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Effects of Some Inhibitors and Carbon Sources on Acetylene Reduction and Hydrogen Production of Isolated Heterocysts of *Anabaena* sp. (Strain CA)

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Metabolically active heterocysts isolated from wild-type *Anabaena* sp. strain CA showed high rates of light dependent acetylene reduction and H_2 evolution. Fructose and erythrose significantly stimulated nitrogenase activity but not H_2 evolution. DCMU and cyanide were not effective. DBMIB significantly inhibited both nitrogenase and nitrogenase-catalysed H_2 evolution. This inhibition was overcome by a catalytic amount of TMPD. These data suggest that in the isolated heterocysts all electrons, irrespective of source, must pass through the plastoquinone pool before reducing ferredoxin, which in turn can reduce dinitrogen to ammonia.

H_2 formation by nitrogenase is irreversible, insensitive to CO, dependent on a supply of electrons from reduced ferredoxin, and requires large amounts of ATP (Houchins & Hind, 1982). *In vitro* three to four molecules of ATP are hydrolysed for the formation of one molecule of H_2, and H_2 production *in vivo* is probably even more energy-consuming. To prevent this loss of energy, blue-green algae can recycle the hydrogen by a hydrogenase (Lambert, Daday & Smith, 1979). Thus hydrogenase can donate electrons to nitrogenase presumably via a photosynthetic electron transport system, feed electrons into a respiratory chain, and reduce O_2 to water by the respiratory pathway (Eisbrenner, Ross & Bothe, 1981; Scherer, Almon & Böger, 1988).

The effects of certain metabolic inhibitors on the nitrogenase and nitrogenase-dependent H_2 evolution by whole cell cultures have been reported (Lex & Stewart, 1973; Spiller *et al.*, 1978; Miyamoto, Hallenbeck & Benemann, 1979). Eisbrenner & Bothe (1979) have studied the role of H_2 in transferring electrons to nitrogenase in isolated heterocysts of *Anabaena cylindrica*. However, there appears to have been no previous work on a possible role of metabolic inhibitors on the kinetics of H_2 evolution by isolated heterocysts. It was the purpose of this work to extend to metabolically active isolated heterocysts the previous observations on acetylene reduction and H_2 evolution by whole cell cultures of cyanobacteria.

MATERIALS AND METHODS

The organism used was *Anabaena* sp. strain CA (ATCC 33047), a filamentous, heterocystous, marine cyanobacterium. Growth and other conditions were the same as described earlier (Smith *et al.*, 1985).

The heterocysts were isolated and assayed for acetylene reducing activity as described earlier (Smith *et al.*, 1985). For hydrogen measurement one 5331 electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) was fitted into a 1·8 ml water-jacketed chamber held at 39°C. The suspensions were continuously mixed with a magnetic stirring bar. The electrode signal was monitored and amplified using model 617 microvoltammeter (Keithley Instruments, Inc., Cleveland, Ohio) and recorded. Actinic light was provided by a projector with DAY-DAK 500-W lamp (Sylvania, Winchester, KY) screened by a No. 34-01-2 hot mirror (Baird Atomic Inc., Bedford, Mass.). The light intensity incident upon the electrode chamber was 1200 $\mu E\ m^{-2}\ s^{-1}$ (model 185 A Quantum meter, Li-Cor, Inc., Lincoln, Nebr.). Gassing of the sample was performed directly in

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the electrode chamber for 2 min before an assay. All solutions were freshly prepared, sterilized and added simultaneously in the electrode chamber or at the start of the experiment.

RESULTS

Figure 1 shows the effect of millimolar concentration range of erythrose and H_2 (90%) on the acetylene reducing activity of the isolated heterocysts of *Anabaena* sp. strain CA. The activity of the heterocysts isolated from cultures grown in 1% Co_2-in-air was 2 and 1 μmole C_2H_4 formed mg dry wt^{-1} h^{-1} under 90% H_2+10% C_2H_2 and 90% Ar+10% C_2H_2 respectively. The response of acetylene reduction to the erythrose concentration was distinctive: it increased with increasing concentrations, becoming highly significant at 2 and 4 mM erythrose (Fig. 1). Acetylene reduction with erythrose supplementation (4 mM) under H_2 atmosphere was even higher (4·8 μmole C_2H_4 mg dry wt^{-1} h^{-1}) than erythrose supplementation under argon gas phase (4·4 μmole C_2H_4 mg dry wt^{-1} h^{-1}). Above this concentration, acetylene reduction declined rapidly. With 20·0 mM erythrose, activity was even less than the endogenous level (0·8 μmole C_2H_2 formed mg dry wt^{-1} h^{-1}).

