The effect of glyoxylate on nitrogenase-catalyzed hydrogen formation in *Anabaena cylindrica*

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1. INTRODUCTION

Photosynthetic bacteria, such as *Rhodopseudomonas capsulata*, when incubated in the light under conditions of nitrogen starvation, produce copious amounts of hydrogen gas via nitrogenase, presumably as a means of regulating their energy charge and/or redox balance and thereby preventing photooxidative damage until growth can begin again [1,2]. A similar role has been suggested for the hydrogenase of the green alga *Chlorella* [3]. Higher plants are now thought to use the reactions of the photorespiratory pathway for a similar purpose in conditions of stress [4,5]. Heterocystous cyanobacteria are capable of both hydrogen gas formation [6] and photorespiration [7,8], although they differ from higher plants in the nature of their further metabolism of glyoxylate [9]. Although there has been considerable controversy concerning the significance of photorespiratory reactions in cyanobacteria [10,11] it now appears that they are not significant except in special circumstances, such as when the organisms are shifted from high to low CO_2, when carbonic anhydrase levels are still low [12]; only under these conditions are significant amounts of glycolate excreted. Hence, reports of glyoxylate functioning to stimulate nitrogenase by inhibiting photorespiration [13,14] were of considerable interest. It seemed to us that if nitrogenase-mediated H_2 formation and photorespiration were alternative or concomitant processes for relieving photoinhibitory effects under conditions of stress due to nitrogen limitation, then glyoxylate might well have an even more substantial effect on nitrogenase-mediated hydrogen gas formation in argon than on acetylene reduction.

Here, we report that glyoxylate markedly stimulates hydrogen formation by *A. cylindrica* but that the stimulation is substantial under conditions where photorespiration would be negligible. The data suggest that glyoxylate stimulates nitrogenase, not as an inhibitor of photorespiration, but rather as an electron supply for nitrogenase in the heterocysts.

2. MATERIALS AND METHODS

2.1. *Algae and their growth*

Anabaena cyclindrica (strain B629) was obtained, grown and incubated as for H_2 formation experiments [15,16]. Cultures were harvested at different ages, as indicated. Sodium glyoxylate (Sigma Chemical Co.) was autoclaved and used as in [13], after adjustment to pH 7.5.

2.2. *Gas analysis*

Hydrogen and ethylene formation were measured gas chromatographically as in [17], 0.2 ml samples being taken from the vials. Dry weights were measured as in [17]. A concentration of 100 Klett units corresponds to 0.33 mg dry wt/ml [18].

3. RESULTS

3.1. *Hydrogen evolution after glyoxylate preincubation in air/CO_2*

In [13] 24 h preincubation with glyoxylate was required for maximal nitrogenase stimulation. Hence we incubated cultures of various ages of log-

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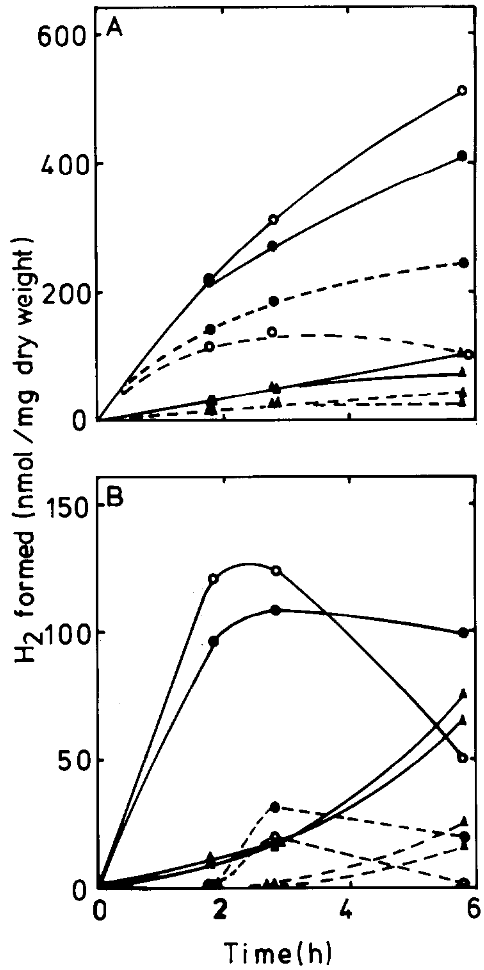

