin labelling approach still suffers from lack of specificity, although significant improvements over previous approaches have been achieved. One factor that has been of concern was the possible lack of antibody activity of the ferritin preparations. However, a new preparation of the ferritin-antibody has now been demonstrated to be active and, thus, can be used in these experiments with greater confidence. The fluorescent labelled antibody staining was successful and specific, the only remaining problem is to get a photographic record of the visual observations. With that, it appears that a comparative study of the localization of nitrogenase in aerobic and anaerobically induced cultures will be possible.

The work on peroxidase localization in the polar plug region is of significant interest in terms of the oxygen protection mechanism of heterocysts. It appears that the peroxidases in the polar regions could serve as a barrier to oxygen diffusion from the vegetative cells. Indeed, this may be a more critical problem to the stability of nitrogenase in heterocysts than oxygen diffusion through the heterocyst cell wall. Further work is planned in this area.

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TABLE 1

EFFECT OF OXIDASE INHIBITORS ON *In Vivo* NITROGENASE ACTIVITY IN AIR-GROWN *A. cylindrica*

Gas Phase	Inhibitor	nME/min/ml	% of Control
Argon		0.455	100
"	10 mM aminotriazole	0.49	105
"	10 mM phenylthiourea	0.036	6.7
Air	--	0.403	100
"	10 mM aminotriazole	0.433	107
"	10 mM phenythiourea	0.011	3.0
"	5 mM Na sulfide	0.054	13.4

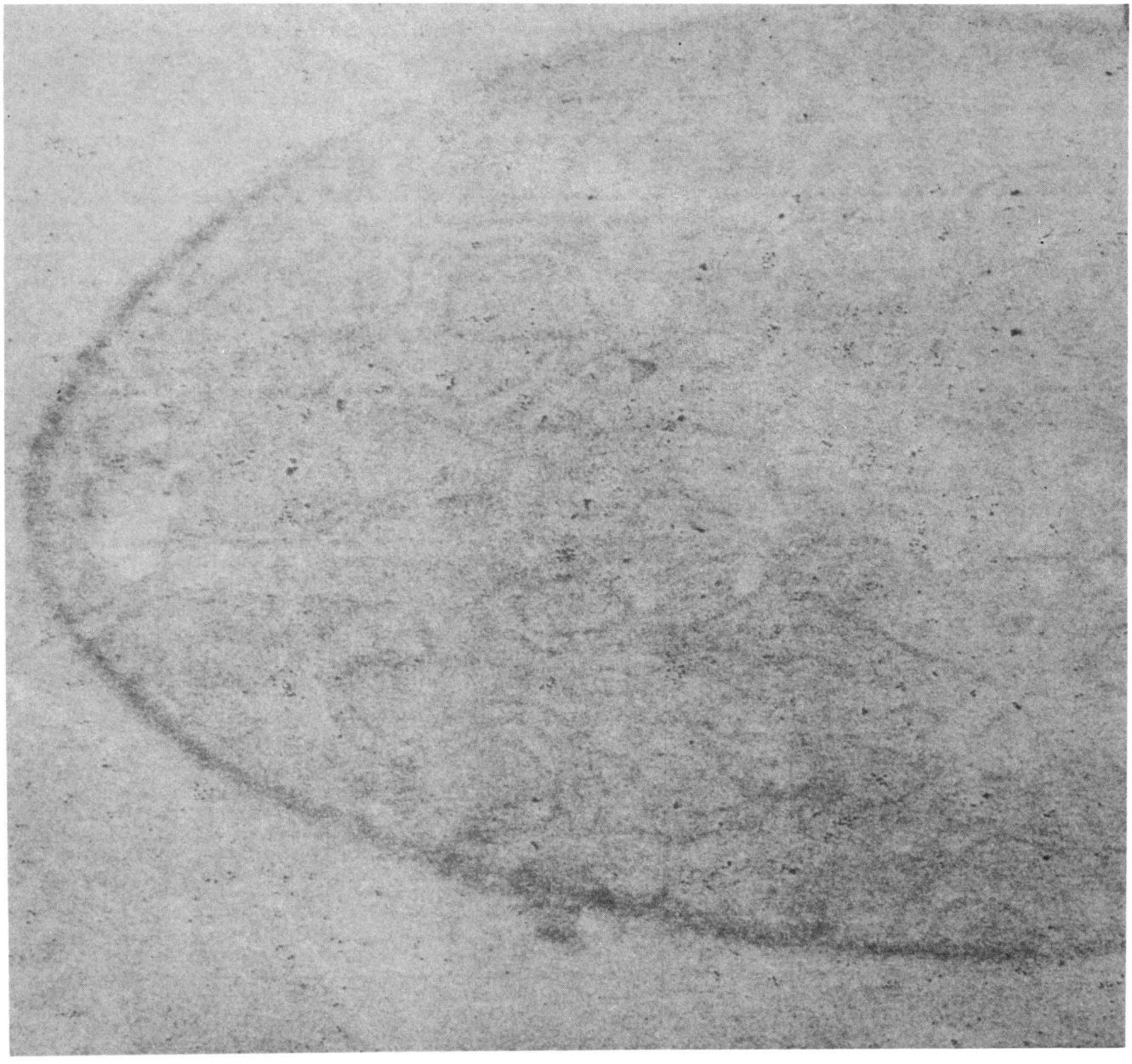


FIGURE 1. ULTRATHIN, FROZEN SECTION OF HETEROCYST OF A. cylindrica STAINED WITH FERRITIN CONJUGATED ANTIBODY ONLY. Magnification 39,000x.

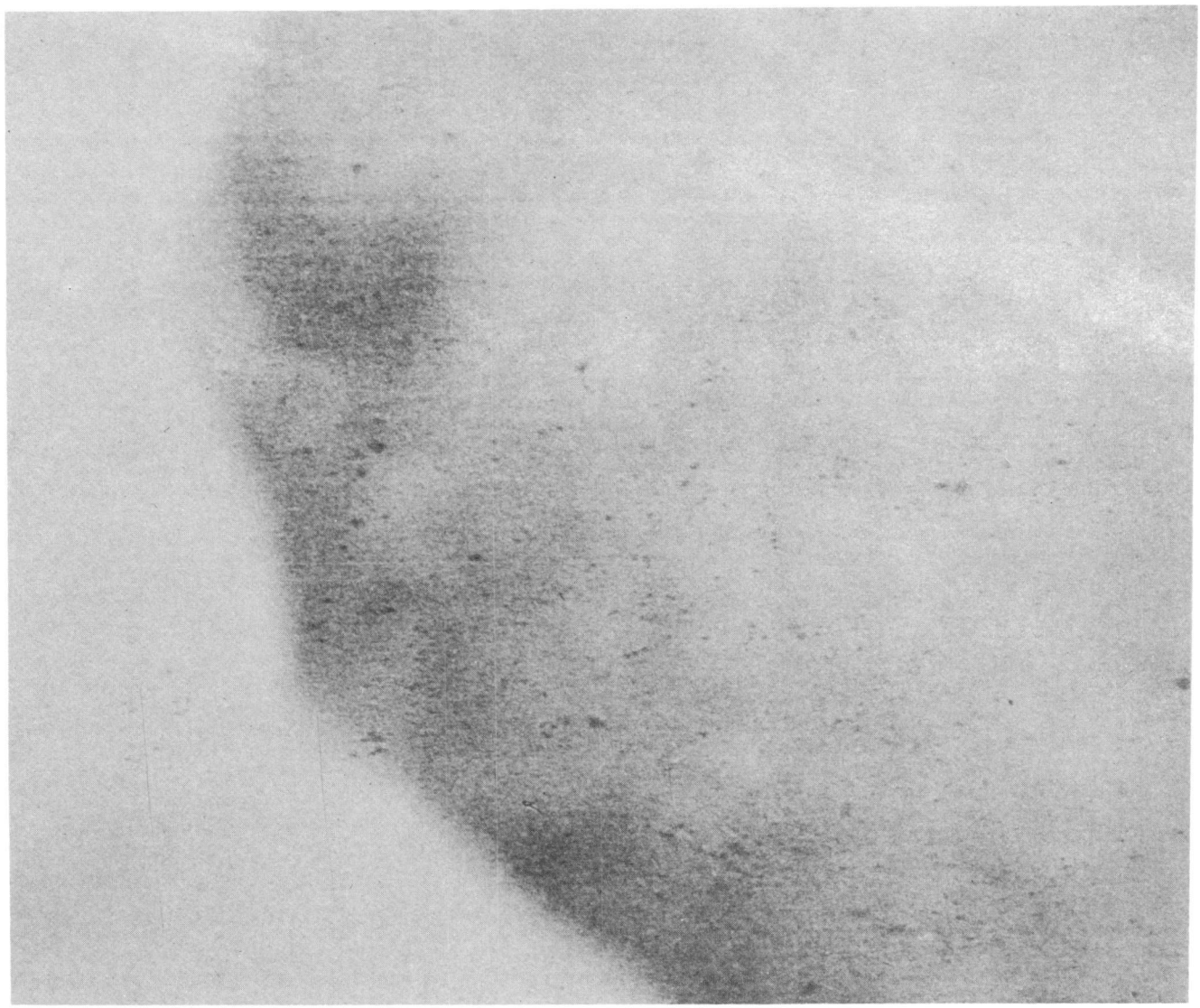


FIGURE 2. ULTRATHIN FROZEN SECTION OF HETEROCYST OF A. cylindrica STAINED WITH FERRITIN-LABELLED ANTIBODY. Magnification 45,000 x.

