Measurement in vivo of Hydrogenase-Catalysed Hydrogen Evolution in the Presence of Nitrogenase Enzyme in Cyanobacteria

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A method was devised that allows measurement in vivo of hydrogenase-catalysed H₂ evolution from the cyanobacterium Anabaena cylindrica, independent of nitrogenase activity, which is also present. Addition of low concentrations of reduced Methyl Viologen (1–10mM) to intact heterocystous filaments of the organism resulted in H₂ evolution, but produced conditions giving total inhibition of nitrogenase (acetylene-reducing and H₂-evolving) activity. That the H₂ formed under these conditions was not contributed to by nitrogenase was also supported by the observation that its rate of formation was similar in the dark or with Ar replaced by N₂ in the gas phase, and also in view of the pattern of H₂ evolution at very low Methyl Viologen concentrations. Conclusive evidence that the H₂ formed in the presence of Methyl Viologen was solely hydrogenase-mediated was its evolution even from nitrogenase-free (non-heterocystous) cultures; by contrast 'uptake' hydrogenase activity in such cultures was greatly decreased. The hydrogenase activity was inhibited by CO and little affected by acetylene. Finally the hydrogenase activity was shown to be relatively constant at different stages during the batch growth of the organism, as opposed to nitrogenase activity, which varied.

H₂ formation can be catalysed by either nitrogenase or hydrogenase enzymes, both of which co-exist in many micro-organisms (Dixon, 1976; Benemann, 1977). In cyanobacteria the hydrogenase appears to exist in two forms, a membrane-bound form that catalyses H₂ consumption and a soluble form that also catalyses H₂ production (Tel-Or et al., 1977, 1978). The membrane-bound form of the enzyme appears to be that which contributes predominantly to the efficiency of nitrogen fixation by recycling nitrogenase-mediated H₂ (Tel-Or et al., 1977, 1978). The soluble enzyme is of primary interest in studies of biophotolysis as a potential means of solar-energy conversion, being ATP-independent and having a higher catalytic capacity than nitrogenase (Benemann, 1977; Lien & Pietro, 1975). Such studies would be aided by the availability of a method of measuring attainable rates of hydrogenase-mediated H₂ production in vivo, independent of that produced by nitrogenase. We have found that the addition of low concentrations of reduced Methyl Viologen to intact filaments of the heterocystous cyanobacterium Anabaena cylindrica results in H₂ evolution that directly reflects the maximal activity of hydrogenase; nitrogenase activity is eliminated under such conditions. We have used this procedure to determine hydrogenase activity at different stages during the batch growth of this organism and have shown that the H₂-evolving form of the enzyme is relatively unaffected by acetylene, an inhibitor of H₂ consumption (Smith et al., 1976; Bothe et al., 1977; Daday et al., 1977). This procedure enabled an investigation of H₂ formation in vivo in heterocyst- and nitrogenase-free cultures.

Experimental

Materials

Flasks were stoppered with Suba-Seal rubber stoppers (William Freeman and Co., Barnsley, Yorkshire, U.K.). Methyl Viologen was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and sodium dithionite was purchased from E. Merck A.G., Darmstadt, Germany. All gases were of the highest available purity and obtained from Commonwealth Industrial Gases, Canberra, A.C.T., Australia.

Methods

Algae and their growth. A. cylindrica (strain B629) and Calothrix membranacea (strain B379), bacteria-free, were obtained from the culture collection of algae, University of Texas at Austin, TX, U.S.A., and heterocystous (nitrogen-fixing) cultures were grown in media free of combined nitrogen as described previously (Daday et al., 1977). All experiments were
carried out with such cultures, except where indicated. When heterocyst-free cultures were required the medium was supplemented with 4 mM-NH₄Cl and was inoculated from stock cultures similarly supplemented several weeks previously. Doubling times were 12–20 h and algae were harvested at a concentration between 70 and 120 Klett units (Mallette, 1969) by settling in measuring cylinders; they were not centrifuged. An algal concentration of 100 Klett units corresponded to 3.3 mg dry wt./10 ml.

**Incubations.** These were carried out with algae at a concentration between 200 and 250 Klett units in flasks as described previously (Lambert & Smith, 1977). The light intensity was 4000 lx. In preparation for the incubations, flasks containing 10 ml of algal suspensions were continuously shaken to remove dissolved gases, while being evacuated via needles inserted through the rubber stoppers. Flasks were then refilled to atmospheric pressure with Ar or N₂. Minor gases, including acetylene, H₂ and CO₂, were added by injection, after prior removal of a sufficient volume of gas (by using Ar-preflushed syringes) to allow restoration of atmospheric pressure. All flasks initially contained 3% CO₂ in the gas phase. Oxidized Methyl Viologen and sodium dithionite were mixed anaerobically and added to the algae simultaneously by injection. Their pH was neutralized by prior addition of a predetermined volume of NaOH.

**Gas analysis.** H₂ and ethylene concentrations in the gas phase were determined by g.l.c., as described previously (Daday et al., 1977; Lambert & Smith, 1977).

