# AN INDUCIBLE HYDROGENASE IN CYANOBACTERIA ENHANCES $N_2$ FIXATION

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## 1. Introduction

$N_2$-Fixing cyanobacteria (blue-green algae) *Nostoc muscorum* and *Anabaena cylindrica* have been reported to contain hydrogenase activity [1,2]. In a recent study from this laboratory it was found that this activity is present both in heterocysts and vegetative cells of aerobically-grown cultures of both species [3,4]. However, it is not known whether the hydrogenase activity in these organisms is inducible. In the earlier studies of Fujita et al. [2] activity was obtained only after activation, similar to activation of hydrogenase activity that has been reported in green algae (cf. Kessler [5]) which occurs after incubation for several hours under $H_2$ gas. Induction of $H_2$-uptake activity has also been reported in chemolitotrophic bacteria by Canevascini and Eberhardt [6].

Various $N_2$-fixing bacteria also possess $H_2$-uptake activity [7,8] and a link between hydrogenase and nitrogenase activity was suggested by Dixon [8] which has received further emphasis in a recent analysis of $H_2$ production in various $N_2$-fixing organisms by Schubert and Evans [9]. Furthermore, Smith et al. [10] and Bothe et al. [11] have reported that hydrogenase can recycle $H_2$ gas lost by nitrogenase.

In the present investigation two questions have been examined: first, whether hydrogenase in *N. muscorum* and *A. cylindrica* is an inducible enzyme and, secondly, whether there is a cooperativity between the uptake of $H_2$ catalyzed by hydrogenase and the rate and efficiency of $N_2$-uptake catalyzed by nitrogenase in aerobically and $H_2$-grown cultures of these microorganisms.

## 2. Materials and methods

### 2.1. *Cells and cultures*

*Nostoc muscorum* strain 7119 kindly made available to us from Dr D. I. Arnon, and *Anabaena cylindrica* Lemn. from the Cambridge culture collection were grown in an Allen and Arnon medium [12] continuously illuminated by fluorescent cool light (3000 lux) on a shaker and gassed with 95% air plus 5% $CO_2$, at 25°C. Cultures grown under $H_2$ were flushed with a mixture of $H_2|N_2|CO_2$ (20:75:5) and illuminated by white light (6000 lux) on a shaker at 25°C.

### 2.2. *Hydrogenase activity*

$H_2$ Consumption was assayed with intact filaments that were washed and resuspended in 1 ml growth medium in 7 ml vials. The vials were sealed with rubber stoppers and preflushed with $N_2$. $H_2$ (2% or 4%) was then added and the vials were incubated at 26 ± 2°C in a specially constructed, illuminated (90 kergs/cm$^2$/s) shaker [13]. Consumption of $H_2$ was compared to control samples containing equivalent amounts of medium and $H_2$ to correct for possible loss of $H_2$ through leakage.

$H_2$ Production was assayed under similar conditions, but with sonicated filaments in the presence of 20 mM dithionite and 5 mM methylviologen. Aliquots (50 μl) were withdrawn from the gas phase of the vials and injected into a Varian Aeorgraph Model 920, provided with a molecular sieve 5A column and thermal conductivity detector. $H_2$ Content was calculated from peak heights of the recorder by reference to a calibration curve.

2.3. *Nitrogenase activity*

Acetylene reduction was assayed with intact filaments washed and resuspended in growth medium under 12% $C_2H_2$ in $N_2$ or 12% $C_2H_2$, 17% $H_2$ in $N_2$. Aliquots (100 μl) were withdrawn from the gas phase and injected into a Varian Gas Chromatograph Model 3700, provided with a Poropak N column, flame ionization detector with $N_2$ as the carrier gas. $C_2H_4$ and $C_2H_2$ content were integrated using a Varian CDS 111 data control system; activities were calculated from a standard curve for $C_2H_4$ concentration.

2.4. *Chlorophyll determination*

Filaments were sonicated and extracted with 80% acetone; chlorophyll *a* concentration was calculated from 663 nm absorbance according to Mackinney [14].

## 3. Results

3.1. *Induction of hydrogenase*

Aerobically-grown filaments from *N. muscorum* and *A. cylindrica* consume molecular $H_2$ at rates between 1–6 μmol/mg chlorphyll/h in the light; sonicated preparations produce $H_2$ at a rate between 1–3 μmol/mg chlorophyll/h. Although Fujita et al. [2] reported that an activation of both $H_2$ consumption and production occurs when filaments of aerobically-grown *A. cylindrica* were incubated for a few hours under $H_2$, we consistently failed to observe any such activation of either activity. However, if cultures of either of these species were grown for several days in a $H_2$-containing atmosphere marked increases were observed in $H_2$ consumption and production as shown in fig.1. Enhancement of hydrogenase activities was generally found between 4–8 days with maximal activity always obtained between 4–6 days of growth in a 20% $H_2$-containing atmosphere based on 10 individual growth experiments with *N. muscorum* and six experiments with *A. cylindrica*. These activities were consistently 5–20-fold higher than the hydrogenase activities of the original aerobically-grown cultures. This indicates that hydrogenase can be induced in these two species of cyanobacteria.