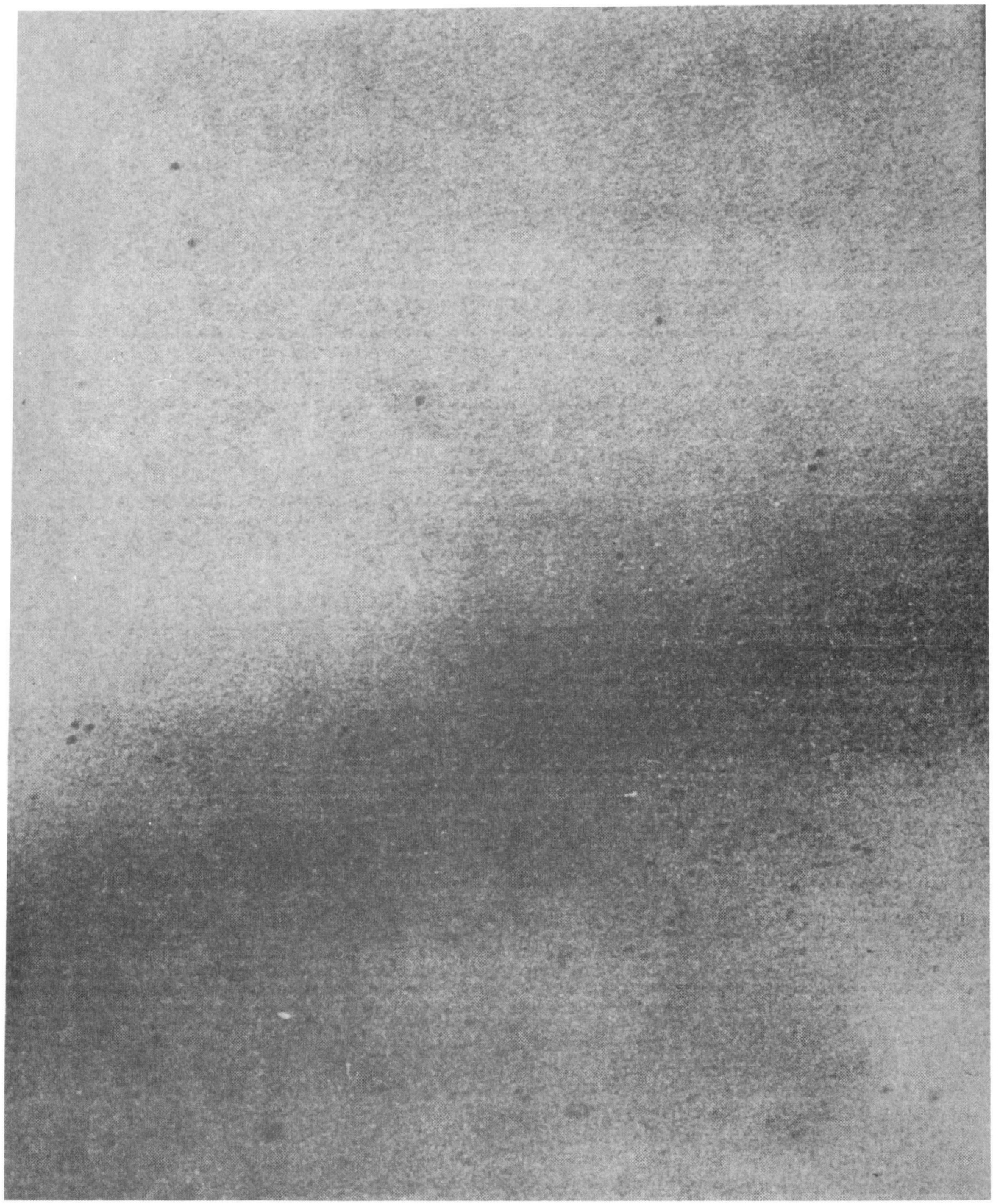


FIGURE 3. FERRITIN-LABELLED ANTIBODY LOCALIZATION OF NITROGENASE IN HETEROCYST OF A. cylindrica. Magnification 180,000 x.

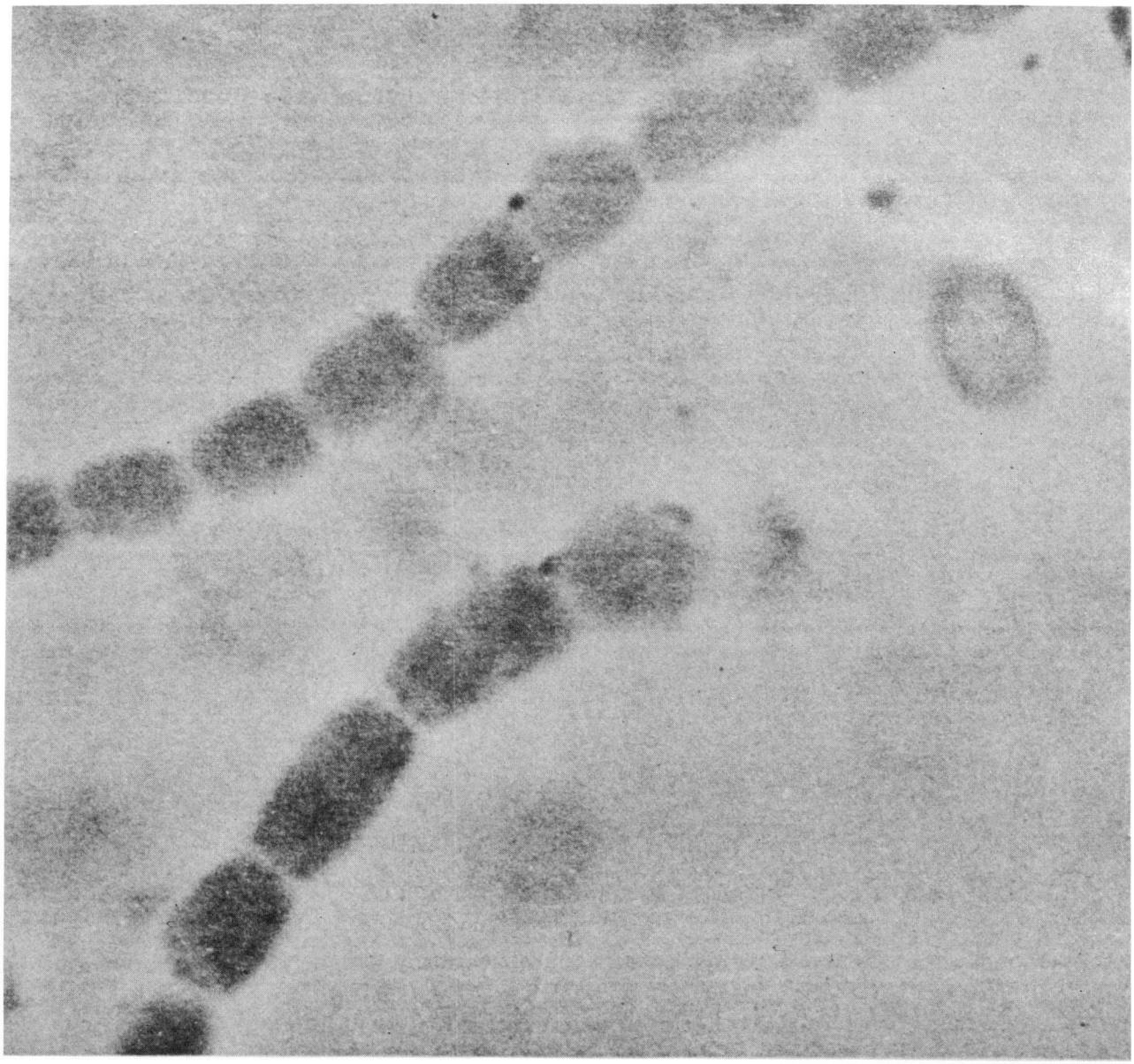


FIGURE 4. UNSTAINED FROZEN SECTIONS (0.5 μ) OF FILAMENTS OF AIR-GROWN Anabaena cylindrica. Note the lack of dark spots in the heterocyst. Polyphosphate granules are apparent in some of the vegetative cells. Magnification approx. 1000 x.

a

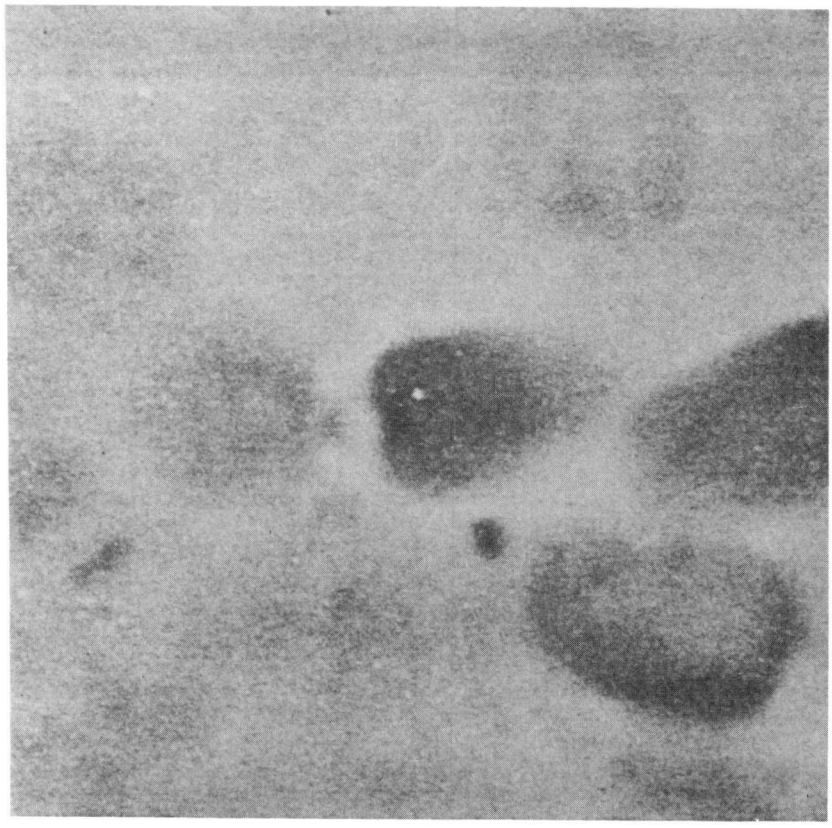
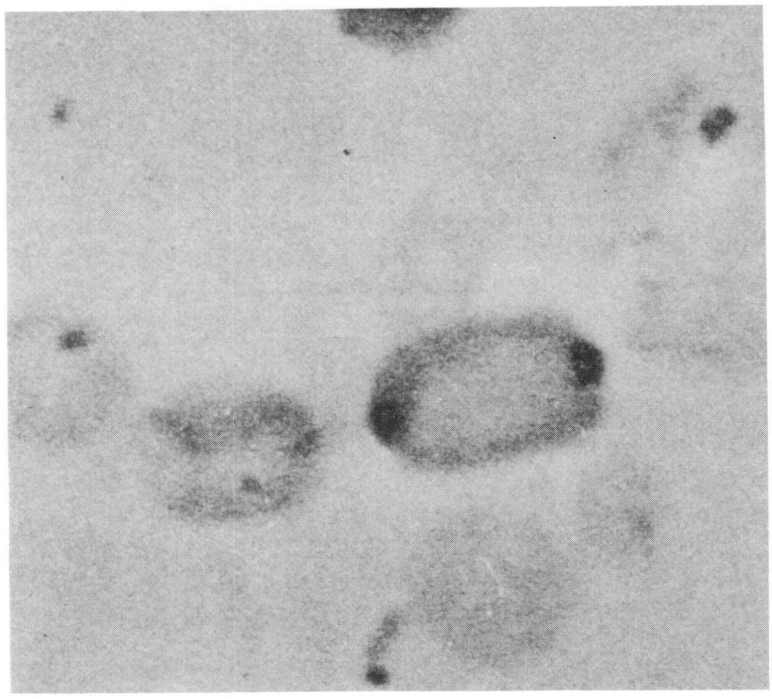
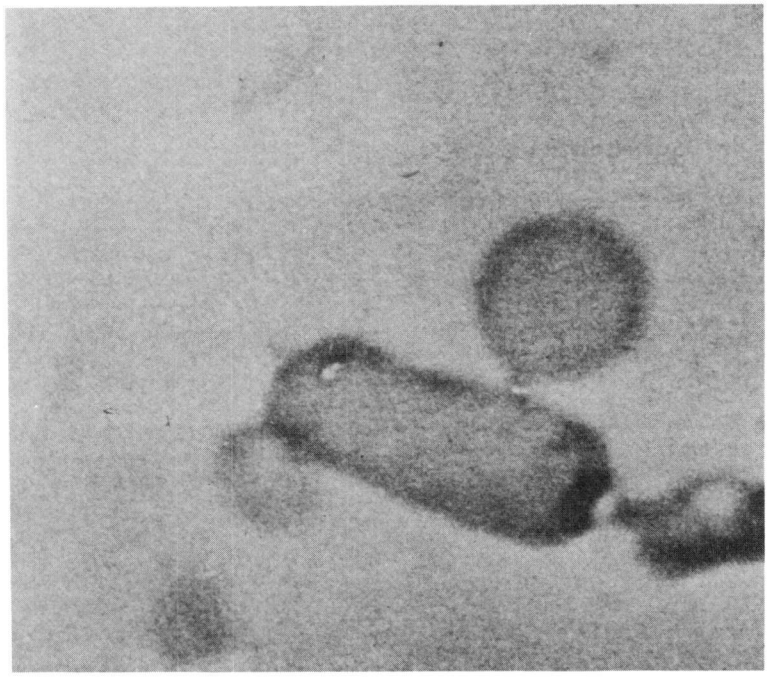


