Nitrogen Fixation by *Gloeotheca* sp. PCC 6909: Respiration and Not Photosynthesis Supports Nitrogenase Activity in the Light

By PAUL S. MARYAN,¹ ROBERT R. EADY,¹ ALAN E. CHAPLIN² AND JOHN R. GALLON^{2*}

¹ AFRC Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, UK

² Department of Biochemistry, University College of Swansea, Singleton Park, Swansea SA2 8PP, UK

(Received 9 April 1985; revised 15 August 1985)

Nitrogenase activity of suspensions of the unicellular cyanobacterium *Gloeotheca* sp. PCC 6909 plotted against the concentration of dissolved O₂ (dO₂) resulted in a bell-shaped curve, both in the light and in the dark, with optima of 25 or 80 µM-O₂ depending on the age of the culture. At the optimum dO₂, nitrogenase activity [typically 4 to 6 nmol C₂H₄ (mg protein)⁻¹ min⁻¹] was similar in the light or in the dark. Alteration of light intensity from zero to 2 klx, or addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), had no effect on nitrogenase activity. At 1 klx the ADP/ATP ratio was 0.2 and showed only a marginal increase as the dO₂ was increased. However, a high level of illumination (30 klx) stimulated or inhibited nitrogenase activity, depending on the external dO₂, presumably as a consequence of changes in the intracellular O₂ concentration; in the presence of DCMU, activity increased twofold, independent of dO₂.

In the dark, the dependence of the rate of respiration on O₂ concentration suggested the presence of three O₂-uptake systems with apparent *K_m* values of 1 µM, 5 µM and 25 µM. The ADP/ATP ratio under anaerobic conditions was 0.47 and showed a marked decrease as dO₂ was increased to 25 µM. A CN-insensitive respiratory activity, which neither supported nitrogenase activity nor was coupled to ATP synthesis, was associated with the system with the apparent *K_m* of 5 µM. The dependence of the specific activity of nitrogenase on dO₂ indicated that both the high affinity (*K_m* 1 µM) and low affinity (*K_m* 25 µM) O₂-uptake systems contributed ATP or reductant for N₂-fixation. KCN (2.5 mM) completely inhibited nitrogenase activity in the dark and at moderate levels of illumination and dO₂. We conclude that respiration is the major source of reductant and ATP for nitrogenase activity both in the dark and in the light, but that photosystem I can contribute ATP at very high levels of illumination.

INTRODUCTION

To grow diazotrophically, cyanobacteria have to overcome the problem of the sensitivity of nitrogenase towards inactivation by both photoevolved and atmospheric O₂. In many species of filamentous cyanobacteria protection is achieved by compartmentalization of nitrogenase into heterocysts (Stewart, 1980). *Gloeotheca* is a unicellular cyanobacterium which fixes N₂ aerobically but does not produce heterocysts. Cultures of *Gloeotheca* grown under alternating periods of light and dark fix N₂ only in the dark periods, at the expense of endogenous carbohydrate reserves accumulated in the preceding light period (Mullineaux *et al.*, 1980, 1981). However, in cultures grown under continuous illumination, N₂-fixation and oxygenic photosynthesis coexist within a single cell (Gallon *et al.*, 1974).

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

The questions arise (Gallon, 1980) whether, under constant illumination, respiration or photosynthesis provides the reductant and ATP necessary for nitrogenase function, and how nitrogenase is protected from inactivation by environmental and photosynthetically generated O_2 . This paper describes an investigation of the relationship between nitrogenase activity and different steady-states of dissolved O_2 concentration (dO_2) in the light and in the dark. We have also determined the effect of dO_2 on the respiratory rate of *Gloeotheca* and the inhibitory effect of cyanide on respiration and nitrogenase activity.

METHODS

Organism and growth conditions. An axenic culture of *Gloeotheca* sp. PC 6909 was a generous gift from Dr R. Rippka, Institut Pasteur, Paris, and was grown at 25 °C under constant illumination (1 klx) in ASM-1 medium free of combined nitrogen (Gallon *et al.*, 1978). Stock cultures were maintained on agar slopes of ASM-1 medium solidified with agar (1%, w/v). Experimental cultures were either sparged or shaken under air. Checks for contamination were made by plating on ASM-1 and also by thorough microscopic examination.