**Dry-weight determinations.** Dry weights were determined by heating 10 ml samples for 16 h at 85°C and correcting for the weight of mineral salts in the medium.

**Results**

**Effects of Methyl Viologen and sodium dithionite on acetylene reduction and H₂ evolution**

Addition of reduced Methyl Viologen (obtained by premixing 5 mM-oxidized Methyl Viologen with 14.4 mM-sodium dithionite) to algae incubated beneath an Ar atmosphere in the light results in a virtually complete inhibition of acetylene reduction by the nitrogenase enzyme (Fig. 1a), but H₂ evolution under the same conditions in the absence of acetylene still occurred, though at a lower rate (Fig. 1b). Dithionite (14.4 mM) and oxidized Methyl Viologen (5 mM), neither of which alone causes H₂ evolution from hydrogenase (Tel-Or et al., 1977, 1978), each gave only partial inhibition of nitrogenase activities as measured by either acetylene reduction or H₂ formation.

Further experiments were carried out to determine the effects of the reductant concentrations on the rates of H₂ evolution and acetylene reduction, and the results are shown in Fig. 2. In the presence of 14.4 mM-sodium dithionite inhibition of acetylene reduction was virtually complete at Methyl Viologen concentrations equal to or above 0.05 mM, and maximal rates of H₂ were attained at concentrations of 1 mM or above. It is noteworthy that the rate of H₂ formation is little affected by the absence of light and unaffected by an N₂ gas atmosphere, and in particular that the rate is unaffected by the presence of 10% acetylene, even when the cultures are preincubated for 3 h with this gas present.

The effects of still lower Methyl Viologen concentrations on acetylene reduction and H₂ formation are

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![Fig. 1. Influence of Methyl Viologen and sodium dithionite on H₂ evolution and acetylene reduction](image)
ASSAY OF HYDROGENASE IN THE PRESENCE OF NITROGENASE

Fig. 2. Dependence on reductant concentration of the rates of H₂ evolution and acetylene reduction
Rates of H₂ evolution were measured at various concentrations of Methyl Viologen (●) in the presence of a fixed sodium dithionite concentration (14.4 mM) and at various concentrations of the latter compound (○) in the presence of a fixed concentration of the former (5 mM). They were compared with the control (■) containing neither reductant. Experiments were also carried out with both reductants present (5 mM Methyl Viologen, 14.4 mM sodium dithionite) in the dark (□) and in the light with 10% acetylene (▲) initially present in the gas phase. An experiment was also carried out with these concentrations of Methyl Viologen and sodium dithionite but with Ar replaced by N₂ gas (▲). Rates of acetylene reduction (10% acetylene initially in gas phase) were measured as a function of Methyl Viologen concentration with 14.4 mM-sodium dithionite (▼) and compared with a control (▼) containing neither reductant.

showed in Fig. 3, compared with control values and values obtained with dithionite alone. It is noteworthy that at certain low concentrations of Methyl Viologen (e.g. 0.1 mM) negligible H₂ evolution and acetylene reduction occurs (Figs. 2 and 3). At lower concentrations inhibition of nitrogenase reactions was incomplete (Fig. 3), but increased with increasing Methyl Viologen concentration (2–10 μM).

CO inhibition of H₂ formation
The putative hydrogenase-catalysed H₂ evolution was sensitive to CO and the dependence of inhibition on CO concentration is shown in Fig. 4.

Hydrogenase activity as a function of algal growth
As discussed below, the above results are consistent with the interpretation that H₂ formation in the presence of reduced Methyl Viologen occurs exclusively by the catalytic action of hydrogenase, and it was decided to measure the activity of this enzyme as a function of algal batch growth time, under which conditions the activity of nitrogenase enzyme is known to vary widely (Weare & Benemann, 1973; Daday et al., 1977). Fig. 5 shows the activity of hydrogenase enzyme measured by this method at different stages during the batch growth of algae. This was compared with the acetylene-reducing and H₂-evolving activities of nitrogenase in vivo (Fig. 5). The hydrogenase-catalysed H₂ formation in the presence of Methyl Viologen is appreciable compared with that catalysed by nitrogenase, particularly at the higher algal concentrations, at which the nitrogenase activity declines rapidly. Algal cultures were

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found to vary somewhat in the proportion of nitrogenase- to hydrogenase-catalysed H₂ formation; some slower-growing cultures, in particular, showed appreciably greater hydrogenase to nitrogenase ratios.

Hydrogenase in nitrogenase-free cultures
Heterocyst-free cultures were grown and shown not to produce H₂ nor to reduce acetylene when incubated beneath an Ar atmosphere (Fig. 6). When reduced Methyl Viologen (5 mM) was added to such cultures, however, they produced H₂ (Fig. 6). This hydrogenase activity was inhibited by CO.
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**H₂ uptake by heterocystous and heterocyst-free cultures**

H₂ uptake by both heterocystous and heterocyst-free algae was measured by incubating the cultures beneath an initial gas atmosphere of N₂ supplemented with 3% CO₂ and 1% H₂ (Fig. 7). Much higher specific rates of H₂ uptake were observed in the heterocystous cultures.