FIGURE 5. FROZEN 0.5 μ SECTIONS OF AIR-GROWN A. cylindrica INCUBATED WITH DIAMINOBENZIDE FOR 5 MIN (a) AND 20 MIN (b and c) AT pH 7.5.

b





c

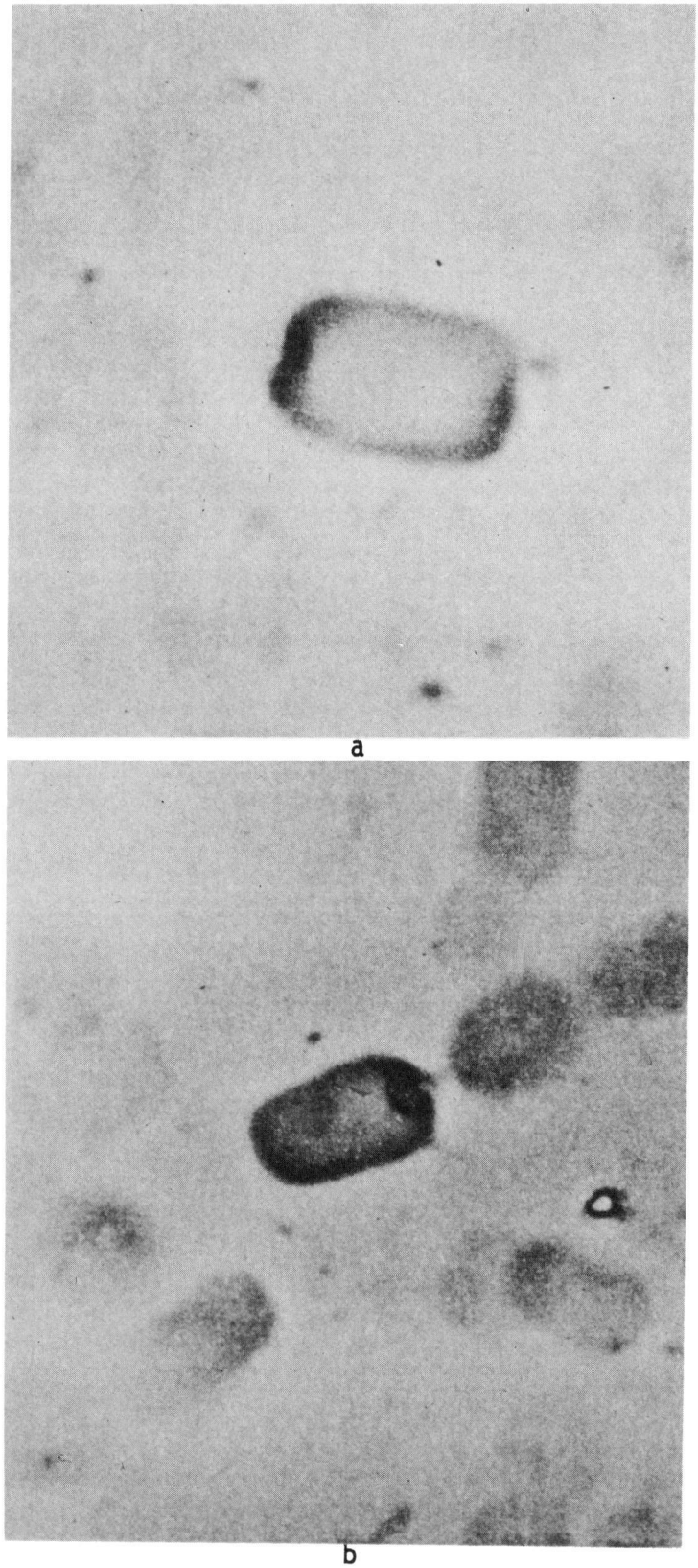


FIGURE 6. FROZEN 0.5 μ SECTIONS OF AIR-GROWN A. cylindrica INCUBATED WITH DAB AND H_2O_2 FOR 5 MIN (a) AND 20 MIN (b) at pH 7.5.

XII. HYDROGEN PRODUCTION BY PHOTOSYNTHETIC BACTERIA

In the short term, the greatest potential for practical applications of photobiological hydrogen production rest with photohydrogen fermentations of wastes by photosynthetic bacteria (1,2). A modest start to the development of such a system was accomplished during the previous year of this project (3). Here only a few additional experiments are described.

The methods and procedures used were the same as previously reported, with the Rhodospirillum rubrum (strain S-1) cultivated on 1 liter of malate/glutamate media of Omerod et al (4) in tall glass cylinders (54 cm high, 5 cm diameter), illuminated with three 150 W General Electric Reflector lights positioned about 26 cm from the culture cylinder. Heat filters were used to maintain the temperature at 32-35°C. Argon was bubbled through the cultures which were axenic. The culture flasks were inoculated with heavy stock culture diluted to a cell density of about 400 Klett units and then incubated in the dark overnight. Maximal hydrogen production rates observed were 740 µℓ hydrogen/liter culture/min after about 24 hr of being put back in the light, at which time a maximal cell density of about 700 Klett units was achieved (due to limiting glutamate). Hydrogen production declined slowly thereafter (see last years final report). A pH optima between 7 and 8 was established. The next experiment was similar to that above but using glucose instead of malate. In that case only about 4% of the above maximal activity was observed. This is not too surprising as glucose is a poor carbon source for these bacteria.

The next set of experiments involved the continuous, rather than batch, addition of a carbon source. The bacteria culture was diluted to a Klett of

200 into a carbon and nitrogen-free media and kept overnight in the dark. In the morning, the light was turned on and a concentrated malic acid solution (1%) fed to the culture by peristaltic pumps controlled by a pH controller held at pH 7.0 (Radiometer Model GK 4032 C). The whole system was axemic. As seen from Figure 1, the hydrogen production by the cultures rapidly declined to a level of 60 µℓ/liter culture/hr or about 8% of that observed previously with the batch culture at pH 7.5. It appears likely that the rate of carbon addition to the culture was a limiting factor. However, this was also a more dilute culture, having only half the density of the batch cultures previously reported. Thus, it appears that considerable improvements in the observed rate of hydrogen production would be possible if the culture density and carbon feed rate were optimized.

The next experiment involved an axenic culture of photosynthetic bacteria mixed with several mL inoculum of sewage effluent. The same protocol as above was carried out with the addition of a 1% glucose solution added continuously to the bacterial culture. The results are shown in Figure 2. A peak of hydrogen production rate of 150 µℓ/liter/hr was observed within 24 hr of placing the culture in the light. Total culture density increased considerably, probably due to a carry over of nitrogen in the inoculum and sewage. After four days, the hydrogen production rate declined and the photosynthetic bacteria were replaced by green, flagellated algae. The pH of the culture increased to 8.6, well above the pH 7.3 at which the controller had been set (thus glucose was thus added continuously). Hydrogen production was stable with this mixed culture for at least nine days (Figure 2). No further experiments were carried out as the pH stat was no longer available.

The above results are obviously of a preliminary nature. They do, however, suggest several interesting aspects that require further study.

The hydrogen production rates in the batch cultures were about six-fold higher (on a cell mass basis) than in a comparative continuous culture. This may reflect the much higher substrate concentration available in the batch culture. Experiments should be carried out with a continuous culture in which a high initial malate concentration is present and the pH stat set much higher (8.0 or even 8.5). The mixed culture system is, of course, the most interesting one for future work. The results demonstrate two points: the need to have a low nitrogen input into the system and the need for selecting an effective and stable mixed culture. As far as the latter is concerned, the non-growing nature of the system will make such a selection difficult. The best approach may be to develop a strategy of selection and adaptation in which even lower dilution rates (lower nitrogen inputs) are used. It may be expected that this will require long-term operation of these cultures. The basic strategies that will be followed are those that have been successfully used in developing mixed cultures for fermentations and single-cell protein production.

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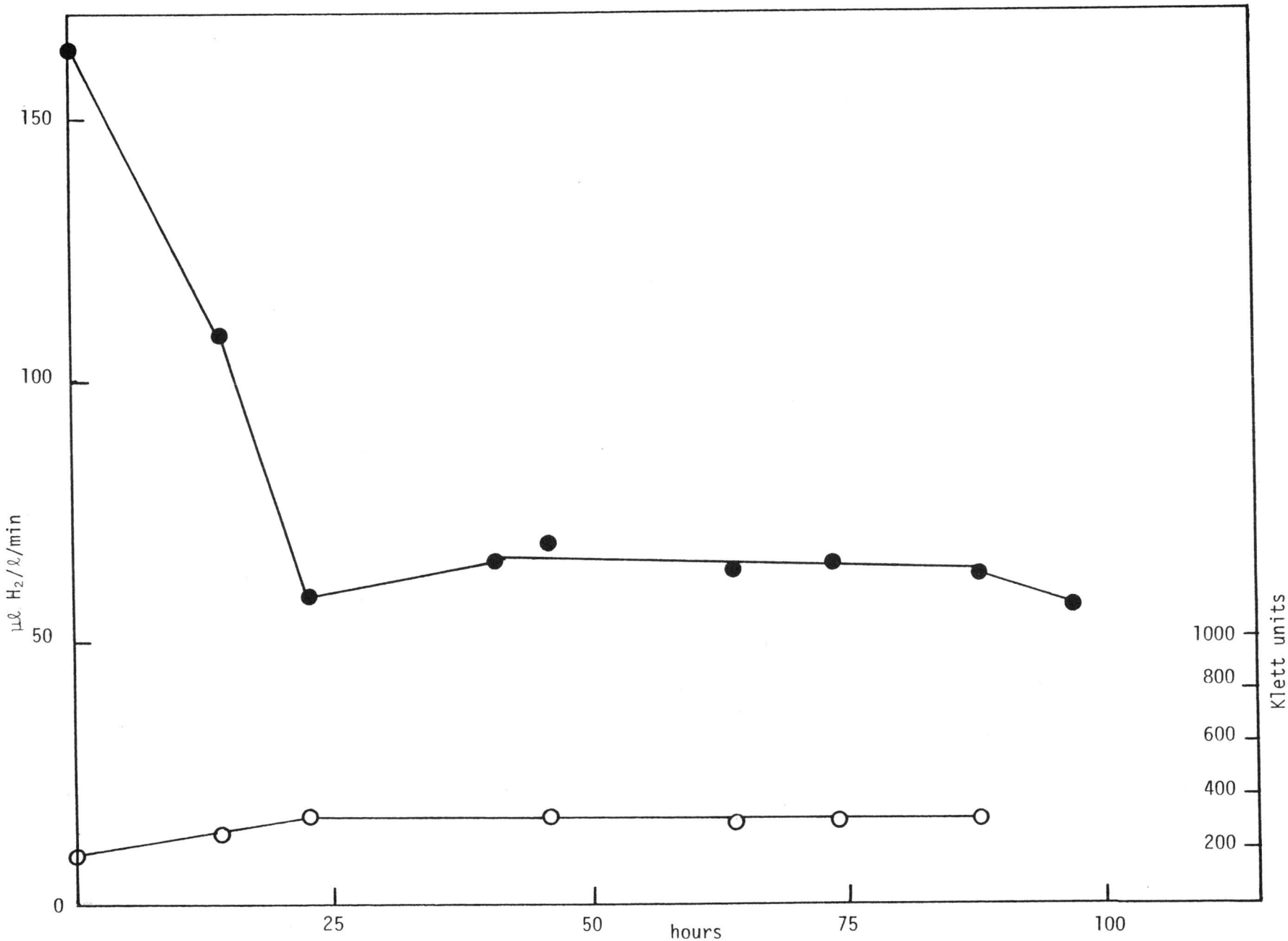

FIGURE 1. HYDROGEN PRODUCTION BY A CONTINUOUS CULTURE OF R. rubrum S-1 FED MALIC ACID AT pH 7.0.