Respiratory O_2 consumption. Stationary phase cultures were concentrated to about 2 mg protein ml⁻¹ by centrifuging and resuspending in fresh medium before they were transferred to a vessel (3 ml) fitted with a Clark-type O_2 electrode (Rank Bros), previously calibrated at low dO_2 by adding small volumes of O_2 -saturated medium. All tests were at 30 °C. After 30 min incubation in the dark under air with constant stirring, the vessel was sealed and the O_2 -uptake measured until the dissolved O_2 was completely utilized. The curves of O_2 concentration vs time were digitized using a Hewlett-Packard plotter type 7220C linked to a PDP1134A computer and converted to rates of O_2 uptake vs O_2 concentration curves by drawing tangents to the digitized data. The resulting curves were then smoothed using a cubic spline algorithm procedure. Typical results are shown in Fig. 4. Values of apparent K_m and V_{max} were calculated using the S/v vs S linear transform (Cornish-Bowden, 1979).

Extraction of nucleotides. A 4 ml sample of culture (typical density between 20 and 35 µg protein ml⁻¹) was mixed with an equal volume of 20% (w/v) trichloroacetic acid in 50% (v/v) methanol. After 5 min, the solution was neutralized by shaking with 20 ml water-saturated ether, which was then removed, avoiding any water loss. This step was repeated three times. Final traces of ether were removed by placing the solution in a water bath at 50 °C for 5 min. The neutralized suspension was then disrupted in a French pressure cell at 69 MPa before centrifugation (10000 g for 10 min at 4 °C) to remove cell debris. The supernatant was collected and 1.5 ml 1 M-Tris/HCl buffer, pH 7.4, added before removing 5 × 1 ml samples for freeze-drying overnight.

Measurement of ATP and ADP. The freeze-dried extracts were redissolved in 0.5 ml water. For measurement of ADP, conversion mixture (50 µl), containing 50 µmol KCl, 6 µmol MgCl₂ and 0.125 µkat pyruvate kinase, was added and the mixture left for 45 min at room temperature. The reaction was then stopped by placing the tubes in a water bath at 90 °C for 5 min.

Crude luciferin/luciferase was extracted from 50 mg dried firefly lanterns (Sigma), suspended in 5 ml water. Insoluble material was removed by centrifugation (18000 g for 10 min, 4 °C). The supernatant was then activated by adding 120 mg calcium phosphate and shaking gently at room temperature for 10 min. The calcium phosphate was removed by centrifugation at 400 g for 10 min at 4 °C and the procedure repeated. To ensure complete removal of the calcium phosphate, the final centrifugation step was done at 18000 g for 10 min at 4 °C. The pale yellow supernatant was removed and kept on ice until required. It was stable for up to 6 h.

ATP was assayed by adding a 50 µl sample to 1 ml assay medium (0.02 M-glycylglycine buffer, 5 mM-sodium arsenate, 4 mM-MgSO₄, pH 8.0) in a bioluminescence tube, which was placed in a luminometer (LKB model 1250 or Berthold Biolumat LB9500). The background reading was taken before 0.1 ml luciferin/luciferase was syringed manually into the tube via the injection port (zero time) and the mV output integrated over a pre-set 10 s counting period.

Bovine serum albumin was added to an ATP solution to a concentration of 25 µg ml⁻¹. This solution was then subjected to the same extraction procedure as the samples. After freeze-drying, the standard ATP was reconstituted and used to construct a calibration curve over the range 0 to 20 pmol ATP.

ATP concentrations were measured directly on unconverted samples and ADP was determined by subtraction of the ATP value from that obtained after conversion of ADP to ATP.

Measurement of nitrogenase activity of cell suspensions. Activity was measured in the system described by Haaker *et al.* (1974) modified to exclude light other than that provided by Osram white-light fluorescent tubes illuminating the entire culture through a small window. A closed gas phase consisting of different partial pressures of O_2 (pO_2) in N_2 was recycled through the culture by an air pump. Dissolved O_2 was measured using a lead-silver electrode (Uniprobe) with a detection limit of 0.5 µM- O_2 . Samples of culture, typically 15–30 µg protein ml⁻¹ (24 ml) were placed in the thermostatted vessel at 30 °C in the dark, and sparged with N_2 until dO_2 was zero. The required dO_2 was imposed by introducing O_2 in the N_2 used to sparge the culture. The vessel was then sealed with a rubber closure and the culture exposed to the chosen light intensity. To start the assay, C_2H_2 (8.5%, v/v) was injected into

