HYDROGEN FORMATION BY MARINE BLUE–GREEN ALGAE

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1. Introduction

Hydrogen formation by biophotolysis in blue–green algae (cyanobacteria) is under investigation as a potential source of fuel. The nitrogen-fixing freshwater alga *Anabaena cylindrica* appears to be particularly suitable by virtue of possessing heterocyst cells which contain nitrogenase [1,2]. However, a possible limitation in the scaling up of such a process is lack of water and/or land area in certain regions and hence there is a need to investigate the process of biophotolysis in marine organisms.

Certain widespread groups of filamentous marine blue–green algae are known to fix nitrogen [3] and hence offered potential as hydrogen producers. The following were chosen for investigation: *Calothrix scopulorum, Calothrix membranacea, Oscillatoria brevis* and *Schizothrix calcicola.* Hydrogen measurements were made with organisms incubated both anaerobically under argon, and aerobically in the presence of carbon monoxide and acetylene; under both of these conditions *Anabaena cylindrica* releases hydrogen gas [4].

2. Materials and methods

2.1. *Algae and their growth*

Anabaena cylindrica (strain B629),
Oscillatoria brevis (strain B1567),
Calothrix membranacea (strain B379) and
Schizothrix calcicola (strain B1817)
were obtained from the Culture Collection of Algae, University of Texas at Austin.

Calothrix scopulorum (strain 1410/5) was obtained from the Culture Centre of Algae and Protozoa at Cambridge.

Each was grown axenically at 25°C in 3 litre glass bottles containing 2 litres Allen-Arnon [5] medium magnetically stirred and sparged with 5% CO_2 in air at 100 ml/min. The light intensity (Philips Fluorescent Daylight lamps TL20/W55) was 7000 lux at the surface of the vessels. *Schizothrix calcicola* showed very poor growth under these conditions and also in seawater and could not be evaluated as a hydrogen producer for this reason.

Algae were harvested by centrifugation (8300 × *g*, 0–4°C, 15 min) at approx. 100 Klett units as measured with a Klett-Summerson colourimeter (660 nm) and concentrated approx. two-fold for experiments. Because *Calothrix scopulorum* stuck very strongly to the walls of the growth vessel its concentration could not be determined in terms of Klett units in the growth vessel. The growth in Allen-Arnon medium of this alga was good but was very slow in seawater, even when supplemented with phosphate at levels equal to those in the Allen-Arnon medium. From Klett measurements the doubling times for *Calothrix membranacea* and *Oscillatoria brevis* were shown to be similar to that of *Anabaena cylindrica* (approx. 20 h). Heterocyst frequencies were determined by counting under a microscope the numbers of each cell type in several filaments. Dry weights were determined by heating duplicate 10 ml samples for 16 h at 85°C and correcting for the weight of mineral salts.

2.2. *Incubations*

Algae (10 ml) were incubated in 36 ml Erlenmeyer flasks fitted with upturned subaseals (W. Freeman and Co.) containing a water trap. An argon atmosphere was ensured by bubbling through inlet and outlet needles inserted through the subaseals and finally evacuating and refilling with argon via a manifold

system [4,6]. CO_2 was injected subsequently as were CO and C_2H_2 when required. Argon/nitrogen mixtures were made by filling flasks to atmospheric pressure with argon, withdrawing a calculated volume and injecting the required volume of nitrogen to restore atmospheric pressure. The cultures were incubated, with shaking, in a light box at 25°C and 4000 lux light intensity. A disposable syringe with all but the rubber gasket of the plunger removed was also inserted into each flask. The gasket responded well to slight pressure changes in the flasks, allowing essentially atmospheric pressure to be maintained.

2.3. *Hydrogen analysis*

Analysis of hydrogen in the flasks was made by taking 1 ml gas samples through the subaseals with disposable syringes (26-gauge needles) as described [4]. Hydrogen was measured by injection into a Pye Katharometer 104 series gas chromatograph [4].