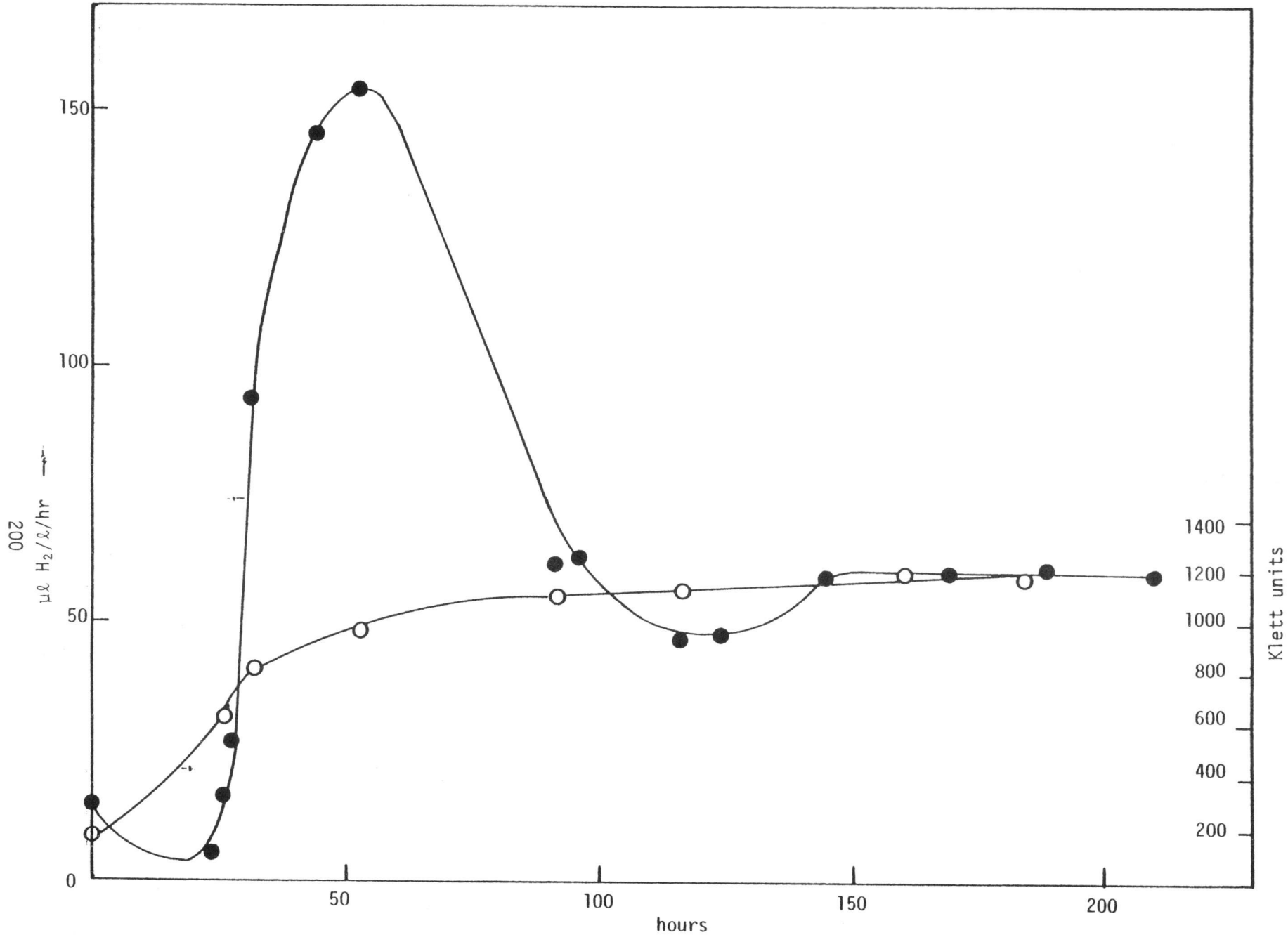

FIGURE 2. HYDROGEN PRODUCTION BY A CONTINUOUS CULTURE OF R. rubrum AND OTHER MICROORGANISMS FED GLUCOSE AT pH ABOVE 7.0.

XIII. APPARENT ATP SYNTHESIS CATALYZED BY NITROGENASE

INTRODUCTION

The requirement for ATP hydrolysis during nitrogenase catalyzed reactions is a well known fact, but is little understood at present. For biophotolysis this ATP requirement is important as it both reduces overall efficiency and allows unidirectional hydrogen evolution. Study of this reaction is also of interest in the long term development of a hydrogenase specifically designed for biophotolysis. One method for studying this ATP requirement is by demonstrating the reverse reaction, ATP synthesis, under suitable conditions. Although nitrogenase catalyzed reactions are commonly considered irreversible, thermodynamically they must be reversible, albeit under non-physiological conditions. Synthesis of ATP by this enzyme would demonstrate ATP synthesis (possibly coupled to electron flow) by a soluble enzyme system. This would constitute a novel alternative to Mitchel's chemiosmotic theory of ATP synthesis.

Here we report the results of initial investigations of ATP synthesis by nitrogenase. ATP synthesis was measured as the incorporation of radioactive phosphate into a form not extractable into benzene as an ammonium molybdate complex. Using such an assay system, we have indeed observed ATP synthesis by nitrogenase containing fractions. However, the present results are not interpretable in terms of any present model of nitrogenase functions. Further work is needed to determine the significance of this effect as regards nitrogenase catalysis.

MATERIALS AND METHODS

Nitrogenase preparations: Anabaena cylindrica nitrogenase fractions were prepared as previously described. Azotobacter nitrogenase was prepared by slight modifications of published procedures, and Rhodospirillum rubrum

nitrogenase was a gift of Dr. Duane Yoch.

Assays: ATP synthesis assay conditions are described in the appropriate places in the results. The n moles of $^{32}PO_4^=$ incorporated was derived from the cpm obtained with an aliquot of the aqueous phase after butanol extraction of the ammonium molybdate phosphate complex and the (determined) specific activity of the $^{32}PO_4^=$ (25.8 cpm/n moles) used. After drying the butanol phase, radioactivity was determined with a planchet counter. Nitrogenase activity in the forward direction (normal assay conditions - ATP hydrolysis) was measured as acetylene reduction as previously described.

RESULTS AND DISCUSSION

Under normal assay conditions, or in vivo, nitrogenase catalyzed substrate reduction is dependent upon both a supply of reductant, and a supply of ATP. Thus, ATP hydrolysis is linked to electron transfer, although highly purified nitrogenase preparations have been found to catalyze, to some extent, a reductant independent APT hydrolysis. Under some conditions, this "uncoupled" ATP hydrolysis can account for up to 30% of the ATP hydrolyzed.

In order to reverse the nitrogenase reaction highly purified nitrogenase components from Anabaena cylindrica were incubated in the presence of an excess of the products of a forward reaction, i.e. ammonia (as ammonium chloride) and ADP (as Mg ADP), potassium ferricyanide was used as the electron acceptor, and glucose and hexokinase were included as a trap for any formed ATP. The results are shown in Table I. A high rate of ATP synthesis, comparable to the rate of acetylene reduction in the forward direction, was observed. However, this ATP synthesis was not linked to either electron transfer or substrate oxidation as omission of NH_4Cl and potassium ferricyanide did not decrease the formation of ATP. Surprisingly, ATP was also formed in the presence of a single component. All other nitrogenase reactions have been demonstrated to have an absolute requirement for the presence of both

components, so this result seems anomalous, and needs to be further investigated.

ATP synthesis was also demonstrated with nitrogenase preparations from Rhodospirillum rubrum and Azotobacter vinelandii as shown in Table II. The ATP synthetase activity of these preparations was also unaffected by the absence of ammonium or potassium ferricyanide. As shown in Table III, this activity was stimulated by the absence of hexokinase, and decreased when ADP was omitted. The activity of an air oxidized sample (known to destroy nitrogenase activity) was somewhat decreased (about 50% of the control) but surprisingly high. As a further control, a reaction stopped at zero time gave no apparent ATP synthesis.

To determine if the observed radioactivity was due to $^{32}PO_4^{=}$, ADP ($^{32}PO_4^{=}$) or the incorporation of $^{32}PO_4^{=}$ into some other substance, the aqueous phases from several assays were combined and, after dilution and adjusting the pH to 8.3, absorbed on a 1.6 x 20 cm column of Ag 1 x 8L previously equilibrated with distilled, deionized water. The column was then eluted with $\sim$ 100 ml of the following solutions. These solutions should elute the indicated compounds (H.V. Bergmeyer, Methods of Enzymatic Analysis, p. 570-571): 0.01 M NH_4Cl (elutes adenosine and adenine); 3 x 10^{-3} NHCl (elutes AMP and inorganic phosphate); 0.02 M NaCl in 10^{-2} N HCl (elutes ADP); 0.2 M NH_4Cl in 10^{-2} N HCl (elutes ATP). The radioactivity was recovered, along with almost all of the A_{260} material, with 0.2 M NaCl in 10^{-2} N HCl. Thus, the radioactivity was not in the form of $^{32}PO_4^{=}$ or ADP $^{32}PO_4^{=}$. It is not known under which conditions glucose-6-phosphate would be eluted from this column, so the radioactive material cannot be positively identified as ATP($^{32}PO_4^{=}$).

Further work needs to be done to determine if this activity is due to nitrogenase or a contaminating protein. It would be desireable to check for ATP synthesis using another methodology, measurement of ATP (incorporated into

glucose-6-phosphate) using fluorometry and glucose-6-phosphate dehydrogenase might be suitable. A time course would also be helpful in determining if the observed activity is indeed due to enzymatic action. It should be noted that at present the demonstration of a reversal of nitrogenase activity has not been proven. This is also true of a recent report by Morowitz and Morowitz in Biochem. Biophys. Res. Comm., (based on our idea and approach) in which the unpurified nitrogenase fractions were used, and the experiments lacked appropriate controls. Thus, no conclusions can be drawn regarding the chemiosmotic theory at present, based on this data. However, these experiments hold promise for elucidating the mechanism of this enzyme and thus allowing the possible development of a hydrogenase more suitable for biophotolysis.

TABLE I

Assays were run in duplicate at 30°C for 40 minutes. Complete reaction mixture contained: K Fe $(CN)_6$ (10 mM), $PO_4^=$ (5 mM), $MgCl_2$ (5 mM), ADP (5 mM), glucose (20 mM), Hexokinase (0.2 mg), HEPES (10 mM), NH_4Cl, except where indicated, (50 mM) and isolated components from A. cylindrica. When combined and assayed in the forward direction, the mixed components reduced 7.5 n moles C_2H_4 per minute.

Reaction Mixture	n moles $^{32}PO_4^=$ Formed
Complete $[NH_4Cl]$	
100 mM	67.5
50 mM	98.5
25 mM	77.8
- NH_4Cl	55.8
- $K_3Fe(CN)_6$	78.3
- Fe-component	43.3
- Fe-Mo component	27.7

TABLE II

Reaction conditions as in Table I.

Reaction Mixture	n moles $^{32}PO_4^=$
A. *Rhodospirillum rubrum*[1]	
complete	1851
- NH_4Cl	1604
- $K_3Fe(CN)_6$	1718
- ADP, and hexokinase system	1804
B. *Azotobacter vinelandii*[2]	
complete	2051
- NH_4Cl	1834
- $K_3Fe(CN)_6$	2223

[1] Partially purified *Rhodospirillum rubrum* nitrogenase, highly enriched in Fe-Mo component. When assayed in the forward direction, this preparation reduced 30.3 n moles C_2H_4/min.