Fig. 1. Effect of dO_2 on nitrogenase activity of (a) stationary phase cultures (25 d old) of *Gloeotheca* sp. PCC 6909 in the light at 2 klx ($\blacktriangle$) and in the dark ($\blacksquare$), and (b) exponential phase cultures (8 d old) in the light at 1 klx ($\blacktriangle$) and in the dark ($\blacksquare$). As a consequence of day to day fluctuations in the specific activity of the cultures the activities obtained in the light and dark were different. However, for a sample from a given culture maintained at a given dO_2 there was no effect on the rate of C_2H_2 reduction on changing the illumination from 0 to 2 klx (Fig. 3). The rates of C_2H_2 reduction were determined at different dO_2 over at least 30 min to establish that linear rates were maintained.

the gas headspace. At intervals, 0.5 ml gas samples were removed by syringe to measure C_2H_4 by gas chromatography. The use of a 24 ml culture volume compared with the 58 ml total gas volume buffered the imposed dO_2 from changes due to O_2 -uptake or photoevolution of O_2 . To obtain saturating light intensities a projector beam was used for illumination.

Measurement of protein content. Cultures were concentrated by centrifuging and protein was estimated by the Lowry method, modified by heating the samples at 100 °C in the alkaline solution for 30 min before assay.

RESULTS

Effect of dO_2 on nitrogenase activity

Nitrogenase activity in the dark. Under strictly anaerobic conditions no C_2H_2 reduction was observed over 2.5 h. The effect of imposing increasing dO_2 on nitrogenase activity is shown in Fig. 1(a). A rapid increase in activity was observed at 2 μM - O_2 followed by a broad optimum, with a maximum rate [typically 4 to 6 nmol C_2H_4 (mg protein) $^{-1}$ min^{-1}] observed near 80 μM - O_2 . Activity was inhibited by higher dO_2 , declining very rapidly in the range 125 to 150 μM . This general pattern was observed consistently, but the optimum dO_2 and the maximum specific activity varied with the age of the culture. Young cultures (2 to 12 d) had lower optima of approx. 25 μM - O_2 and were completely inhibited by 120 μM - O_2 (Fig. 1b). For all cultures tested, time courses of nitrogenase activity at inhibitory dO_2 values were linear over the 20 min assay period usually used, even at the highest dO_2 tested.

Nitrogenase activity in the light. In the light, substantial rates of C_2H_2 reduction were observed in the absence of added O_2 . The rate depended on the light intensity, increasing over the range 0.5 to 2 klx (Fig. 2). In contrast, when a positive dO_2 was imposed, the rate of C_2H_2 reduction was independent of the light intensity up to 2 klx (Fig. 3) and the pattern observed (Fig. 1a, b) with increasing dO_2 was similar to that in the dark.

At these light intensities no increase in dO_2 as a consequence of photosynthetic O_2 evolution was detected during the 30 min over which measurements were made. At saturating light intensities (~30 klx), however, a small increase in dO_2 occurred but the effect on nitrogenase activity depended on the initial dO_2 . Cultures with an optimum dO_2 for activity of 25 μM , maintained at a sub-optimal dO_2 (7.2 μM), showed an increase in specific activity from 1.6 to

Fig. 2

Fig. 3

Fig. 2. Effect of varying the light intensity on C_2H_2 reduction by a culture of *Gloeotheca* sp. PCC 6909 maintained at zero external dO_2 . Results are from a single experiment but are typical.

Fig. 3. Effect of varying light intensities on C_2H_2 reduction by suspensions of *Gloeotheca* sp. PCC 6909 maintained at a dO_2 of $8 \mu M$. Results are from a single experiment but are typical.

$2.7 \text{ nmol } C_2H_4 \text{ produced min}^{-1} (\text{mg protein})^{-1}$, whilst those maintained at a higher than optimal dO_2 ($82 \mu M$) showed a decrease in activity from 0.5 to $0.35 \text{ nmol } C_2H_4 \text{ produced min}^{-1} (\text{mg protein})^{-1}$ compared with the rate observed at 2 klx .

When photosynthetic O_2 evolution was inhibited with $10 \mu M$ -DCMU, saturating light intensities stimulated nitrogenase activity twofold compared with the rate in the dark over the range 5 to $220 \mu M$ - O_2 . In contrast, illumination at 2 klx had little effect on nitrogenase activity.