**Discussion**

Three main conclusions can be drawn from this work. First, the addition of reduced Methyl Viologen (1–10 mM) to intact heterocystous filaments of the cyanobacterium *Anabaena cylindrica* enables the H₂-evolving capacity of hydrogenase to be distinguished from that of nitrogenase, even in the light. Presumably nitrogenase activity is inhibited because the very strong reductant Methyl Viologen interferes with photosynthetic electron transport and hence photophosphorylation, resulting in cessation of the ATP supply to nitrogenase. Methyl Viologen nevertheless can function as a substrate for hydrogenase (Peck & Gest, 1956; Gogotov & Kosyak, 1976).

That the H₂-evolving activity under these conditions is hydrogenase-mediated and not in any part due to nitrogenase is substantiated by the observed constancy of production under an atmosphere of N₂ (Fig. 2), a potent inhibitor of nitrogenase-catalysed H₂ evolution (Benemann & Weare, 1974), and in the absence of light (Fig. 2; Bothe et al., 1977), and also by the observation that low concentrations of Methyl Viologen (e.g. 0.1 mM) resulted in negligible H₂ evolution (Figs. 1 and 2), presumably being sufficient to inhibit nitrogenase, but providing negligible substrate for hydrogenase. Furthermore, the apparent *Kₚ* in *vivo*, approx. 0.4 mM (Fig. 2), is in good agreement with the value (0.31 mM) determined in *vitro* with Methyl Viologen (Tel-Or et al., 1978). Conclusive evidence that the H₂ formed in the presence of reduced Methyl Viologen was solely hydrogenase-catalysed came from measurements of its formation even in nitrogenase-free (non-heterocystous) cultures (Fig. 6).

Variation of Methyl Viologen concentration is a useful tool for studies *in vivo* in that in its absence H₂ production is nitrogenase-mediated (Bothe et al., 1977; Jones & Bishop, 1976; Daday et al., 1977), at very low concentrations both nitrogenase- and hydrogenase-mediated H₂ formation are effectively inhibited (Figs. 1 and 2) and at higher concentrations H₂ production is hydrogenase-mediated and thus a measure of hydrogenase activity (Fig. 2). The fact that H₂ production and inhibition of acetylene reduction were constant over a broad range of concentrations of dithionite plus Methyl Viologen (Fig. 2) indicates that the H₂ production reflects the maximal catalytic H₂-evolving capacity of the enzyme present; the membrane-bound H₂-uptake form of the enzyme (Tel-Or et al., 1978) would presumably not contribute to the H₂ evolution. Fig. 1 shows that in fact there is a slight decline in activity as the Methyl Viologen concentration (at fixed dithionite concentration) is increased above 1 mM. This is probably due to the increasing dark-blue colour of the algal solutions due to the presence of Methyl Viologen itself, which would decrease light-penetration into the solution. Presumably the slight increase in rate as the dithionite concentration is increased (at a fixed Methyl Viologen concentration) simply reflects the increasing reduced/oxidized Methyl Viologen ratio as the total reductant concentration is increased.

This hydrogenase-catalysed H₂ evolution was sensitive to CO (Fig. 1), as is the hydrogenase enzyme *in vitro* (Tel-Or et al., 1977, 1978), but not significantly sensitive to acetylene (Fig. 1). The hydrogenase-catalysed consumption of H₂ is thought to be potently inhibited by acetylene (Smith et al., 1976; Bothe et al., 1977; Daday et al., 1977), so that the insensitivity to this substance of H₂ evolution in the presence of Methyl Viologen may serve as a distinguishing feature of the soluble form of this enzyme.

The second main conclusion is that in the presence of a suitable reductant hydrogenase is capable of catalysing H₂ formation *in vivo* at appreciable rates (Fig. 2) in relation to nitrogenase-catalysed rates, and much greater than previously supposed (Benemann, 1977). Given the aforementioned advantages of an ATP-independent hydrogenase activity over nitrogenase for biophotolysis and the fact that hydrogenase activity is sustained to higher algal concentrations than is nitrogenase-mediated H₂ production (Fig. 5), the reversible hydrogenase would appear to be well suited to application in solar-energy bioconversion. Two problems would be to increase the already significant activity of this enzyme and link it *in vivo* to the reductant supply from photosynthesis within the ideal anaerobic environment of the heterocyst.

Thirdly, in non-heterocystous cultures, hydrogenase is expressed (Fig. 6) despite the absence of nitrogenase (Fig. 6) and virtual absence of 'uptake' hydrogenase (Fig. 7) activities. This hydrogenase may therefore have a physiological role unrelated to nitrogen fixation. Alternatively, it may simply be a phylogenetic relic that is constitutively expressed (Kessler, 1974).

This procedure for determination of the activity of reversible hydrogenase should prove useful in further studies of hydrogenase in nitrogen-fixing micro-organisms. This activity has been measured similarly in the marine blue-green alga, *Calothrix membranacea* (A. Daday, G. R. Lambert & G. D. Smith, unpublished work).
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References


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