[2] Partially purified *Azotobacter vinelandii* nitrogenase. When assayed in the forward direction, this preparation reduced 111.6 n moles C_2H_4/min.

TABLE III

Reaction Mixture[1]	n moles $^{32}PO_4^=$
Complete	840
- hexokinase	1846
- ADP	447
Air oxidized (3hr) nitrogenase	711
Reaction stopped at zero time	0

[1] Conditions as before. A Rhodospirillum rubrum nitrogenase preparation was used.

XIV. BIOENGINEERING ASPECTS OF BIOPHOTOLYSIS

INTRODUCTION

The only direct solar energy conversion process by which fuels (rather than heat or electricity) can be produced is bioconversion, or the production of fuels from biomass [1]. Of the large number of bioconversion technologies, biophotolysis combines in a simple concept the attractiveness of biological solar energy with the prospects of a future solar hydrogen economy.

The term "biophotolysis" was first used by Krampitz, who reported six years ago on a bacterial-algal system capable of converting light energy to hydrogen and oxygen [2]. At about the same time it was reported that spinach chloroplasts could produce a small amount of hydrogen from water when coupled to a bacterial hydrogenase enzyme [3]. In the intervening years a number of different biophotolysis systems have been proposed, based on current knowledge of the biochemistry of photosynthesis, hydrogen metabolism, and algal physiology (see 4-7 for reviews). Two basic approaches to biophotolysis can be considered: single-stage systems in which photosynthetic oxygen and hydrogen production occur in the same vessel (although not necessarily simultaneously) and two-stage processes in which the two reactions are spatially separated but linked through an exchange of metabolites. The critical problem to be solved in a single-stage system is how to protect the very oxygen-sensitive hydrogen-evolving reaction(s) from the oxygen generated by photosynthesis; while in the two-stage systems, the problem is how to effectively generate, transport, and utilize an oxygen-stable reductant that will couple the two stages. Table 1 lists the various biophotolysis systems proposed or being studied.

Table 1. Proposed Biophotolysis Systems

	Single-Stage Systems	Status*	Reference
1.	Chloroplast-ferredoxin-hydrogenase	laboratory	3, 13-17
2.	Primary electron acceptor	conceptual	4,5
3.	Heterocystous blue-green algae	outdoor	8-12
4.	Alternating H_2 and O_2 evolution	conceptual	4,5
	Two-Stage Systems		
5.	O_2 stable intermediate	laboratory	2,18
6.	Algal-culture cycles between stages	conceptual	4,5
7.	Organic wastes/photofermentations	laboratory	4,7
8.	Reversible O_2 trap	experimental	19
9.	Membrane separation of reactions	conceptual	20,7

*Conceptual: no experimental work, based on known biochemistry and physiology
Experimental: not yet demonstrated; however, laboratory experiments are in progress
Laboratory: shown in the laboratory; not yet scaled up
Outdoor: demonstrated with a model converter under outdoor conditions.

The most successful biophotolysis system, in terms of current state of development, is based on the use of nitrogen-limited cultures of heterocystous blue-green algae [8]. The ability of those algae to simultaneously produce hydrogen and oxygen in the laboratory [8], the demonstration of prolonged hydrogen (and roughly stoichiometric) oxygen production by nitrogen-limited cultures [9], and the successful operation of such a biophotolysis system outdoors [10-12], make this system the most advanced at present.

A bioengineering analysis of biophotolysis has not yet been attempted. It is required to assess the prospects for achieving a practical and economic biophotolysis process for solar energy conversion. In this review we discuss the various approaches to biophotolysis and the common bioengineering problems likely to be encountered in developing them past the conceptual or laboratory stages. In particular we address the materials and operations limitations encountered in designing a cost-effective biophotolysis system. We conclude that biophotolysis is a long-term solar energy option with some possible near-term practical applications in specific areas. We start with a brief review of relevant biochemical and physiological information on hydrogen producing enzymes and photosynthetic microbes. A recent review should be consulted for more details in these areas [19].

HYDROGENASES: BIOCHEMISTRY AND PHYSIOLOGY

Hydrogen metabolism is catalyzed by three distinct types of enzymes: the "uptake" hydrogenase, the "reversible" hydrogenase, and nitrogenase, which can catalyze hydrogen evolution (Table 2). All three enzymes are sometimes found in the same photosynthetic microorganisms, complicating the study of hydrogen metabolism. The "uptake" hydrogenase is generally a

Table 2. General Properties of Hydrogenases

Type	Molecular Weight (approximate)	Active Site	Inhibitors	Function
Reversible	60,000	FeS	CO, O_2	fermentation
Uptake	unknown	FeS	CO	H_2 recycling
Nitrogenase	200,000	Mo(FeS)	O_2, N_2	nitrogen fixation

a membrane-bound enzyme, although a soluble uptake hydrogenase has also recently been found in the strictly anaerobic Clostridium pasterianum [21]. It should be noted that this enzyme does exhibit a small degree of hydrogen exchange or evolution. One physiological role of the uptake hydrogenase is to recycle the hydrogen formed in a side reaction of nitrogenase [22,23]. This has been clearly demonstrated in the blue-green alga, Anabaena cylincrica where hydrogen supports nitrogenase activity in an uptake hydrogenase mediated reaction [23]. However, in biophotolysis this enzyme may pose a serious problem as it results in hydrogen uptake and, thus, lowers overall rates of hydrogen production.

The "reversible" hydrogenase has a redox potential similar to that of the hydrogen couple and the ferredoxin-flavodoxin group of electron carriers (≃400 mv). Thus, it catalyzes hydrogen uptake and evolution at similar rates. Although some reversible hydrogenases are easily inactivated in the presence of oxygen, others, specifically those in microalgae, are only inhibited, not inactivated, by oxygen [25].

In algae which possess this enzyme, a period of 2-20 hours of incubation under dark anaerobic conditions results in an activation of the reversible hydrogenase activity. (The enzyme itself is apparently constitutive but not active under normal photosynthetic, aerobic conditions.) The enzyme can catalyze hydrogen evolution in the dark and at low light intensities, or hydrogen uptake, with or without carbon dioxide fixation or oxygen consumption. At high light intensities photosynthetic oxygen evolution commences and results in inhibition. The physiological role of the reversible hydrogenase in algae appears to be in maintaining a proper cellular redox level, particularly after anaerobic conditions which inhibit photosynthesis [24]. The enzyme has been isolated from blue-green algae and appears similar to the clostridial type except for a much higher resistance to inactivation by O_2 [25].

Nitrogenase--the enzyme responsible for nitrogen fixation--is, like hydrogenase, also an iron sulfur protein but of a much larger size and complexity. It is made up of two proteins and contains molybdenum. It not only reduces molecular nitrogen to ammonia but also catalyzes the reduction of acetylene to ethylene (this reaction being used as an assay), hydrogen evolution, hydrogen exchange, and a variety of reductions of triple bonded molecules [26]. In biophotolysis the hydrogen evolution reaction is of greatest interest. It has the advantage of being unidirectional and, thus, no back reaction need be considered. It suffers from the disadvantages of a rather low turnover number (~ 150 versus 8,200 for the reversible hydrogenase) and an absolute requirement of at least 5 ATP's per hydrogen produced. These lower the achievable efficiency of a biophotolysis system based on this enzyme.

PROPOSED BIOPHOTOLYSIS SYSTEMS

The simplest, conceivable biophotolysis system would combine, in a single process, decomposition of water to hydrogen and oxygen. Photosynthesis can be visualized as a "green box" divided into two compartments, each absorbing light energy, with oxygen being evolved in one and a strongly reducing electron generated in the second, at what is termed the "primary electron acceptor" (PEA) [27]. Although the PEA could thermodynamically generate hydrogen by reacting with protons (always present in water), none has been detected [28]. Interestingly, this electron acceptor is also very resistant to oxidation by oxygen; it will donate electrons only to ferredoxin or a similar natural or artificial electron carrier. The possibility of imparting to the PEA a hydrogenase activity (through either biochemical or genetic manipulations) has been suggested [5]. Another single-stage process for biophotolysis involves the chloroplast-ferredoxin hydrogenase system [3]. has been the subject of a great deal of laboratory experimentation [5,13-17]. This system combines the photosynthetic apparatus and the natural electron acceptor (ferredoxin) with a reversible hydrogenase (bacterial or algal) to catalyze the overall reaction of $H_2O \rightarrow H_2 + 1/2\ O_2$. The initial study [3]

demonstrated only low rates of hydrogen production which were maintained for periods of less than one hour. Addition of an oxygen trap (glucose-glucose oxidase) increased the rates and stability of the system about six-fold. Recent work has extended and improved these findings [13 - 17], concentrating on problems of stability of chloroplasts and hydrogenase [29-31]. However, the critical factors still remain: the inhibition of the hydrogenase reaction by oxygen, the autooxidation of ferredoxin, and lack of net oxygen production by these systems. One interesting concept is that of using a reversible oxygen absorber which is cycled to a desorption stage for regeneration (System 8, Table 1) [19].

The heterocystous blue-green algal biophotolysis system (System 3, Table 1) is based on the microscopic separation of the oxygen and hydrogen evolving reactions into (respectively) the vegetative and heterocyst cells of these filamentous algae. The more numerous vegetative cells are responsible for photosynthetic carbon dioxide fixation (and oxygen evolution). The heterocysts are specialized for nitrogen fixation, lacking the oxygen-evolving reactions of photosynthesis, and being protected from oxygen by a diffusion barrier at the cell wall and an active rate of respiration. Thus, the problem of oxygen inhibition is overcome. However, the metabolic energy required for differentiating and maintaining heterocysts and supplying them with reductant must be significant (although not yet quantitatively determined). More importantly,current development of this system has emphasized the use of the nitrogenase enzyme for hydrogen production, an energetically unfavorable reaction as discussed above. Although the presence of the reversible hydrogenase in heterocysts was inferred from *in vivo* experiments [8] and shown *in vitro* [32,33], only recently has its functioning in these cells been demonstrated conclusively [34] and future work should explore the potential of this enzyme in this system. As this biophotolysis system is the only one in an advanced stage of development, it is analyzed in detail below. Figure 1 shows a model of hydrogen metabolism in these algae. H_2 production by these algae requires exclusion of molecular N_2 which can be achieved by gassing the cultures with argon.

A final, presently only conceptual, single-stage biophotolysis system is based on a cyclic production of hydrogen and oxygen (System 4, Table 1). This idea is that algal photosynthesis results in the accumulation of a reductant, following which hydrogenase is activated and a period of hydrogen production ensues,depleting stored reductant. The key aspect requiring experimental demonstration is the procedure by which the culture is switched from one physiological state to the other. A short period of anaerobiosis must suffice, and inhibition of oxygen production must be maintained until reductant reserves are drawn down. One possibility is to couple this process with the diurnal light cycle.

Concurrently with the development of the chloroplast-ferredoxin-hydrogen system, two-stage systems based on both isolated subcellular components and on whole organisms have been studied (System 5, Table 1) [2, 18]. The basic concept was to generate in a photosynthetic stage an oxygen-stable reductant such as NADPH or an organic metabolite which would be separated and transferred to a hydrogen-evolving stage where it would serve as reducing agent before being recycled to the first stage. The photosynthetic stage could contain isolated chloroplasts (and other required enzymes) or algae capable of excreting the desired compounds. The hydrogen-producing stage contained either the hydrogenase enzyme or suitably treated bacteria capable

of metabolizing the reductant provided by the first stage. Transfer between stages was accomplished through dialysis fibers, allowing free passage to the reductants. This approach has recently been the subject of a U.S. patent application [35].