Fig.1. The effect of glyoxylate on hydrogen formation by *A. cylindrica*, under a gas atmosphere of argon alone (○) or argon supplemented with 5% CO_2 (●), 20% O_2 (△) or 5% CO_2 plus 20% O_2 (▲). The solid lines refer to cultures preincubated with glyoxylate (2 mM), the controls being represented by the dashed lines. (A) Cultures were grown to 25 Klett units before addition of glyoxylate and grew to 62 Klett units before harvesting for the experiments. (B) Cultures were grown to 110 Klett units before addition of glyoxylate and grew to 288 Klett units for the experiments. For all experiments cultures were sparged with air/0.3% CO_2 (170 ml/min) during the 24 h preincubation with glyoxylate in the light (7000 lux). Note the difference in scales between A and B.

arithmic growth in glyoxylate whilst sparging with a gas mixture of air/0.3% CO_2 (170 ml/min) for 24 h. Cultures were then assayed for H_2 formation in the presence and absence of CO_2 (5%) and/or O_2 (20%) in argon. The results for cultures of different ages in logarithmic growth are shown in fig.1. With both young and older cultures glyoxylate stimulated H_2 formation, but the effect was much more dramatic with the older culture, where a very significant stimulation of H_2 formation was observed. The presence of CO_2 (5%) was, in general, slightly stimulatory but in O_2 (20% of the gas phase) the rate and extent of H_2 formation was markedly reduced in all cases, even when CO_2 was present at concentrations (5%) sufficient to inhibit photorespiratory reactions. This apparent inhibitory effect of O_2 was less with younger than older cultures. With still older cultures (preincubation commenced at 288 Klett units; harvested at 470 Klett units) glyoxylate was able to stimulate some H_2 formation even when none was observed in controls (not shown). Experiments were also performed to verify that the long glyoxylate preincubation was necessary for its effect [13]; no stimulation was observed when glyoxylate was added immediately prior to the assay (not shown).

3.2. *Hydrogen evolution after glyoxylate preincubation in argon*

Experiments similar to those in fig.1 were done after sparging the cultures with argon during the glyoxylate preincubation. Photorespiration would not be possible under such anaerobic conditions. The results of both H_2 formation and acetylene reduction measurements are shown in fig.2 for a relatively young culture with an active nitrogenase. Again glyoxylate stimulated nitrogenase activity, measured either as acetylene reduction or H_2 formation. Also O_2 was inhibitory, whether CO_2 was present or not, but the stimulatory effect of CO_2 was more pronounced, particularly after longer incubation times. After preincubation for 24 h in both air and argon, cultures to which glyoxylate had been added had a frequency of heterocysts and proheterocysts markedly greater than control cultures. Incubation in argon led to a yellowing of cultures at the lower concentrations but not at higher concentrations. Much mucilage was formed in argon, particularly with cultures at high concentrations.

The dependence of both acetylene reduction and H_2 formation on glyoxylate concentration for batch cultures of different age is shown in fig.3. The stimulation after argon preincubation was not saturated