Light dependent enhancement of nitrogenase activity of isolated heterocysts by erythrose under hydrogen or argon appears to be a novel observation. At lower light intensity, stimulation of nitrogenase activity was less than at the higher light intensity (Fig. 2).

Certain electron transport inhibitors known to block at defined steps were used to delineate the path of electrons from hydrogen, fructose and erythrose to nitrogenase. The effects of these inhibitors on acetylene reduction by isolated heterocysts are summarized in Table I. Some inhibitors were dissolved in redistilled ethanol, which was also added in the control sets. Not only H_2 but fructose, erythrose and even endogenously supported acetylene reducing activity were sensitive to 10 μM of DBMIB and

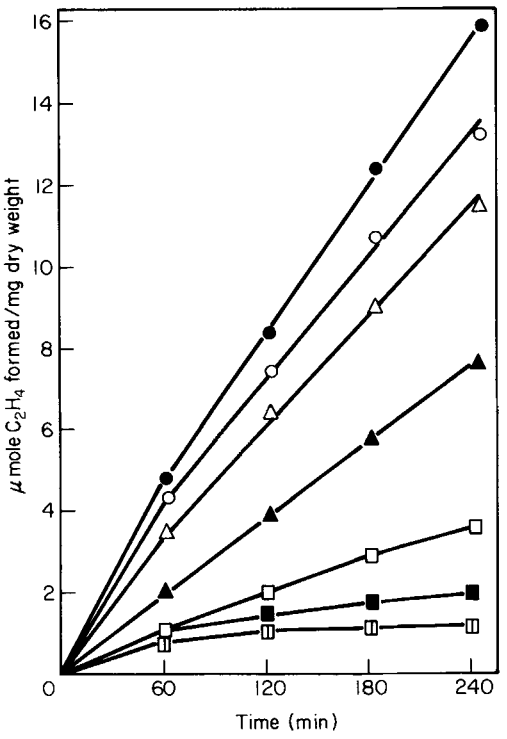

FIG. 1. Effect of erythrose on hydrogen and endogenously supported acetylene reducing activity of isolated heterocysts of *Anabaena* sp. strain CA. Assays were performed as described in the text. (▲), 90% H_2+10% C_2H_2; (●), 4 mM erythrose+90% H_2+10% C_2H_2; (○), 4mM erythrose+90% Ar+10% C_2H_2; (△), (□), (▥), 2, 10 and 20 mM erythrose+90% Ar+10% C_2H_2; (■), 90% Ar+10% C_2H_2.

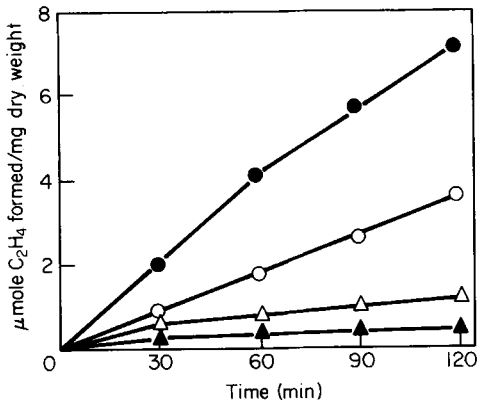

FIG. 2. Effect of light intensity on erythrose supported acetylene reduction activity of the isolated heterocysts under argon atmosphere. (●), 4 mM erythrose under 300 μE m^{-2} s^{-1}; (○), 4 mM erythrose under 12 μE m^{-2} s^{-1}; (△), 90% Ar+10% C_2H_2 under 300 μE m^{-2} s^{-1}; (▲), 90% Ar+10% C_2H_2 under 12 μE m^{-2} s^{-1}.