Another system (System 6, Table 1) to be considered involves the cycling of an algal culture between a shallow oxygen-producing stage and a deeper hydrogen-producing stage. As the algae accumulate reductant, they are transferred to the anaerobic stage where the greater depth results in less light per cell and, thus, conditions propitious for reversible hydrogenase induction and function. It should be relatively simple to experimentally demonstrate this concept.

One approach, termed "photofermentation" [4] uses photosynthetic bacteria in hydrogen production [19]. Photosynthetic bacteria do not produce oxygen, but do evolve hydrogen through a nitrogenase catalyzed reaction [7, 36, 37]. The use of photosynthetic bacteria has the advantage of overcoming the limitations of dark fermentative hydrogen production because light energy can be used to drive the hydrogen-producing reactions to completion. Indeed, the use of preformed organic compounds allows a very high rate of hydrogen production per unit area. The use of photosynthetic bacteria for hydrogen production from organic wastes, as an alternative to methane fermentations, has been proposed [4] and is being investigated (see below). Such a system (System 8, Table 1) stretches the definition of biophotolysis although, of course, the organic wastes originated with photosynthesis.

The final two biophotolysis systems presented in Table 1 involve either a trapping of the oxygen produced by photosynthesis, with subsequent release in a second stage, or an oxygen impermeable membrane which separate the oxygen and hydrogen-producing reactions. These concepts are still in the very initial stages of development. This review emphasizes the *in vivo* systems of biophotolysis as their practical development appears relatively more feasible at this time than the *in vitro* systems.

EFFICIENCY AND ECONOMICS OF BIOPHOTOLYSIS

Any analysis of biophotolysis must start with an examination of the potential efficiency of such processes. The conversion of sunlight into stored chemical energy by photosynthesis is subject to a number of limitations which reduce the theoretically achievable efficiency well below that possible with photovoltaics or concentrating mirrors. One major limitation is that only the visible (400-700 nm) wavelengths of sunshine can be used (although photosynthetic bacteria can use up to 850 nm). Thus, 55% of total solar energy is not used. The biggest limitation, however, is the 10 (or higher) quantum requirement of photosynthesis for the fixation of one carbon dioxide molecule. This results in a maximum utilization of 25% of photosynthetically active radiation absorbed by the photosynthetic system. As some sunshine is reflected, much is ineffectively absorbed, respiration and maintenance must be considered and solar insolation is either too high or too low much of the time, overall maximum photosynthetic efficiencies are between about 3 to 6% total solar radiation [38,39]. Certain other inherent limitations of the biophotolysis system, itself, must be considered: light reflection by any glass cover over the system, metabolic energy losses if nitrogenase is used. Therefore, a 3% conversion efficiency is the best that may be hoped for with the heterocystous

blue-green algal system. Achieving even this efficiency will require a considerable research and development effort, including genetic manipulation (e.g. maximize induction of reversible hydrogenase in the heterocysts). A more precise estimate is presently not possible. The best sustained efficiencies thus far recorded in outdoor operations of the heterocystous algal systems are about 0.2% (see below). However, process optimization has not yet been carried out and may be expected to result in significant improvements. It should also be pointed out that a 0.2% efficiency already corresponds to the average achieved in agricultural production.

The efficiency achievable with green algal systems, involving a cycling between periods or stages of oxygen and hydrogen production, may be expected to be somewhat higher than with the blue-green algal systems, although efficiencies exceeding 4% are not presently likely. In these systems other constraints may exist; however, these are not predictable. If the energy content of the organic substrate is ignored, the "efficiency" of hydrogen production with photosynthetic bacteria could exceed 100%. This is because the actual energy theoretically required for hydrogen evolution is rather minimal. Unfortunately, however, the hydrogen production is catalyzed by nitrogenase, resulting in the 5 ATP/hydrogen requirement. However, it may be possible to induce the reversible hydrogenase in these bacteria. In vitro systems are thought to have an ultimate potential efficiency of 10% [7]; however, the inherent limitations of any photosynthetic biological system make this appear too optimistic. Artificial systems may, however, achieve such efficiencies. The above calculations do not consider the question of net energy efficiency (see below).

The intensity of sunlight falling at the earth's surface is well known. A biophotolysis system operating in the southwestern U.S. (receiving about 500 Langleys per day at an average 3% efficiency) would produce yearly about 5,400 Kcal/m^2. At 1979 imported oil prices, the total value of the energy produced is about \$0.54/m^2/year. However, it may be expected that in real 1979 prices, fossil fuel (oil, gas) will rise to almost \$30/barrel (\$20/10^6 Kcal) well before the end of the century and that bulk petrochemical substitutes such as hydrogen may be worth \$50/10^6 Kcal. An externalized cost-benefit analysis may increase even this price to \$100/Kcal for hydrogen from biophotolysis in some local applications. Thus, at least an order of magnitude range in price may be estimated for hydrogen from biophotolysis from about \$0.5/m^2/yr for the current value of a hydrogen/oxygen fuel mixture produced by heterocystous blue-green algae to \$5/m^2/yr for the future value of a local, pure hydrogen source produced by any of the conceptual systems proposed in Table 1. If different assumptions about solar insolation and conversion efficiency are made, these numbers obviously change. For a photosynthetic-bacterial system, credits for waste treatment may exceed revenues from the hydrogen and allowable costs per unit area could well be as high as \$50 /m^2/yr [41]. Thus, to a first approximation, the economic constraints on the capital costs of biophotolysis systems can be estimated from the expected annual returns. Assuming a 15% capital charge and a 2:1 ratio of capital to operations cost, each \$1/m^2 annual return would allow a \$4.4/m^2 capital investment.

CONCEPTUAL DESIGNS OF BIOPHOTOLYSIS SYSTEMS

The hardware required for a biophotolysis system can be specified in general terms from the description of the processes given above. Thus, for

example, single-stage systems are required to be completely enclosed in a hydrogen-impervious vessel, which means glass, at least, for the top surface. Two-stage systems need only be covered in the hydrogen stage which is much smaller (10% or less) than the size of the oxygen-producing stage. The lowest-cost glass material available, already widely used in solar energy converters and eminently suitable for biophotolysis applications, are glass tubes such as are being manufactured for fluorescent light fixtures. Manufacturers [42] quotations (last quarter 1978 dollars) are $0.125 for a tube of 3.2 cm diameter, 244 cm in length, and 2 mm glass thickness. This is equivalent to $1.40/$m^2$. Somewhat larger tubes are also available at $ 3 /m^2. These tubes could be assembled in a closely spaced array with a plastic distribution pipe at both ends of the array. The glass tubes are already manufactured with a small indentation at each end, facilitating assembly by means of plastic pipes into an array. These should be arranged vertically, facing south, and inclined to meet the sun at the most favorable angle. This arrangement maximizes the amount of sunlight intercepted per unit of covered area, improving the overall economics of the system. The overall capital costs of a 5 m^2 array (glass, plastic) may be estimated at $3-4 per m^2 of flat area.

For the two-stage system an open-pond type may be considered, similar to the "high-rate pond" designs used in wastewater treatment and microalgal biomass production [43]. The lowest possible cost system may be constructed for as little as $1 m^{-2} for large-scale systems [44]. However, for the purposes of biophotolysis, a smaller-scale system would be required and greater process control is needed. A plastic cover may perhaps be advantageous, as it would prevent water evaporation, allow control of carbon dioxide supply, and permit greater control over the algal culture. The cost of the smaller covered algal growth systems is likely to be $4 m^{-2}. Together with the necessary second stage (which could be relatively more expensive as only a small area needs to be covered), the costs of such a system are likely to equal those of a single-stage system, particularly if the cost of the pumping unit that connects the stages is considered. As the size of the pumping units cannot be specified because the cycle times are undetermined, this factor must be considered uncertain. A detailed parametric study should, however, be feasible.

In addition to the cost of the basic collector-converter units, significant costs will be incurred in the accessory and peripheral equipment for any biophotolysis system. These include gas collection/distribution (piping), recirculating pumps, gas clean-up, culture production and recycling, process control systems, etc. It is difficult to estimate the costs of these peripheral systems; it may be expected to be at least as much as that of the basic collector-converter unit itself. There would be a definite economy of scale for these peripheral systems, suggesting that a minimum scale for biophotolysis may be several thousand square meters. Such a scale would also reduce operational costs which are a major unknown. They are the subject of the bioengineering analysis presented below. This scale would be appropriate for smaller industrial applications, suggesting that as one of the nearer-term markets for biophotolysis systems. A more detailed engineering design appears to be warranted to resolve some of these uncertainties. However, even considering the wide spread in potential value of the hydrogen produced by such systems, it appears that the costs of biophotolysis systems will be in the upper range of those permissible with foreseeable energy economics.

The marginal economics of biophotolysis, even with optimistic assumptions, suggest that this type of technology will find only specialty applications and/or require a long-term research and development effort. One alternative is to integrate biophotolysis with some other process so that the costs of the overall system are lowered. Two specific proposals can be made at this stage: incorporation of biophotolysis into a flat-plate solar-thermal collector, and integration of biophotolysis with waste treatment. In the first proposal, thermophilic algae operating at about 50°C would be used both as a biophotolysis converter and as a heat collector-working fluid. Although such a system would work at a relatively low temperature (and therefore efficiency) compared to flat-plate collectors, the thermal heat collected could be used for some applications. It may be estimated that the heat collected would be actually worth more than the hydrogen produced, strengthening the overall economics considerably. The use of photosynthetic bacteria in the production of hydrogen gas from organic wastes has been mentioned above. At present, such schemes would be limited to wastes containing relatively little nitrogen because the nitrogenase enzyme responsible for hydrogen production is repressed by fixed nitrogen.

HYDROGEN PRODUCTION BY HETEROCYSTOUS BLUE-GREEN ALGAE

As reviewed above, only the heterocystous blue-green algal system has been studied in any detail and, thus, can be subjected to a more detailed analysis of the operational factors that must be considered in any detailed design of a biophotolysis system. Table 3 summarizes presently available data on hydrogen production by Anabaena, the heterocystous blue-green algae genus which has been used in most studies. It would be preferable to convert the data to rates of hydrogen production per unit area per unit light energy, but it is not possible to do so at present. It should be noted that rates of hydrogen evolution per unit packed cell volume, dry weight or even chlorophyll (in these phycocyanin-containing organisms) are not directly interpretable in terms of light energy conversion. Also, in short-term experiments, hydrogen production is dependent on the previous history of the culture. This was shown clearly in experiments comparing exponentially growing with light-limited cultures (2nd Entry Table 3), which showed much lower rates of hydrogen production in the "older" (light-limited) batch cultures. This fact can explain very low rates of hydrogen production observed by several authors [47,48,50]. A number of factors are responsible for this low rate of hydrogen production by light-limited cultures, including increased activity of the uptake hydrogenase, reductant limitation, increased oxygen sensitivity of the reaction, and regulatory inhibition of nitrogenase biosynthesis. It is one of the challenges of the physiological-biochemical studies of these algae to devise procedures for overcoming some of the regulatory processes involved, allowing light-limited cultures to channel most of the reductant to the site of hydrogen production--the heterocysts.