Effect of dO_2 on respiration

O_2 -uptake by concentrated suspensions of *Gloeotheca* was measured in a closed system in the dark. The rate of respiration, measured continuously as the dO_2 decreased from air saturation ($220 \mu M$) to $0.5 \mu M$, was non-linear, since the rate increased rapidly up to $30 \mu M$ - O_2 and then increased more gradually up to $200 \mu M$ (Fig. 4). The initial rapid increase was a complex response, suggesting the presence of two types of O_2 -uptake activity with different affinities for O_2 (Fig. 4, inset). The data up to $30 \mu M$ - O_2 fit best to the sum of two hyperbolic concentration-activity curves with approximate K_m values of $1 \mu M$ - and $5 \mu M$ - O_2 , and V_{max} values of 1.32 and $2 \mu \text{mol min}^{-1} (\text{mg protein})^{-1}$, respectively. The gradual increase at higher dO_2 was associated with a lower affinity O_2 -uptake system with an apparent K_m of $25 \mu M$, and a V_{max} of $2.2 \mu \text{mol min}^{-1} (\text{mg protein})^{-1}$. Similar data were obtained for cultures that showed optimum rates of nitrogenase activity at $25 \mu M$ - or $80 \mu M$ - O_2 . The complex response of respiration rate as a function of dO_2 is not due to exhaustion of reductant during the duration of the experiments (typically 20 to 40 min) since the rate of C_2H_2 reduction was linear, at all dO_2 tested, over this period. Comparison of Figs 1(a) and 1(b) with Fig. 4 indicates that, in the dark, nitrogenase activity increases over a range of dO_2 where both the high and low affinity O_2 -uptake systems could function to provide ATP for nitrogenase activity (see also Fig. 6).

Effect of KCN on respiration and nitrogenase activity

Respiration in the dark was inhibited 85% by 2.5 mM -KCN but the residual O_2 -uptake rate was linear above $15 \mu M$ - O_2 (Fig. 5). An analysis of the rate of O_2 -uptake as a function of dO_2 below $15 \mu M$ indicated that the CN^- insensitive respiration was associated with the O_2 -uptake system with an apparent K_m of $5 \mu M$ - O_2 . In the dark, KCN totally inhibited C_2H_2 reduction over the range 10 to $120 \mu M$ - O_2 . This inhibition was not due to substrate competition between C_2H_2 and CN^- for nitrogenase since no CH_4 was detected. In the light, a more complex pattern was

Fig. 4

Fig. 5

Fig. 4. Computer-generated curves showing the rates of respiration as a function of O_2 concentration by suspensions of *Gloeotheca* sp. PCC 6909. A normal progress curve of O_2 -uptake at $30^\circ C$ was digitized and tangents were drawn to the data to obtain rates. The curves obtained were then smoothed (see Methods for details). The inset shows the expanded scale used to analyse the initially rapid response to low dO_2 . Results are from a single experiment but are typical of N_2 -grown cultures.

Fig. 5. Computer-generated curve showing the rate of respiration as a function of O_2 concentration by a suspension of *Gloeotheca* sp. PCC 6909 in the presence of 2.5 mM-KCN. A typical progress curve of O_2 -uptake at $30^\circ C$ was digitized and tangents were drawn to the data to obtain rates. The curve obtained was then smoothed (see Methods for details). Results are from a single experiment but are typical of N_2 -grown cultures.

Fig. 6. Effect of dO_2 on the ADP/ATP ratio in extracts of *Gloeotheca* sp. PCC 6909 in the light at 1 klx (▲) and in the dark (■). Cultures were maintained at a fixed dO_2 and samples (4 ml) removed for nucleotide analysis as described in Methods. Each extract was divided into 5 samples (1 ml) which were assayed in duplicate. The data shown are therefore the mean of 10 assays and the error bars show the standard deviation from the mean.

observed. The ability of CN^- to inhibit nitrogenase depended on both the dO_2 and the intensity of illumination. Below $80 \mu M$ - O_2 and at light intensities below 2.5 klx, CN^- completely inhibited nitrogenase activity of a culture which had optimum activity at $25 \mu M$ - O_2 . However, above $80 \mu M$ - O_2 and at light intensities above 2.5 klx, a low rate of CN^- -insensitive C_2H_2 reduction [$0.08 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$] occurred, which was not inhibited by $10 \mu M$ -DCMU.