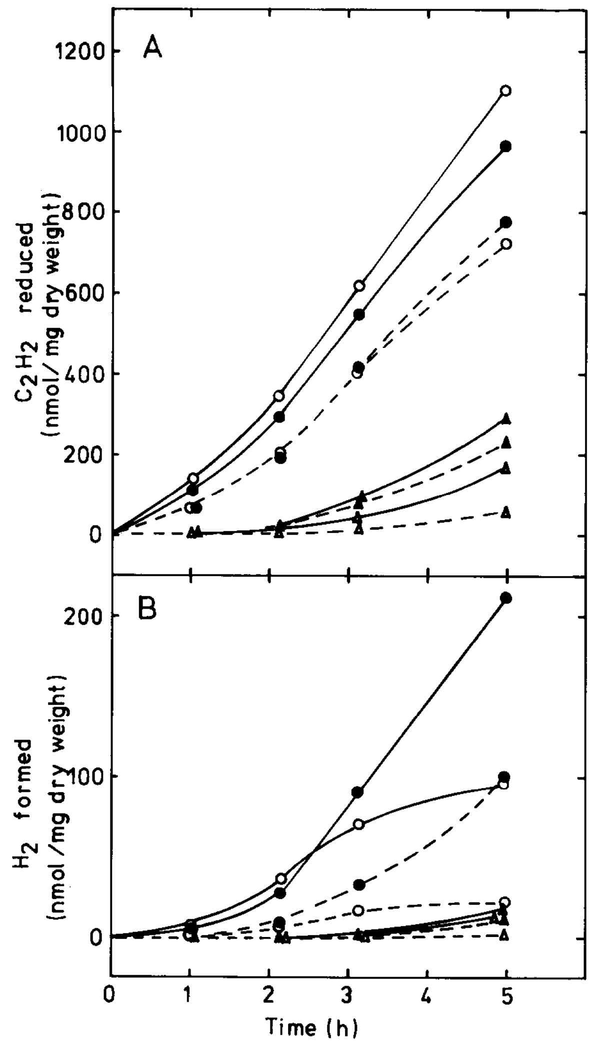

Fig.2. The effect of glyoxylate on acetylene reduction (A) and hydrogen formation (B) by *A. cylindrica*, the glyoxylate preincubation (24 h) being done while sparging with argon in the light (7000 lux). The gas atmospheres in the experiments were argon alone (○), or argon supplemented with 5% CO_2 (●), 20% O_2 (△) or 5% CO_2 plus 20% O_2 (▲). The solid lines refer to cultures preincubated with glyoxylate (6 mM), the controls being represented by dashed lines. Cultures were grown to 113 Klett units and no further growth occurred during the glyoxylate preincubation.

even at 10 mM glyoxylate. Again it is seen that the relative stimulation of nitrogenase activity is much greater with older cultures.

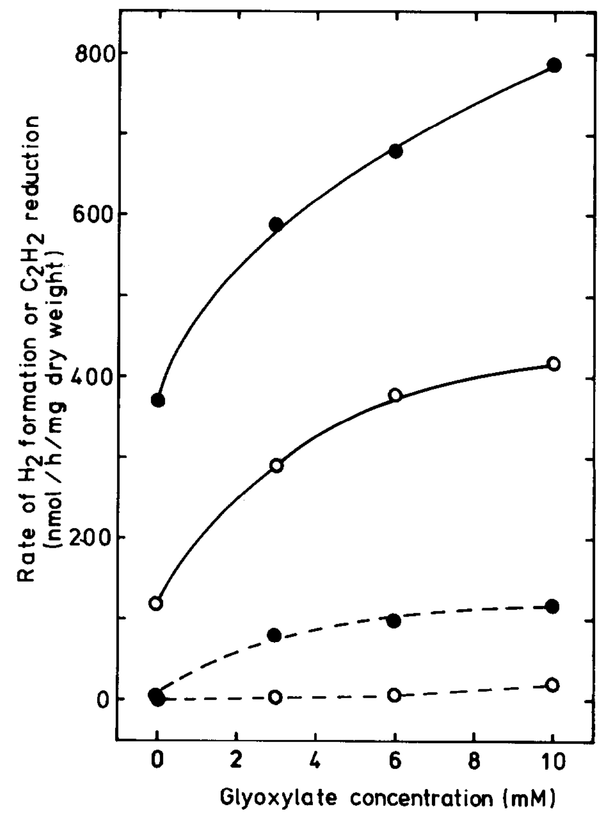

Fig.3. Rates of acetylene reduction (●) and H_2 formation (○) as a function of glyoxylate concentration with cultures of different ages of batch growth (89 Klett units (——) and 200 Klett units (---) as indicated). With the older cultures a slight lag of 1–2 h was observed with the H_2 formation and the reported values refer to the rates measured after such time.

Experiments (not shown) in which DCMU was added with the glyoxylate during the 24 h preincubation, to prevent even the presence of photosynthetically produced O_2, were not useful because nitrogenase was totally inhibited whether glyoxylate was present or not.

4. DISCUSSION

We interpret these results as showing that glyoxylate, although capable of stimulating nitrogenase under conditions where photorespiration is active [19], is also capable of stimulating nitrogenase activity in the absence of photorespiration. The effect of glyoxylate is observed (fig.1) after sparging cultures with air supplemented with CO_2 to a concentration (0.3%) known to abolish photorespiration in *A. cylindrica* [19] or after incubation in the absence of O_2 (fig.2) where photorespiration would also not occur. Furthermore, in incubations supplemented with CO_2 to a concentration of 5%, O_2 was inhibitory to nitrogenase-mediated H_2 formation, whether or not glyoxylate was present. Assuming that O_2 has no direct inhibitory effect on nitrogenase (due to the protective nature of the heterocyst [7]), oxygen must have some other metabolic effect. In these experiments, when nitrogenase assayed by H_2 formation, H_2 uptake in the oxyhydrogen reaction [20] could partly explain the reduced H_2 formation in the presence of O_2.