The best rates of hydrogen production were obtained with nitrogen-starved cultures continuously bubbled with an argon gas stream containing a small amount of carbon dioxide (0.3%) and nitrogen gas (about 1%) (Table 4). This regimen provides a continuous supply of carbon dioxide and a sufficient supply of nitrogen to allow cell repair while keeping the algae at a very slow growth rate. The rates of hydrogen production were equivalent to about a 2.5% light conversion efficiency [55], or about one-fifth the rate of light energy conversion into cellular biomass observed with continuous cultures of the non-heterocystous blue-green algae Spirulina [57]. This difference must be ascribed in the inefficiencies inherent in the heterocyst system, the lack of optimization

TABLE 3

SUMMARY OF HYDROGEN PRODUCTION BY HETEROCYSTOUS BLUE-GREEN ALGAE

H_2 Production as Reported	H_2 production* µl/mg dry wt·h	Comments	Reference
1.24 nm/min/ml	11.9	Use of logarithmic phase culture	8
1.05 nm/min/ml	14.6	Logarithmic phase culture	
0.15 nm/min/ml	2.08	Light-limited culture (both activities unchanged by CO)	23
1.0 l/ l PCV/h	5.0		45
2.5-3.8 µl/mg dry wt·h	2.5-3.8	Anabaena cylindrica Oscillatoria brevis	46
0.0-0.7 µmoles/mg chl/h	0.09	Light-limited cultures	47
3.0 µmole/mg chl/h	0.3	Measured in the presence of CO and C_2H_2 which are reported to inhibit hydrogen consumption	48
3.05 µl/mg dry wt·h	3.05	Measured in the presence of 3% CO C_2H_2 reduction 6 x H_2	49
5.9 µmole/ml PCV·h	0.72	Nostoc muscorum, activities somewhat (30%) higher in the presence of DCMU due to inhibition of H_2 consumption	50
		Azolla, Anabaena azollae	
7.1 µl/mg chl/hr	0.036	N_2 grown	51
30.5 µl/mg chl/hr	0.15	NO_3 grown	
13.5 nm/mg chl/min	0.10	Azolla, Anabaena azollae	
50 nm/mg chl/min	0.37	Isolated Anabaena azollae	52

*To allow comparison between different laboratories, all production data were converted to the preferred units of µl/mg dry wt·h [6]. PCV (packed cell volumes) were connected to mgs chlorophyll using the conversion factor given by [50]. Mgs chlorophyll were converted to mg dry wt using the conversion factor given in [9]. Where possible, rates were converted using conversion factors given in the refs.

TABLE 4

HYDROGEN PRODUCTION BY NITROGEN-STARVED CULTURES OF BLUE-GREEN ALGAE

H_2 (μℓ/mg dry wt•h)	Comments*	Reference
32 (maximum)	artificial illumination average efficiency 0.8%	9
14 (averaged over 18 days)	maintained with ammonium additions artificial illumination	9
14-20	artificial illumination	53, 54
22.8 (averaged over 18 days)	artificial illumination efficiency 1.5%	11
6.0 (averaged over 24 days	sunlight, efficiency 0.1-0.2% maintained with low [N_2]	11
32 (maximum) 16 (averaged over 16 days)	artificial illumination efficiency 2.5% maintained with low [N_2]	55
19 (maximum)	Mastigocladus laminosus	56
11 (averaged over 18 days)	sunlight, maximum efficiency 0.2%	12
32 (averaged over 3 days, assuming 1% chlorophyll)	Miami BG-7 strain, reported not to be a heterocystous species	57

*all Anabaena cylindrica except where noted

of conditions, and the regulation of photosynthetic metabolism in nitrogen-starved cultures. Future work should concentrate on the latter aspect to increase the rates of hydrogen production by this system.

The outdoor experiments used a simple glass tube (5 cm diameter by 50 cm height) filled with one liter of algal culture. A set of glass tubes was exposed to sunlight by positioning them at an angle of 35°, facing south. The temperature was controlled at 25-29°C. The cultures were sparged with a similar gas mixture (0.3% CO_2, 0.5% N_2, balance argon) to that used in the indoor experiments. The culture of Anabaena cylindrica was grown in the laboratory on air/CO_2 using a standard blue-green algal media. After the cells had reached a cell density of 350 mg/L, they were harvested and resuspended at various concentrations and placed into the outdoor glass tube. The optimal algal concentration was about 200 mg/L during the winter. Figure 2 shows the results of one such experiment.

During the 36 days during which hydrogen production was measured, several distinct phases could be recognized: an induction phase during which hydrogen production is very low and oxygen production declines by over 90% is observed during the first two to three days of the experiment. In the second phase, hydrogen production rose sharply, achieving a high H_2/O_2 ratio (6:1) for a three to four-day period. Most of this hydrogen production took place at the expense of preformed reductant. Following this, the production of oxygen slowly increased, while that of hydrogen declined, resulting in a fairly long phase during which H_2:O_2 ratios were about 1:1. In the last phase, after about one month of operations, a steady decline of hydrogen, but not oxygen, production took place. The variability of the gas production by the algae was, in part, due to the variability in sunlight. This is most clearly seen in the case of oxygen production. However, hydrogen production also correlates (with some delay) with oxygen production. These different phases are probably characterized by different limiting factors: nitrogenase biosynthesis and starch accumulation in the induction phase, filament breakdown in the terminal phase, and, probably, reductant limitation in intermediate periods. However, this is speculative at present and requires further study. The rates of hydrogen production observed during the 36-day experiment shown in Figure 3 correspond to an averaged 0.2% total solar energy conversion efficiency, or about one-fifth of that observed in microalgal biomass production by outdoor algal cultures during this period. This difference is similar to that observed in the laboratory. Thus, it appears that the laboratory studies can be extrapolated fairly readily to an outdoor situation.

CONSIDERATIONS IN OPERATING A BIOPHOTOLYSIS CONVERTER

In this section the factors that must be considered in operating a heterocystous blue-green algal system are examined in detail and then extrapolated to other systems. This analysis will direct the future research required for the development of these systems.

The first problem is that of providing the culture for stocking the converters and inducing it to produce hydrogen at high rates. It is clear that this should be done in situ with air provided as the nitrogen source until the culture has achieved an optimal cell density for induction. This process will require several days and must be monitored carefully. The algal denisty at which the culture should be induced may not be the same at which it should be operated, so that a complex induction process may be eventually developed

to optimize both requirements. Overall, however, the induction phase is not a critical factor as it is rather short when compared to the desired period of hydrogen production which should be several months.

The effect of insolation on converter performance is the most critical factor. Hydrogen production by Anabaena cylindrica is directly proportional to light intensity up to a saturating value which depends on the algal density, light path, and algal physiology. More importantly, hydrogen production responds quickly to changes in light intensity, decreasing to zero within minutes after sunset and assuming a new steady-state value within one hour of changed light intensity. Since maximal hydrogen production rates are achieved at lower than full sunlight intensities [12], it would probably be optimal to decrease incident sunlight by placing the converter panels at a steeper angle to the sun and packing them closer together. This would increase costs per unit area, but result in higher efficiencies. Sunlight reflection from the curved glass panels must be considered in such a design. One interesting observation is that relatively long pulses of intermittent light increased the yield of hydrogen production [54]. This should be verified and studied further.

Temperature effects in hydrogen production have not been studied extensively yet. The temperature dependence of acetylene reduction by A. cylindrica has been studied as a function of light intensity and oxygen tensions [10]. The temperature optima for acetylene reduction by A. cylindrica is around 30°C while that for Mastigocladus laminosus is about 45°C. In the biophotolysis converter proposed in Figure 2, temperatures will depend on the heat budget which will be a complex function of ambient temperature, insolation, wind speed, converter placement, and operations. They are easier determined experimentally than predicted. However, significant temperature variations appear tolerable, as no significant difference was observed between two parallel outdoor hydrogen-producing cultures, one thermostated, and one not.

The supply of optimal concentrations of metabolizable nitrogen appears to be an important factor in the long-term maintenance of an active culture. The use of ammonia as a nitrogen source has been reported, however, with contradictory results [9 ,53]. The use of a small amount of nitrogen in the gas phase is a better, and potentially cheaper, method of supplying sufficient nitrogen to maintain cellular repair mechanisms. Determining proper nitrogen concentration requires the determination of the volumetric transfer coefficients ($k_L a$) in the converter tubes. These coefficients are also important in carbon dioxide supply and the removal of hydrogen and oxygen. As the ratios of mass transfer coefficients of two gases are related to the square root of the ratios of their diffusivities in water; by determining the coefficient for oxygen (which are easily determined), the others can be calculated. This was done for the converters used in both the indoor and the outdoor experiments described above. As mass transfer = $k_L a\ (C_i - C_o)$ (where C_i is gas concentration in the gas phase and C_o in solution), the nitrogen supply rate could be calculated as a function of partial pressure [55]. A rather broad optimum nitrogen supply, from 15 to 35 μmoles nitrogen/L · hr, was determined to maintain maximal hydrogen production in a culture. This amount of nitrogen does, however, result in slow growth of the culture. This growth compensated for some of the decline of hydrogen production on a dry-weight basis observed in the experiments (Fig. 2).

In practice, the operations of the converter would involve periodic replacement of a small fraction of the culture or operated as a very slow dilution continuous culture to remove dead cell material and maintain optimal

culture density. This would introduce another complication into the operation of the converter. Whether the same lines used for gas supply and removal could be used in the supply of media and removal of the algal culture must be answered by a more detailed engineering analysis.

Mixing of the culture is another of the critical factors in biophotolysis. Mixing will maintain an evenly suspended culture and remove hydrogen and oxygen as they are formed. The most appropriate mixing method is through gas recirculation. It can be readily shown that if a converter is not mixed, as would be the case for the flate-plate, horizontal converters previously proposed [6], mass transfer of nitrogen and carbon dioxide to and hydrogen and oxygen from the algae settled on the bottom of the converter would be the limiting factors. From the equation: mass transfer rate = - D_L (C_g-C_o/L) where D_L is diffusivity, L is path length, and C_g is concentration in the gas-liquid interface, assuming C_o - concentration in the liquid - to be zero; it can be calculated that a 25% nitrogen gas phase would be required for supplying a similar amount of nitrogen as 0.5% nitrogen provides in the vertical converter. The calculation was based on a solubility of nitrogen of 0.62 mmole/L atm at 25°C and a diffusivity of 1.9 x 10^{-5} cm^2/sec. Similarly, an overpressure of carbon dioxide would be required during the algal culture growth phase. More critically, a horizontal converter would be subject to instabilities, as gas bubbles form due to local overpressures of hydrogen and oxygen. Thus, only mixed vertical converters can be considered in biophotolysis applications.

Of course, the energy required in bubbling gas through the converter becomes the critical problem. The pressure drop in a vertical glass tube with a 1 m head is estimated at 0.1 kg/cm^2. Assuming a 50% increase in pressure drop due to pipe resistance, about 15 glass tubes per m^2 and a 1.5 L/hr gassing rate for each tube, then about 10,000 m^2 of converter area would be supplied by a single 5.0 HP (shaft power) blower delivering 5 m^3/min of gas. This would require about 10% of the hydrogen output from the system, assuming an insolation of about 500 Langleys, a 3% solar conversion efficiency, operation of the blower during daytime only, and an overall efficiency of 25% for conversion of hydrogen to shaft power. Obviously, these are crude calculations and must be backed up by careful measurements of the pressure drops involved and the minimum allowable gassing rates. Nevertheless, the calculation suggests that it may be feasible to operate, in principle, a net energy producing biophotolysis system. This calculation also sets the operational limits to a practical biophotolysis system using algal cultures.

One factor that is important in the utilization of the hydrogen produced is its concentration in the gas phase. As hydrogen is produced simultaneously with oxygen, a too low or too high concentration would result in poor utilization or danger of explosion. The relatively high cost of separating the hydrogen gas suggests that it be combined directly with oxygen in a thermal process. Thus, a partial pressure of about 3-30% would be the range for hydrogen desired, depending on the design of the combustion system. At present, the hydrogen gas concentrations in the outflow from the converters are only 0.1-0.2%. Thus, about a 100-fold increase would be desirable. This could best be achieved by a gas recirculation pump. The essentially irreversible nature of the nitrogenase and the adaptive character of the oxygen-resistance mechanisms of heterocysts suggests that the heterocystous blue-green algal system could be operated at just about any desirable partial pressure of hydrogen and oxygen. However, this needs to be verified.

