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Factors Influencing Dark Nitrogen Fixation in a Blue-Green Alga

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Nitrogen-fixing activity declines first rapidly and then more gradually when *Anabaenopsis circularis* is transferred from light into dark conditions. The rate and duration of dark acetylene reduction (nitrogen fixation) depend upon conditions prevailing during the preceding light period. Factors (such as light intensity, CO_2 concentration, and supply of glucose), which in the light affect photosynthesis and the accumulation of reserve carbon, have a profound effect on dark nitrogen fixation. Glucose greatly promotes nitrogen fixation in the light and supports prolonged nitrogenase activity in the dark. The results suggest that heterotrophic nitrogen fixation by blue-green algae in the field may be important both under light and dark conditions.

In blue-green algae the main requirements for the enzymatic reduction of nitrogen, an electron donor and adenosine 5'-triphosphate, can be generated in light and dark reactions, and a carbon source for the assimilation of ammonia can be provided equally via photosynthesis and through light-independent metabolic pathways (3-5, 8, 16, 21). Certain blue-green algae can fix nitrogen and grow in the dark in the presence of suitable organic substances (2, 8, 15, 20). Reports on measurement of nitrogen-fixing activity under natural conditions indicate that nitrogen fixation in blue-green algae is light dependent, exhibits a typical diurnal variation, and may continue at a decreased rate and for a limited period in the dark (7, 11-14, 19). Although it has been shown that dark nitrogen fixation depends upon previous exposure to light (7, 13), little experimental information is available about this and other factors that determine both the rate and the duration of dark nitrogenase activity in blue-green algae. The present study, in which some of the factors involved were tested under relatively simple laboratory conditions, was initiated as a preliminary exploration before investigating the more complex field situation.

MATERIALS AND METHODS

Anabaenopsis circularis was grown axenically in an inorganic medium free from combined nitrogen (1) at a temperature of 20 ± 2 C in aerated culture vessels, shaken continuously at 90 oscillations/min and illuminated with white fluorescent light of 1,200 lx. Using an inoculum equivalent to about 50 mg of dry alga/liter of medium, the yield obtained was about 200 mg of dry algal material after 4 days.

For studies on the effect of light pretreatment on nitrogenase activity in the dark, a 4-day-old culture was divided into three and incubated at light intensities of 1,500, 3,000 and 6,000 lx, respectively, but under otherwise identical conditions for 24 h. Then the cultures were darkened (by means of black plastic covers), and samples were removed at intervals for nitrogenase assay.

For experiments on the effect of carbon nutrition upon nitrogenase activity in the dark, the alga was grown for 4 days as outlined previously. This was followed by a variety of experimental treatments: CO_2, at atmospheric concentration and at a concentration of 1% in air, and glucose, at a concentration of 0.03 M, were supplied separately and in combination to the alga during a 24-h incubation period in the light (Table 1). At the end of this period acetylene reduction (nitrogenase activity) was measured in the light (1,500 lx) and/or in the dark, and with the alga suspended in the same incubation medium, except for treatments 4 and 6, for which the alga was collected by centrifugation, washed free of glucose and transferred into a glucose-free supernatant medium prior to the acetylene assay. The latter medium was obtained from a culture in which the alga was grown for 4 days under air and the algal material was removed by filtration with membrane filters (Millipore Corp., 0.45-μm pore size). This medium was used to eliminate potential decrease in activity due to the lack of complexing substances in the medium (10).

Algal material for dry weight determination was collected and washed on Whatman GF/C glass-fiber filter pads, dried at 110 C for 2 h, and stored in a desiccator at room temperature.

Nitrogenase activity was measured by the acetylene-ethylene assay utilizing the nitrogenase-catalyzed conversion of acetylene to ethylene (18). The incubation time for the assay was 30 min. Gas sam-

ples were analyzed for ethylene in a Varian-Aerograph 1200 gas chromatograph fitted with a Porapak R column and a hydrogen flame ionization detector.

RESULTS

Effect of light intensity. In general enzyme activity in the dark, after light pretreatment at different intensities, declined exponentially at first, reaching a value a little above 50% of the initial value after 30 min of incubation in the dark (Fig. 1). The exponential rate of decline was about the same for all three light intensities (Fig. 1, insert). The rate of decline then gradually slowed down. Preincubation at higher light intensity resulted in both higher rates and longer periods of enzyme activity in the dark. Whereas activity was undetectable after 4 h in the dark after light preincubation at 1,500 lx, it was still significant after 10 h in the dark in cultures exposed to 6,000 lx during the light period.