3. Results

3.1. *Characteristics of hydrogen formation*

Hydrogen gas formation by each of the algae was measured both anaerobically under agon, and aerobically in the presence of the inhibitors carbon monoxide and/or acetylene. The results of a typical experiment under the latter conditions with *Oscillatoria brevis* are given in fig.1. It is seen that, whereas in air supplemented with CO_2 alone no hydrogen formation could be detected, hydrogen was formed in the presence of CO or C_2H_2; its rate of formation was further enhanced in the presence of both gases. After an initial lag period of 2–4 h a constant rate of formation was sustained for at least 32 h in each case, but this had slowed or ceased by 60 h. The rates, which were calculated in the period 12–36 h, after the lag, are presented in table 1 together with the rate obtained in argon supplemented with CO_2. Under argon no lag period was observed.

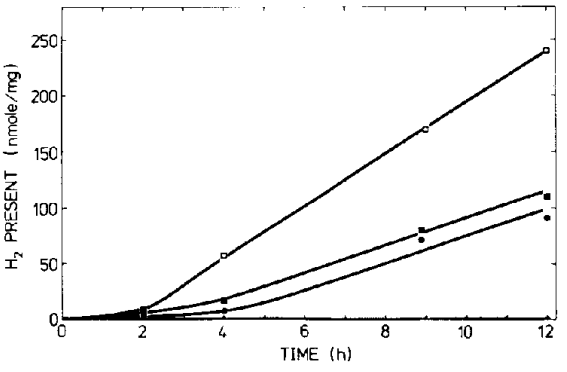

Fig.1. Formation of hydrogen as a function of time by *Oscillatoria brevis*. Algae were incubated in Allen-Arnon medium [5] below the following gaseous atmospheres: air/3% CO_2 (○), air/3% CO_2/2% CO (●), air/3% CO_2/10% C_2H_2 (■) and air/3% CO_2/2% CO/10% C_2H_2 (□). Concentrations of gases refer to initial times throughout.

Similar experiments were performed with *Calothrix scopulorum, Calothrix membranacea* and, for comparison, *Anabaena cylindrica* (table 1). For the sake of comparison each of the experiments was done with algae suspended in the medium of Allen and Arnon [5]. Table 1 also shows the results of experiments

Table 1
Formation of hydrogen gas by marine algae[a]

Gas atmosphere[b]	*Anabaena cylindrica*	*Oscillatoria brevis*	*Calothrix membranacea*	*Calothrix scopulorum*	*Calothrix scopulorum*[c]
Argon	103	168	108	128	47
Air or Dinitrogen	0	0	0	0	0
Air/2% CO	11	14	13	12	8
Air/10% C_2H_2	23	12	13	15	6
Air/2% CO/10% C_2H_2	56	28	26	24	15

[a] The rates of hydrogen formation are given as nmol/h/mg dry wt algae – All experiments were done with algae suspended in Allen-Arnon medium [5] except those specified

[b] Each of the atmospheres was supplemented with 3% CO_2

[c] Experiments performed with algae grown and suspended in seawater

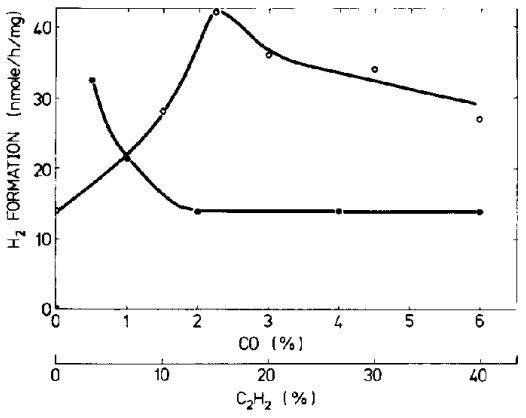

Fig.2. Effects of CO and C_2H_2 concentrations on hydrogen formation by *Oscillatoria brevis* suspended in Allen-Arnon medium with a gas atmosphere balanced by argon/3% CO_2. Effect of CO concentration (•) and effect of C_2H_2 concentration with 2% CO also present (○). Experiments and measurements as for fig.1.