Effect of dO_2 on the ADP/ATP ratio

ADP/ATP ratio in the dark. Under anaerobic conditions in the dark, where ATP can be generated only by substrate-level phosphorylation, nitrogenase activity was not detected and the ADP/ATP ratio of suspensions of *Gloeotheca* was 0.47 ± 0.1 (SD). When suspensions were maintained at a positive dO_2 the ratio initially decreased rapidly with increasing dO_2 , followed

by a more gradual decrease at higher dO_2 (Fig. 6). Comparison of Fig. 4 with Fig. 6 indicates that both high and low affinity O_2 -uptake systems couple to ATP production. However, the major change in the ADP/ATP ratio correlates with the range of dO_2 where the activity of the high affinity system predominates, and where nitrogenase activity shows the most marked dependence on dO_2 (Fig. 1*a, b*). The KCN-insensitive O_2 -uptake system, which does not support nitrogenase activity, also does not couple effectively to ATP synthesis since the ADP/ATP ratio in the dark increased from 0.18 ± 0.05 (SD) to 2.0 ± 0.21 (SD) after addition of 2.5 mM-KCN.

ADP/ATP ratio in the light. In the light (1 klx) and in the absence of added O_2 , the ADP/ATP ratio was 0.2 (Fig. 6). The ratio showed a marginal increase at the lowest positive dO_2 imposed, but thereafter remained constant with increasing dO_2 . The data of Fig. 6 are directly comparable with the effect of dO_2 on nitrogenase activity (Fig. 1*b*), since the samples analysed for ADP/ATP ratio were taken from the culture on which activity measurements were made. It is clear that, in the light, changes in nitrogenase activity do not correlate with changes in the ADP/ATP ratio.

In the presence of $10 \mu\text{M}$ -DCMU, at $11 \mu\text{M}$ - O_2 and under saturating light, nitrogenase activity was stimulated twofold compared with the rate in the dark, and the ADP/ATP ratio decreased from 0.26 ± 0.05 (SD) to 0.14 ± 0.03 (SD).

DISCUSSION

With few exceptions (Hochman & Burris, 1981; Post *et al.*, 1983; Jensen & Cox, 1983; Klugkist & Haaker, 1984) studies of the effects of O_2 on N_2 -fixation *in vivo* have used either different pO_2 or varied shaking rates to alter the dO_2 . With heterotrophic organisms the dO_2 is dependent both on the respiratory rate and on the solution rate of O_2 . In the case of organisms which photoevolve O_2 the actual dO_2 is even more uncertain. In our experiments, different dO_2 were imposed by varying the composition of the gas phase in an apparatus which allowed nitrogenase activity and dO_2 to be measured simultaneously.

Nitrogenase activity plotted against dO_2 resulted in a bell-shaped curve characteristic of that of all aerobic diazotrophs. In the dark no activity was observed under anaerobic conditions, but it increased rapidly as the external dO_2 was increased to $10 \mu\text{M}$ - O_2 . The optimum dO_2 was the same in the light and in the dark. This general pattern is similar to that reported by Gallon & Hamadi (1984) for *Gloeotheca* sp. ATCC 27152, but differs in that Gallon & Hamadi (1984) observed a rate under anaerobic conditions in the dark which was approximately 60% of the maximum. This apparent difference most probably resides in the incomplete removal of O_2 in the earlier experiments since we have subsequently found an O_2 requirement for nitrogenase activity in *Gloeotheca* ATCC 27152. This emphasizes the importance of the measurement of dO_2 in cultures of organisms, such as *Gloeotheca*, which have a high affinity O_2 -uptake system that can couple to nitrogenase.

In both strains of *Gloeotheca* activity is more susceptible to inhibition by supraoptimal dO_2 in the dark than in the light but strain ATCC 27152 was more O_2 -tolerant since activity was still detected in the light and in the dark at a pO_2 of 0.7 atm (Gallon & Hamadi, 1984). Since these strains originate from the same isolate this difference may result from the adventitious selection of a variant during their independent maintenance. In agreement with this hypothesis, strain ATCC 27152, unlike PCC 6909 (Kallas *et al.*, 1983), is capable of growth and N_2 -fixation at light intensities of 2.5 klx.