TABLE 1. Effects of some inhibitors and electron donors on acetylene reduction by isolated heterocysts of *Anabaena* sp. strain CA. Experiments were performed in 8 ml vacutainer tubes at 39°C. Light intensity was 300 μE m^{-2} s^{-1}. DCMU (10 μM), KCN (75 μM), DBMIB (10 μM), TMPD (60 μM), erythrose (4 mM) and fructose (350 mM) were used. Nitrogenase activity was expressed as μmole C_2H_4 formed mg dry wt^{-1} h^{-1}. Heterocysts were isolated as described in the text. Values are means of three replicates. Standard errors for the means are given in parentheses

Electron donors/ inhibitors	Gas-phase	Acetylene reducing activity
Control	90% H_2 + 10% C_2H_2	2·2 (±0·10)
KCN	" "	2·1 (±0·10)
DCMU	" "	2·1 (±0·15)
DBMIB	" "	0·05 (±0·01)
DBMIB + TMPD	" "	1·0 (±0·14)
Control	90% Ar + 10% C_2H_2	1·0 (±0·09)
KCN	" "	0·9 (±0.05)
DCMU	" "	1·0 (±0.12)
DBMIB	" "	0·02 (±0.001)
DBMIB + TMPD	" "	0·5 (±0.05)
Erythrose (light)	" "	4·4 (±0.20)
Erythrose + KCN	" "	4·3 (±0.15)
Erythrose + DCMU	" "	4·3 (±0.20)
Erythrose + DBMIB	" "	0·05 (±0.00)
Erythrose + DBMIB + TMPD	" "	2.2 (±0.14)
Erythrose (dark)	" "	None
Fructose (light)	" "	1·8 (±0.05)
Fructose + KCN	" "	1.7 (±0.15)
Fructose + DCMU	" "	1·7 (±0.10)
Fructose + DBMIB	" "	0·05 (±0.00)
Fructose + DBMIB + TMPD	" "	1·0 (±0.09)
Fructose (dark)	" "	None

this inhibition was overcome by addition of TMPD (60 μM) without ascorbate. This also appears to be a novel observation. Under H_2 or an argon atmosphere supplemented with fructose (350 mM) or erythrose (4 mM), acetylene reduction by isolated heterocysts was found to be insensitive to DCMU (10 μM) or KCN (75 μM). Neither fructose nor erythrose enhanced nitrogenase activity in the dark (Table I).

We have previously reported that the kinetics of H_2 production by isolated heterocysts are biphasic with an initial burst, which lasts only about 1–2 min, followed by a constant steady state rate (Smith *et al.*, 1985). Erythrose (4 mM) increased the steady state rate of H_2 evolution by about 16%, but fructose (50–350 mM) was ineffective. The initial H_2 burst, which lasted only for 1 min, was unaffected by either of the two sugars (Fig. 3). There was no effect of erythrose or fructose on the H_2 uptake activity of the isolated heterocysts (Fig. 3). DBMIB (10 μM) significantly inhibited the initial burst of H_2 production, steady rate of H_2 production and H_2 uptake activity (Fig. 3). KCN at lower concentration (25 μm) caused only very slight inhibition of the initial H_2 burst and the steady state rate was unaffected. Higher concentration (75 μM) of KCN produced significant inhibition of both the initial burst (42%) and the steady state rate (59%) of H_2 production. H_2 uptake activity was inhibited marginally (13%). DCMU affected neither H_2 production nor H_2 uptake activities of the isolated heterocysts.

DISCUSSION

Erythrose appears to be most active in stimulating nitrogenase activity of the isolated heterocysts, followed by hydrogen and fructose. The stimulation of acetylene reducing activity was strictly light dependent and DBMIB sensitive which was overcome