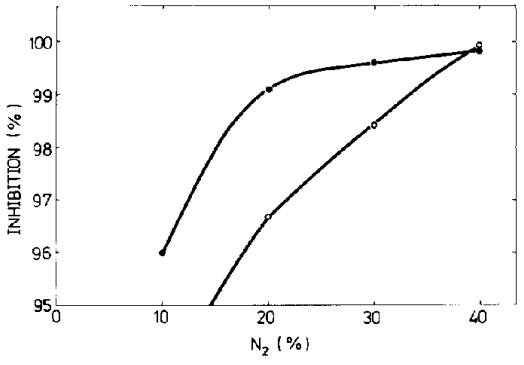

Fig.3. Inhibition by dinitrogen of hydrogen formation by *Oscillatoria brevis* (•) and *Calothrix membranacea* (○). The gas phase was balanced with argon/3% CO_2. The ordinate refers to inhibition of the rate of hydrogen formation.

performed with *Calothrix scopulorum* suspended in seawater; under each of the conditions tested the rates were lower than in Allen-Arnon medium. Controls performed in the dark with *Calothrix membranacea* and *Oscillatoria brevis* showed negligible hydrogen formation under argon.

3.2. *Rates of hydrogen formation as a function of CO and C_2H_2 concentrations*

The concentrations of CO and C_2H_2 used in the above experiments (2% and 10%, respectively) were those selected for previous work with *Anabaena cylindrica* [4]. Figure 2 shows the dependence on CO and C_2H_2 concentrations of hydrogen production by *Oscillatoria brevis*. It is seen that the optimal concentration of CO is less than 1% and that of C_2H_2 (in the presence of 2% CO) is approx. 15%.

3.3. *Inhibition of hydrogen formation in argon by dinitrogen*

To provide evidence that the hydrogen was produced by the catalytic action of nitrogenase the rates of hydrogen formation by *Oscillatoria brevis* and *Calothrix membranacea* in argon were measured as a function of nitrogen concentration in the gaseous atmosphere. After an initial lag the rates were constant and were measured from 3–12 h of incubation (fig.3). It is seen that low concentrations of nitrogen were highly inhibitory.

4. Discussion

The major conclusions from this work are that the marine filamentous heterocystous blue–green algae *Oscillatoria brevis*, *Calothrix scopulorum* and *Calothrix membranacea* produce hydrogen and that the characteristics of the process are similar to those previously established for the freshwater alga *Anabaena cylindrica* [1,2,4,7,8]. That hydrogen is formed without prior anaerobic adaptation (table 1) and is inhibited by dinitrogen (fig.3, table 1) is indicative that the process is mediated by nitrogenase in the heterocysts of the organisms. This conclusion is supported by the aerobic hydrogen formation observed in the presence of CO and C_2H_2 (fig.2), inhibitors of (the nitrogen-fixing function of) nitrogenase and uptake hydrogenase, respectively [7,9]. The lack of hydrogen evolution in air alone (table 1) as is known to occur in certain rhizobia lacking an uptake hydrogenase [9] is additional evidence for the presence of this latter enzyme.

Although the general characteristics of each of the tested algae, including *Anabaena cylindrica*, were similar, differences in rates were observed. These are presumably due to physiological and

biochemical differences between the algae. In addition, although all of the organisms studied had a heterocyst frequency of approx. 5%, it was observed that they exhibited variable fragility at the heterocyst/vegetative cell junction. For example *Calothrix membranacea* tended to break readily, which may have affected hydrogen formation. The observation of heterocysts in *Oscillatoria brevis* was surprising in view of the reported lack of such cells in members of the family Oscillatoriaceae [10], although is consistent with aerobic growth in the absence of a combined nitrogen source.

As shown in table 1 the rates in air/CO/C_2H_2 were invariably lower than those in argon. Possibly the former rates could be enhanced by optimizing the CO and C_2H_2 concentrations. Each alga may vary somewhat in sensitivity to these gases. The lower rates for *Calothrix scopulorum* in seawater than in Allen-Arnon medium are not explicable at this stage but warrant further investigation.

In summary the marine blue–green algae examined appear to have all the attributes of the freshwater alga *Anabaena cylindrica* for a possible practical biophotolytic system and their obvious advantage of being marine organisms makes them particularly deserving of further attention.

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