In the range of dO_2 over which nitrogenase activity was inhibited, the time course of C_2H_2 reduction was linear, indicating that the inhibition was not due to inactivation of nitrogenase by O_2 , but more probably to oxidation of the electron donor to nitrogenase. The optimum dO_2 of $80 \mu\text{M}$ supporting maximum nitrogenase activity, and the 0.1 atm pO_2 reported by Gallon & Hamadi (1984), are both higher than that of most aerobic diazotrophs, where typical values range from $1 \mu\text{M}$ for cowpea *Rhizobium* strain 32H1 to $20 \mu\text{M}$ for *Azotobacter chroococcum* (see Robson & Postgate, 1980). Nitrogenase activity in other non-heterocystous cyanobacteria can

also tolerate high dO_2 values since light-limited continuous cultures of *Plectonema boryanum* were established growing on N_2 at $40\ \mu M$ - O_2 (Pearson & Howsley, 1980).

The expression of aerobic nitrogenase activity by *Gloeotheca* is not a consequence of respiration decreasing the dO_2 to very low levels. The effective coupling of the high affinity O_2 -uptake system to both ATP synthesis and nitrogenase activity also suggests that protection of nitrogenase from inactivation by O_2 does not involve an O_2 diffusion barrier, as is observed in the heterocystous cyanobacterium *Anabaena variabilis* (Jensen & Cox, 1983) and in *Frankia* sp. (Murry *et al.*, 1984). Thus, the extensive extracellular slime capsule of *Gloeotheca* does not appear to act as a diffusion barrier. This is in agreement with the observations that nitrogenase activity of a capsuleless mutant of *Gloeotheca* is no more sensitive to O_2 than the parental strain (Kallas *et al.*, 1983), and that activity of strains which overproduce slime is as sensitive to O_2 as the wild-type (J. R. Gallon, unpublished observation).

The high affinity O_2 -uptake system of *Gloeotheca* (K_m $1\ \mu M$) is coupled to nitrogenase activity and ATP synthesis, since at $6\ \mu M$ - O_2 nitrogenase activity was approximately 90% of that at the optimum dO_2 and the ADP/ATP ratio was 140% of the minimum value at saturating dO_2 . This O_2 -uptake system would be effectively saturated at the optimum dO_2 of $25\ \mu M$, a value well below that of air-saturated medium. Natural populations of *Gloeotheca*, often found as colonies on limestone (Whitton & Sinclair, 1975), would therefore have the potential for N_2 -fixation throughout the colony where a gradient of dO_2 might be expected to exist.

In the dark, reductant for nitrogenase activity arises from endogenous carbon reserves (Mullineaux *et al.*, 1980) and ATP from O_2 -dependent respiration. At low dO_2 , where the ADP/ATP ratio showed a marked decrease with increasing dO_2 , nitrogenase activity increased with the ADP/ATP ratio, whilst addition of KCN ($2.5\ mM$) increased the ADP/ATP ratio from 0.18 to 2 and completely inhibited nitrogenase activity. Total inhibition by KCN was also observed at moderate levels of illumination and dO_2 , indicating that respiration provided ATP for nitrogenase activity even in the light. The similarity of the dependence of nitrogenase activity on dO_2 in the light and in the dark supports this view. Furthermore, when cultures were maintained at a fixed dO_2 , the rate of C_2H_2 reduction was independent of the level of illumination from darkness to $2\ klx$ regardless of the cell density.

The independence of nitrogenase activity from illumination differs from earlier reports where increased rates were observed in the light (see Gallon, 1980). However, both light intensity and the prevailing dO_2 (in relation to the optimal dO_2 of the culture) are critical parameters in determining the effect of illumination. For example, under anaerobic conditions, nitrogenase activity was stimulated by light which could well be a consequence of an increased intracellular dO_2 rather than a direct effect of increased photophosphorylation. However, under saturating light intensities ($\sim 30\ klx$) in the presence of DCMU the observed twofold stimulation of nitrogenase activity is consistent with the view that activity is normally limited by the availability of ATP, since the generation of reducing equivalents derived from photosynthesis is inhibited under these conditions.

Our broad conclusion is that respiration provides ATP for N_2 -fixation by *Gloeotheca* sp. PCC 6909 both in the dark and in the light. In addition, its ability to conduct aerobic N_2 -fixation does not involve respiratory protection of the kind observed in *Azotobacter*, where high rates of respiration decrease the ambient dO_2 to low levels (see Robson & Postgate, 1980).