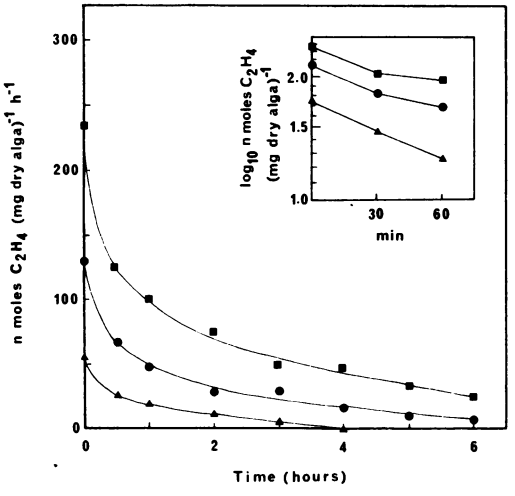

FIG. 1. *Effect of light pretreatment of 1,500 (▲), 3,000 (●), and 6,000 (■) lx intensity on nitrogenase activity of A. circularis in the dark. Insert: logarithmic plot of the rate of acetylene reduction during the initial 1 h in the dark.*

Effect of carbon nutrition. *A. circularis* is known for its ability to grow and fix nitrogen in the dark at the expense of an organic carbon source (20). In the following series of experiments the effect of carbon nutrition in light upon subsequent nitrogen fixation by the alga in the dark was examined. It is clear from Table 1 that a higher concentration of CO_2 in the gas phase (treatment 2) or the presence of glucose in the medium (treatment 3) greatly enhanced nitrogenase activity, both when measured in the light and in the dark. Enzyme activity was increased about eightfold when CO_2-enriched air was used and 25-fold when glucose was added, as compared with the activity of the control culture in which the alga was grown under air without glucose (treatment 1). When 1% CO_2 and 0.03 M glucose were provided simultaneously (treatment 5), the rates of acetylene reduction were significantly lower than those obtained in cultures supplied with glucose alone (treatment 3) and were only slightly higher than in cultures aerated with CO_2-enriched air and not supplemented with glucose (treatment 2). It appears that higher concentration of CO_2 at some point impedes the process by which glucose is utilized for nitrogen fixation. Transfer of alga from a glucose-containing medium into a glucose-free medium caused a considerable fall of nitrogenase activity (see treatment 4 versus treatment 3 and treatment 6 versus treatment 5).

Duration of nitrogenase activity in the dark. The previous experiments were extended further to test nitrogenase activity during a prolonged dark period (up to 72 h) that followed the 24-h pretreatment of the alga in the light. Dark incubation was in all cases at 20 ± 2 C under air and with continuous agitation of the

TABLE 1. *Effect of carbon assimilation in the light upon nitrogenase activity of A. circularis in the light and in the dark*

Treatment no.	Pretreatment in light[a]		C_2H_4 (nmol/mg of dry alga/h)	
	Gas phase	Carbon source	In light	In dark
1	Air	CO_2	39	26
2	1% CO_2 in air	CO_2	320	209
3	Air	CO_2 + glucose[b]	957	620
4	Air	CO_2 + glucose[b] → glucose free[c]	295	315
5	1% CO_2 in air	CO_2 + glucose[b]	392	380
6	1% Co_2 in air	CO_2 + glucose[b] → glucose free[c]	275	237

[a] Light pretreatment at 1,500 lx for 24 h.

[b] Glucose concentration, 0.03 M (glucose in the medium was decreased at the end of the pretreatment period by 10.4% when air was used and by 9.1% when 1% CO_2 in air was used to aerate the culture).

[c] The alga was transferred into glucose-free medium prior to the acetylene-ethylene assay.

culture suspension. Rates and duration of nitrogenase activity varied a great deal and were clearly related to preceding and prevailing conditions of carbon nutrition (Fig. 2). Lowest activity was found in control cultures pretreated in light under air with no additional carbon source provided (treatment 1); significant acetylene reduction was detected for only a couple of hours in the dark. Highest rates of acetylene reduction were recorded in cultures supplemented with 0.03 M glucose (treatment 3); as expected (20), considerable activity was present in these cultures even after 72 h of dark incubation. Rates and duration of nitrogenase activity in cultures transferred into glucose-free medium prior to dark incubation (treatment 4) indicate that the provision of glucose in the medium during light pretreatment lead to the accumulation of reserve carbon sufficient to support nitrogen fixation even after 60 h in the dark. The fluctuations of enzyme activity (initial fall on transfer to glucose-free medium, rapid increase during the first 3 h in the dark, followed by a similar dramatic decline) probably reflect the metabolic adaptation of the alga to suddenly changed conditions (+glucose to −glucose and light to dark transition). Rates of ethylene production by algae subjected in the light to treatments 2 (1% CO_2 in air), 5 (1% CO_2 in air plus 0.03 M glucose), and 6 (as 5 but transferred into glucose-free medium) were intermediate between those previously described.