Other factors may also be considered in the operation of a biophotolysis system but should not be critical. Thus, the pH, controlled by the carbon dioxide pressure and alkalinity, did not affect hydrogen production between 7.4 and 9.4 in one study [54]. Minor nutrients could always be supplied in optimal amounts in a closed system like the one proposed. Comparing axenic and non-sterile cultures in the laboratory did not reveal any significant differences, suggesting that contaminants may not present any problems. Scaling up problems should be rather minor due to the modular nature of the system. Perhaps the most important consideration, after the ones discussed above have been studied and optimized, is that of process control of the system. This will probably require sophisticated, modern control techniques. However, many problems of algal physiology need to be resolved before this issue needs to be addressed.

CONCLUSIONS

Almost all work on biophotolysis has emphasized the biochemical and physiological mechanisms involved in such processes. The development of practical systems will require a greater emphasis on the bioengineering aspects discussed above. Contrary to general belief, biophotolysis need not be a very long-term R & D activity; some specific near-term options are available. In particular, photosynthetic bacterial systems could be developed which produce hydrogen while degrading low-nitrogen wastes. This will, however, be a limited option, restricted by the availability of suitable wastes. The production of hydrogen from water for small-scale industrial uses will be a much larger market. The prospective value of the hydrogen appears to be in line with the probable capital and operating costs of a biophotolysis system. Fabricating and operating biophotolysis panels such as those described above and carrying out a detailed engineering-economic analysis should be of high priority. This should resolve the doubts that presently exist about the practicality of such systems and justify expansion of the R & D activities necessary to achieve the 2-4% solar conversion efficiencies required for commercial feasibility.

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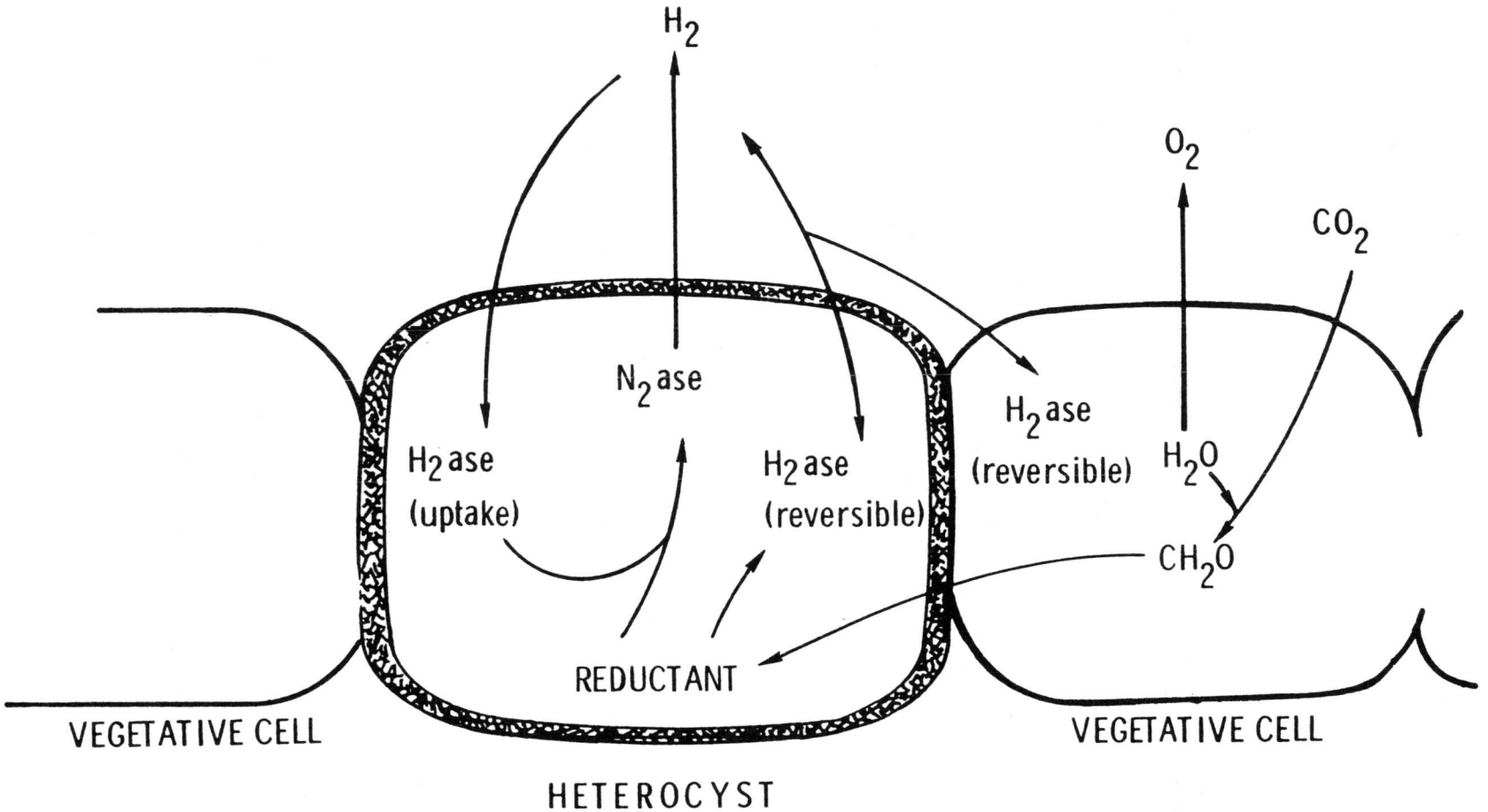

FIGURE 1. MODEL OF HYDROGEN METABOLISM IN HETEROCYSTOUS BLUE-GREEN ALGAE

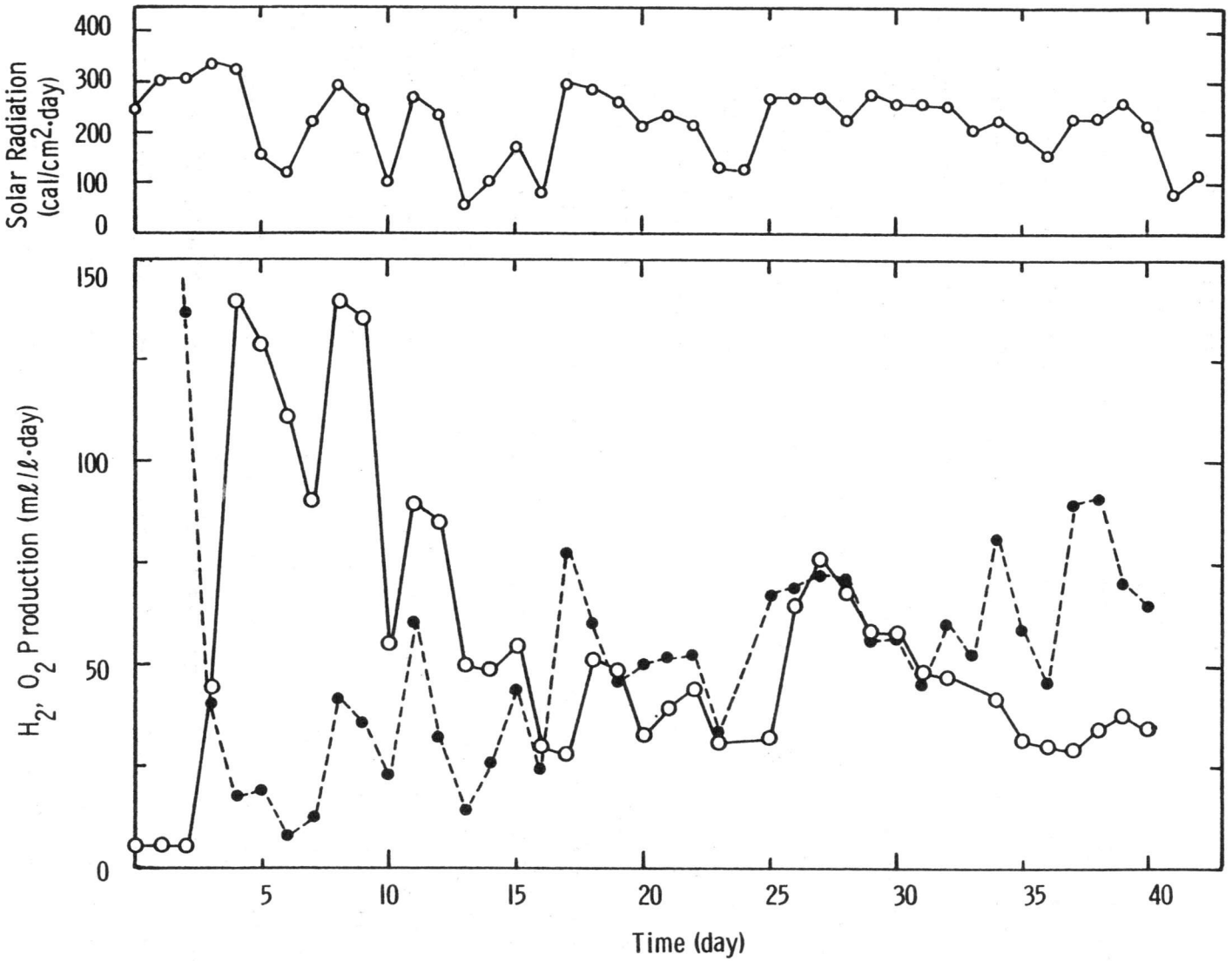

FIGURE 2. HYDROGEN PRODUCTION BY Anabaena cylindrica IN AN OUTDOOR CONVERTER.

Cultures were placed in 1-L glass tubes and thermostated at 26.5°C (± 1.5°C) and inclined at 35° facing south. Argon gas was bubbled through the column to mix the culture and remove any H_2 and O_2 formed, which were determined by gas chromatography (open circles H_2, closed circles O_2). Daily solar radiation is also shown in the upper part of the graph. Algal loading was kept constant at about 1.6 mg/mL throughout the experimental period (Nov. 6-Dec. 16, 1978).

XV. CONCLUSIONS AND FUTURE WORK

The basic research, outdoor testing and bioengineering analyses presented above, represent a significant advance in the development of a practical biophotolysis system based on blue-green algae. Nevertheless, it is clear that this goal will require a long-term research plan as many difficulties will need to be overcome. Two parallel efforts will be required: the development of an algal strain capable of a high sustained rate of hydrogen production and the engineering of a low-cost converter system. The first will necessitate a careful study of the relevant physiology and biochemistry of these algae, in particular, the effects of nitrogen starvation and regulation of hydrogenase, nitrogenase, and their oxygen-protection mechanisms (see Chapters VII - XI). This must be followed by selection and genetic improvement of the most suitable strains of algae. The converter development can proceed, in part, independently of this effort as it is, to a large extent, a materials and control problem. Such a converter would have applications to some other biophotolysis systems, as they have similar requirements. Indeed, as discussed in the previous chapter, such systems could have somewhat nearer-term potential. Thus, such systems as photosynthetic bacteria should receive a greater emphasis in the near future.

The continuation proposal of this project emphasizes only the conclusion of the work on nitrogenase regulation and localization. It is expected that work can be completed during the summer of 1979. Thereafter, the project is proposed to be continued outside the University of California with a shifted emphasis toward the effects of nitrogen starvation on the photosynthesis and metabolism of the heterocystous algae, biophotolysis converter development, and study of alternative systems of biophotolysis.

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