We gratefully acknowledge the provision of an SERC CASE award to P.S.M. and the award of Scientific Investigation Grants from the Royal Society to A.E.C. and J.R.G. We also thank Dr D. J. Lowe for assistance with computing and Professor J. R. Postgate for constructive criticism of the manuscript.

REFERENCES

- CORNISH-BOWDEN, A. (1979). *Fundamentals of Enzyme Kinetics*. London: Butterworth.
- GALLON, J. R. (1980). Nitrogen fixation by photoautotrophs. In *Nitrogen Fixation*, pp. 197–238. Edited by W. D. P. Stewart & J. R. Gallon. London: Academic Press.
- GALLON, J. R. & HAMADI, A. F. (1984). Studies on the effects of oxygen on acetylene reduction (nitrogen fixation) in *Gloeotheca* sp. ATCC 27152. *Journal of General Microbiology* **130**, 495–503.
- GALLON, J. R., LARUE, T. A. & KURZ, W. G. W. (1974). Photosynthesis and nitrogenase activity in

- the blue-green alga *Gloeocapsa*. *Canadian Journal of Microbiology* **20**, 1633–1637.
- GALLON, J. R., UL-HAQUE, M. I. & CHAPLIN, A. E. (1978). Fluoroacetate metabolism in *Gloeocapsa* sp. LB795 and its relationship to acetylene reduction (nitrogen fixation). *Journal of General Microbiology* **106**, 329–336.
- HAAKER, H., DE KOK, A. & VEEGER, C. (1974). Regulation of dinitrogen fixation in intact *Azotobacter vinelandii*. *Biochimica et biophysica acta* **357**, 344–357.
- HOCHMAN, A. & BURRIS, R. H. (1981). Effect of oxygen on acetylene reduction by photosynthetic bacteria. *Journal of Bacteriology* **147**, 492–499.
- JENSEN, B. B. & COX, R. P. (1983). Effect of oxygen concentrations on dark nitrogen fixation and respiration in cyanobacteria. *Archives of Microbiology* **135**, 287–292.
- KALLAS, T., RIPPKA, R., COURSIN, T., REBIERE, M.-C., DE MARSAC, N. T. & COHEN-BAZIRE, G. (1983). Aerobic nitrogen fixation by nonheterocystous cyanobacteria. In *Photosynthetic Prokaryotes*, pp. 281–302. Edited by G. C. Papageorgiou & L. Packer. Amsterdam: Elsevier.
- KLUGKIST, J. & HAAKER, H. (1984). Inhibition of nitrogenase activity by ammonium chloride in *Azotobacter vinelandii*. *Journal of Bacteriology* **157**, 148–151.
- MULLINEAUX, P. M., CHAPLIN, A. E. & GALLON, J. R. (1980). Effects of a light to dark transition on carbon reserves, nitrogen fixation and ATP concentrations in cultures of *Gloeocapsa* (*Gloeotheca*) sp. 1430/3. *Journal of General Microbiology* **120**, 227–232.
- MULLINEAUX, P. M., GALLON, J. R. & CHAPLIN, A. E. (1981). Acetylene reduction (nitrogen fixation) by cyanobacteria grown under alternating light–dark cycles. *FEMS Microbiology Letters* **10**, 245–247.
- MURRY, M. A., FONTAINE, M. S. & TJEPKEMA, J. D. (1984). Oxygen protection of nitrogenase in *Frankia* sp. HFPAr13. *Archives of Microbiology* **139**, 162–166.
- PEARSON, H. W. & HOWSLEY, R. (1980). Concomitant photoautotrophic growth and nitrogenase activity by cyanobacterium *Plectonema boryanum* in continuous culture. *Nature, London* **288**, 263–265.
- POST, E., KLEINER, D. & OELZE, J. (1983). Whole cell respiration and nitrogenase activities in *Azotobacter vinelandii* growing in oxygen controlled continuous culture. *Archives of Microbiology* **134**, 68–72.
- ROBSON, R. L. & POSTGATE, J. R. (1980). Oxygen and hydrogen in biological nitrogen fixation. *Annual Review of Microbiology* **34**, 183–207.
- STEWART, W. D. P. (1980). Some aspects of structure and function in N_2 -fixing cyanobacteria. *Annual Review of Microbiology* **34**, 497–536.
- WHITTON, B. A. & SINCLAIR, C. (1975). Ecology of blue-green algae. *Science Progress* **62**, 429–446.