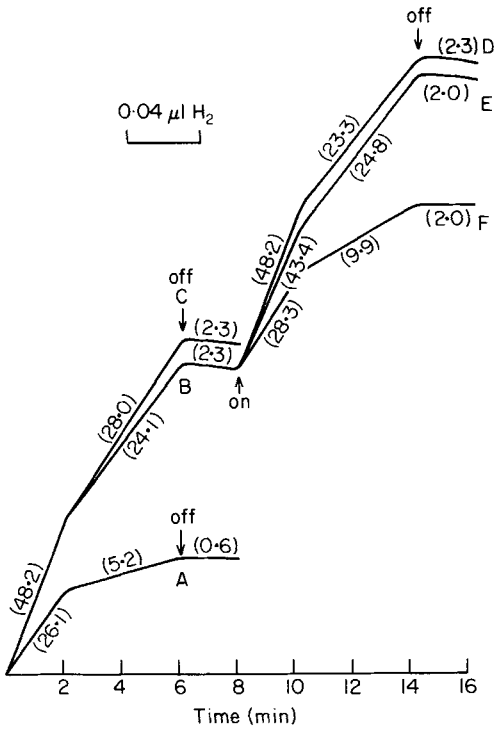

FIG. 3. Electrode records (reduced in scale) of hydrogen production by the isolated heterocysts. Several aliquots were prepared from the same preparation of the isolated heterocysts. Heterocysts were incubated at 39°C under argon atmosphere for 20 min before assay. Heterocysts were then transferred directly to the electrode chamber, where they were gassed with argon for 2 min. Inhibitors and sugars were added individually directly to the electrode chamber. All tracings are from different aliquots of isolated heterocysts. A, H_2 production under 10 μM DBMIB (2,5-dibromothymoquinone); B, H_2 production under argon atmosphere (control), and fructose-supplementation (50–350 mM); C, H_2 production under 4 mM erythrose; D, E, and F, H_2 production by isolated heterocysts with the supplementation of 10 μM dichlorophenyl dimethyl urea (DCMU), 25 μM and 75 μM potassium cyanide (KCN) respectively. All measurements were performed under argon atmosphere. Light was on at time zero and arrow up ↑ indicates light ON; arrow down ↓ indicates light OFF. All experiments are independent ones. Values shown in parentheses are H_2 production or uptake in μl H_2 mg dry wt^{-1} h^{-1}.

by TMPD. This indicates that the electrons originating from all sources pass through the plastoquinone pool (Bowyer *et al.*, 1980). An electron then may continue through the electron transport route and, in the light, eventually reduce ferredoxin which in turn can supply electrons to nitrogenase (Privalle & Burris, 1984). The stimulation of acetylene reducing activity of the isolated heterocysts by hydrogen, fructose and erythrose has been reported (Privalle & Burris, 1984; Neuer & Bothe, 1985). However, the efficacy of fructose and erythrose in enhancing nitrogenase activity of isolated heterocysts differs from organism to organism. The observed enhancement of acetylene reduction by fructose at only higher concentrations (> 200 mM) questions the requirement for such a high concentration. At present we are unable to explain the requirement of higher concentration of fructose for enhancement. In contrast to the observations of Privalle & Burris (1984), the heterocysts isolated from *Anabaena* sp. strain CA do not show any inhibition of acetylene reduction under hydrogen by erythrose. In fact, nitrogenase activity was higher under hydrogen than under argon supplementation. Heterocysts from strain CA are some 10 to 15 times more active than those isolated from *Anabaena* sp. strain 7120 (Privalle & Burris, 1984), and this may be part of the explanation for the differences in the results. Our observation that hydrogen supported activity was KCN insensitive, indicates that the electrons from hydrogen are probably routed to PS I rather than to cytochrome oxidase.

Smith *et al.* (1985) have shown the biphasic nature of H_2 evolution from isolated heterocysts of *Anabaena* CA. The present observations on the effect of different inhibitors as well as erythrose and fructose on the kinetics of H_2 evolution constitute novel observations. Fructose did not enhance the H_2 production. Erythrose only affected the steady state rate (16%), not the initial burst. The significant enhancement of acetylene reduction in isolated heterocysts by fructose (190%) and erythrose (above 400%) and the slight stimulation of H_2 production by erythrose only indicate that proton reduction may occur at a site different from that of N_2 reduction. As expected, DCMU had no effect on H_2 production. The lower concentration (25 μM) of KCN was slightly inhibitory whereas the higher concentration (75 μM) inhibited the rate drastically. KCN appears to be non-specific, as it blocks cyto-

chrome oxidase activity and can act as an alternative substrate to nitrogenase (Rivera-Ortiz & Burris, 1975). DBMIB affected both the initial burst and the steady state rate of H_2 production significantly. The inhibition of H_2 production by DBMIB may be related to the inhibition of nitrogenase activity in the isolated heterocysts. H_2 uptake activity was also inhibited both by DBMIB and cyanide, and in this respect our data are in accord with earlier observations (Neuer & Bothe, 1985; Privalle & Burris, 1984).

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