DISCUSSION

The results obtained in this study are consistent with earlier findings (8, 20) on stimulation by organic substrates of nitrogen fixation in blue-green algae. Photoassimilation of glucose at a light intensity limiting for photosynthesis resulted in higher rates of nitrogenase activity in *A. circularis*. The provision of glucose in the medium greatly increased enzyme activity in light at low (atmospheric) concentration of CO_2 but had no significant effect when the alga was incubated under a gas phase containing 1% CO_2 in air.

It has been shown that carbon reserves accumulated during a period of nitrogen starvation can be used to support dark nitrogen fixation in *Anabaena cylindrica* (3, 4, 16). Dark nitrogenase activity is O_2 dependent, indicating that the requirements for nitrogenase are generated in terminal respiration (6, 21). No activity was found in the dark when the alga was subjected to a treatment of carbon starvation in the light period (5). Present observations with *A. circularis* suggest that both the rate and the duration of enzyme activity in the dark are affected by the rate of photosynthesis and carbon assimilation during the light period. The initial rapid decline of nitrogenase activity after the transfer of the alga from light to dark, and apparently associated with the speedy depletion of requirements for nitrogenase reaction, sug-

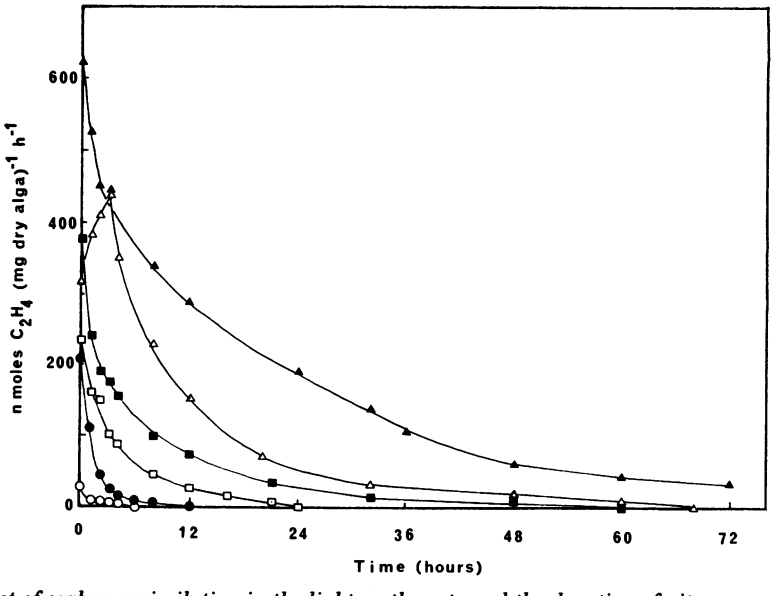

FIG. 2. *Effect of carbon assimilation in the light on the rate and the duration of nitrogenase activity of A. circularis in the dark. Pretreatment in the light was as described in Table 1. Symbols: ○, treatment 1; ●, treatment 2; ▲, treatment 3; △, treatment 4; ■, treatment 5; □, treatment 6.*

gests that the pools of reductant and adenosine 5'-triphosphate are in rather short supply at the site of enzyme activity. The decline of nitrogenase activity was less rapid during the later period in the dark. Continued dark nitrogen fixation would have to depend on the mobilization of reserve products and on the rate at which carbon moves from its reservoir to the site of nitrogenase activity. It is noteworthy that the decrease of enzyme activity was less dramatic when alga was supplemented with glucose, and indeed provision of glucose could support nitrogen fixation for a prolonged period in the dark.

Field studies have indicated that the most important source of reductant and energy for nitrogen fixation in the aquatic environment is photosynthesis, and that the rate of dark fixation depends upon previous exposure to light, when both the intensity of light and length of exposure are equally important (7, 11-13). Whereas the present study confirms these conclusions, it also shows that light-dependent and dark assimilation of organic substances could be an additional or alternative source of the requirements for nitrogenase. The correlation between the abundance of blue-green algae, rates of nitrogen fixation, and concentration of dissolved organic matter in lake waters is well documented (7, 13, 17). Although the modes by which organic substances stimulate growth of blue-green algae may be varied and complex (10), the enhancement of nitrogen fixation through direct assimilation of organic substrates is a real possibility. Preliminary field investigations suggest that organic substances are more important to freshwater blue-green algae than previously thought (B. M. Guest and P. Fay, unpublished data). The ability to utilize organic substances in dim light and in the dark in support of nitrogen fixation may be of considerable ecological advantage. It is thus essential to consider the stimulatory effect of organic substrates on light and dark nitrogen fixation when estimating amounts of fixed nitrogen in natural situations.

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