Using $H_2$-induced cultures, the dependence of $H_2$ consumption on light and $CO_2$ was examined. Table 1 shows that light and light plus $CO_2$ enhance $H_2$ consumption. Thus, once hydrogenase has been induced,

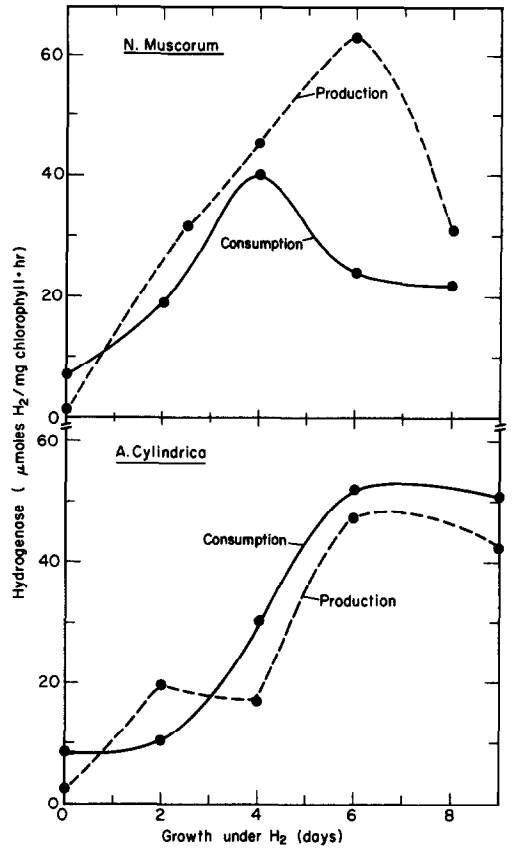


Fig.1. Hydrogenase activity of $H_2$-grown cultures of *N. muscorum* and *A. cylindrica*. Filaments containing 5–20 μg chlorophyll were harvested at the indicated times, suspended and assayed for $H_2$ consumption (with 4% $H_2$ in $N_2$) and $H_2$ production.

the activity can be enhanced 2–3-fold further in the presence of light plus $CO_2$. Other experiments have shown that photosynthetic electron transport inhibitors such as dichlorophenyl-dimethyl urea (DCMU), dibromothymoquinone (DBMIB), heptal-hydroxy-quinoline-*N*-oxide (HOQNO) and disalicylidene-propanediamine (DSPD) partially inhibited the rate of $H_2$ consumption in the light. These findings indicate an involvement of the light-induced electron transport and reactions initiated by $CO_2$ in the stimulation of $H_2$ consumption activity.

3.2. *Effect of $H_2$ on nitrogenase*

To determine whether $H_2$ consumption could sup-

Table 1
Effect of light and $CO_2$ on $H_2$ consumption

| | $H_2$ Consumption (μmol/mg chlorophyll/h) | | | |
|---|---|---|---|---|
| | *N. muscorum* | | *A. cylindrica* | |
| 2% $H_2$ in $N_2$ | | | | |
| dark | 10.6 | (4) | 8.9 | (3) |
| light | 25.8 | | 16.3 | |
| 2% $H_2$ in $N_2$ | | | | |
| light 2% $H_2$, 5% $CO_2$ in $N_2$ | 28.8 | | 20.5 | (3) |
| light | 48.0 | | 30.0 | |

Filaments of 4–8 days $H_2$-grown cultures, containing 5–20 μg chlorophyll/ml. Values in brackets represent number of experiments averaged.

port the reductive process of $N_2$ fixation, we have followed the effect of $H_2$ on nitrogenase with filaments of aerobically-grown cultures of *N. muscorum* and *A. cylindrica*. In the presence of $H_2$ gas the rate of nitrogenase and extent of $N_2$ fixation, as measured by the acetylene reduction assay, is more than doubled (fig.2). Since $H_2$ consumption is much greater in $H_2$-grown cultures, we have followed in parallel the effect of induction of hydrogenase on nitrogenase activity. Figure 3 presents typical results with *N. muscorum* showing a progressive increase in rate and yield of $N_2$ fixed by the filament preparations over a period of eight days growth. Indeed, nitrogenase activity is