The most likely reason for the effect of glyoxylate is that it provides reducing equivalents in the heterocysts. This is consistent with its particularly marked effect on nitrogenase in cultures starved in argon (fig.2) and also with its substantial stimulation of nitrogenase in older cultures grown in air/CO_2 (fig.1). There is evidence that the glyoxylate cycle is particularly active in heterocysts of *A. cyclindrica*, and the presence in these cells of the reserve compound poly(β-hydroxybutyrate), which is a source of acetyl-CoA, is consistent with its operation [21]. This cycle could be a means by which glyoxylate supplies reducing equivalents to the nitrogenase. The long preincubation time required for glyoxylate to exert its effects [13] could well be a result of an induction time for synthesis of enzymes of the glyoxylate cycle. The possibility of differences in glyoxylate metabolism between heterocysts and vegetative cells may also be indicated by earlier studies with total extracts of *A. cylindrica* [9].

In these experiments the cyanobacterial concentration was a very important determinant of the results obtained. This may be related to the changes in the specific activity of nitrogenase during logarithmic batch growth [18]. Age variation may be responsible for apparent differences between our results and those in [13], using cultures harvested in 'late exponential phase'. This may explain, for example, our observation that 2 mM glyoxylate was sufficient to induce substantial increases in H_2 formation (fig.1) whereas this concentration was shown to have little effect on nitrogenase or photosynthetic activity in [13]. In our experience it is extremely important to define the culture age and growth conditions precisely for this type of work.

The results are consistent with the interpretation that, in general, photorespiratory reactions in *A. cylindrica* do not represent an 'electron sink' for protecting the organism against photooxidative damage during nitrogen starvation in the light.

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REFERENCES

[1] Gest, H. (1972) Adv. Microbial Physiol. 7, 243–282.
[2] Hillmer, P. and Gest, H. (1977) J. Bacteriol. 129, 732–739.
[3] Sayre, R.T. and Homann, P.H. (1979) Plant Physiol. 63, 153a Suppl.
[4] Andrews, T.J. and Lorimer, G.H. (1978) FEBS Lett. 90, 1–7.
[5] Heber, U. and Krause, G.H. (1980) Trends Biochem. Sci. 5, 32–34.
[6] Benemann, J.R. and Weare, N.M. (1974) Science 184, 174–175.
[7] Fogg, G.E., Stewart, W.D.P., Fay, P. and Walsby, A.E. (1973) in: The Blue–Green Algae, Academic Press, London, New York.
[8] Stanier, R.Y. and Cohen-Bazire, G. (1977) Annu. Rev. Microbiol. 31, 225–274.
[9] Codd, G.A. and Stewart, W.D.P. (1973) Arch. Mikrobiol. 94, 11–28.
[10] Lloyd, N.D.H., Canvin, D.T. and Culver, D.A. (1977) Plant Physiol. 59, 936–940.
[11] Birmingham,B.C. and Colman, B. (1979) Plant Physiol. 64, 892–895.
[12] Ingle, R.K. and Colman, B. (1976) Planta 128, 217–223.
[13] Bergman, B. (1980) Physiol. Plant 49, 398–404.
[14] Bergman, B. (1981) Planta 152, 302–306.
[15] Lambert, G.R., Daday, A. and Smith, G.D. (1979) Appl. Environ. Microbiol. 38, 521–529.
[16] Lambert, G.R. and Smith, G.D. (1980) Arch. Biochem. Biophys. 205, 36–50.
[17] Daday, A., Platz, R.A. and Smith, G.D. (1977) Appl. Env. Microbiol. 34, 478–483.
[18] Daday, A., Lambert, G.R. and Smith, G.D. (1979) Biochem. J. 177, 139–144.
[19] Lex, M. Silvester, W.B. and Stewart, W.D.P. (1972) Proc. Roy. Soc. Lond. B. 180, 87–102.
[20] Bothe, H., Distler, E. and Eisbrenner, G. (1978) Biochimie 60, 277–289.
[21] Carr, N.G. and Bradley, S. (1973) Soc. Gen. Microbiol. Symp. 23, 161–188.