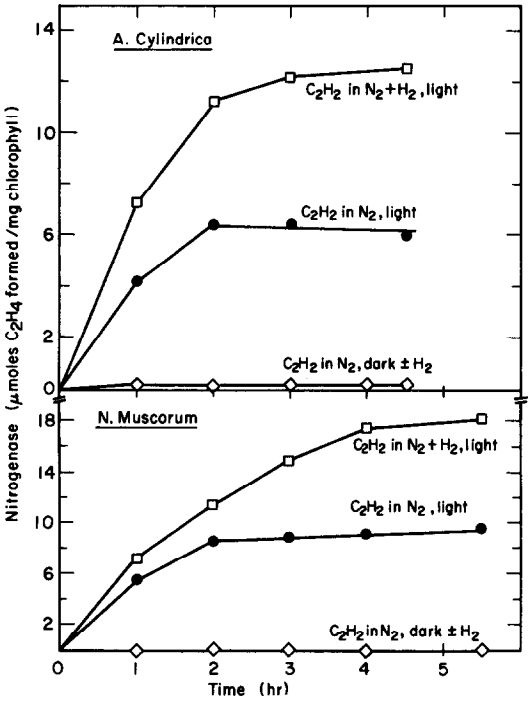


Fig.2. Effect of $H_2$ on nitrogenase activity in aerobically-grown *N. muscorum* and *A. cylindrica*. Filaments containing 8.6 and 5.7 mg chlorophyll, respectively, were assayed for nitrogenase activity.

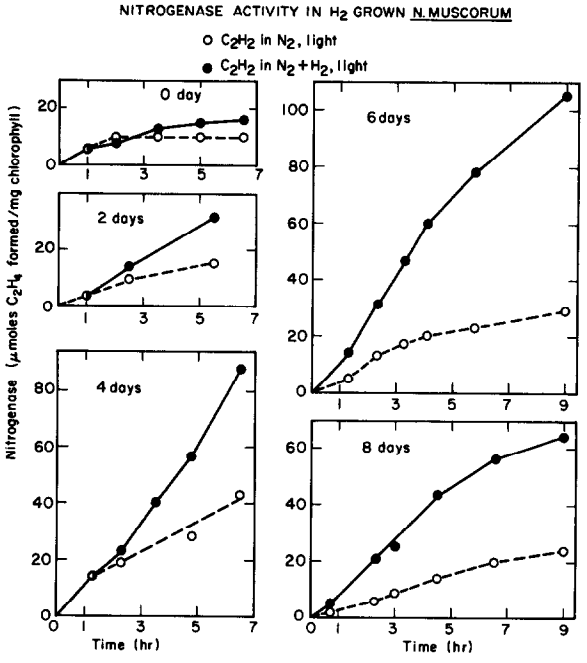


Fig.3. Nitrogenase activity of $H_2$-grown *N. muscorum*. Filaments contained 5–20 mg chlorophyll.

enhanced in the $H_2$-grown cultures even when $H_2$ is not present during assay of nitrogenase. If $H_2$ is present during the assay, the rate of nitrogenase activity in the $H_2$-induced cultures is 16 μmol ethylene formed/mg chlorophyll/h with a total yield of up to 100 μmol ethylene/mg chlorophyll which represents a 3–5-fold enhancement in nitrogenase activity. This indicates that nitrogenase is induced in $H_2$-grown cultures. Similar results are observed in $H_2$-grown cultures of *A. cylindrica*.

## 4. Discussion

The studies reported in this investigation appear to resolve three questions with respect to two heterocystous species of cyanobacteria.

First, whether hydrogenase is activated or induced; we found no evidence for activation of either consumption or production of $H_2$ in aerobically-grown cultures, but both of these activities increased 5–20-fold when cultures are grown under $H_2$ gas. On the other hand, hydrogenase-catalyzed consumption of $H_2$ is stimulated by light and/or light plus $CO_2$ in hydrogenase-induced cultures.

Secondly, nitrogenase activity appears to be induced in cultures grown under $H_2$.

Thirdly, our studies unambiguously establish that in $H_2$-induced cultures hydrogenase manifests a cooperativity with nitrogenase. In the presence of $H_2$ the activity of nitrogenase is stimulated 3–5-fold such that rates of about 3 μmol $N_2$ fixed/mg chlorophyll/h are obtained if the method of Peterson and Burris [15] is used to convert acetylene reduction data to equivalents of $^{15}N_2$ fixation to ammonia.

These findings provide evidence to support earlier suggestions that:

(i) There is a link between hydrogenase and nitrogenase, Dixon [8].

(ii) That hydrogenase may serve to recycle $H_2$ gas lost by nitrogenase, Smith et al. [10] and Bothe et al. [11].

(iii) That $H_2$-supported $N_2$ fixation in reductant-limited cultures (grown in the presence of DCMU) of *A. cylindrica*, Benneman and Weare [16].

(iv) That an important role for hydrogenase, which is poised to function unidirectionally towards consumption [3], is in supporting nitrogenase in cyanobacteria species and this may also be the case in various $N_2$-fixing bacteria as examined recently by Schubert and Evans [9].

It therefore seems reasonable to suggest that it may be feasible to increase the capacity of $N_2$ fixation in the heterocystous cyanobacteria by the simple expedient of growing cultures in the presence of $H_2$ gas. These findings if exploited will enhance the importance of cyanobacteria for agriculture, i.e., ammonia fertilizer and food production.

## Acknowledgements

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