Cultures of Gloeothecae sp. ATCC 27152 did not reduce acetylene when exposed to O₂ concentrations greater than 0.7 atm. However, following exposure to 1 atm O₂ for up to 12 h or to 0.8 atm O₂ for up to 14 d, the ability to reduce acetylene recovered rapidly when cultures were returned to air. Complete recovery required active protein synthesis, probably for de novo synthesis of nitrogenase. Respiratory O₂ consumption by cultures of Gloeothecae was stimulated under elevated concentrations of O₂. This respiration may contribute to the protection of nitrogenase from inactivation by O₂ at concentrations up to 0.4 atm. The role of Ca²⁺ in nitrogen fixation may be related to respiratory protection.

**INTRODUCTION**

Among the various organisms that fix N₂, the properties of nitrogenase (EC 1.18.2.1) are remarkably constant. For example, nitrogenase is extremely sensitive to inactivation by O₂ and it is now well-documented that N₂-fixing organisms have evolved a variety of mechanisms in order to protect this enzyme from damage by O₂ (Gallon, 1981). Cyanobacteria are unique among N₂-fixing organisms in that they evolve O₂ as a result of their photosynthesis. Accordingly, N₂-fixing cyanobacteria are able to protect nitrogenase from both photoevolved and atmospheric O₂. Precisely how this protection is achieved is not understood, but it appears to involve a combination of different mechanisms (Gallon, 1981; Gotto et al., 1979).

Gloeothecae (formerly Gloeocapsa) is a unicellular cyanobacterium capable of aerobic N₂ fixation. It does not possess the differentiated heterocysts that characterize most aerotolerant N₂-fixing cyanobacteria (Haselkorn, 1978). In Gloeothecae, N₂ fixation and photosynthetic O₂ evolution coexist within a single, undifferentiated cell type. Although cultures of Gloeothecae, grown under an alternating cycle of light and darkness, effect an almost complete temporal separation of photosynthesis and N₂ fixation (Mullineaux et al., 1981), cultures grown under constant illumination evolve O₂ and fix N₂ simultaneously. Like heterocystous cyanobacteria, Gloeothecae appears to use a variety of strategies in order to protect nitrogenase from O₂ damage. Among these are a superoxide-metabolizing system (Tözüm & Gallon, 1979) and an unidentified Ca²⁺-dependent mechanism (Hamadi & Gallon, 1981). However, these may well be supplemented by other systems.

In this paper, we describe a more detailed study of additional mechanisms by which the nitrogenase of Gloeothecae sp. ATCC 27152 might be protected from inactivation by O₂. In addition, we attempt to identify the role of Ca²⁺ in O₂ protection.

**METHODS**

_Growth of cultures._ Gloeothecae sp. ATCC 27152 (American Type Culture Collection, Rockville, Md., USA) was grown aerobically in 15 litres of sterile medium, free of combined nitrogen (Tözüm & Gallon, 1979). Plectonema boryanum UTEX594 (University of Texas Culture Collection, Austin, Texas, USA) was grown similarly, either

*Abbreviation:* Disulfiram, tetraethylthioperoxydicarbonic diamide.
acribically in medium containing 2 mm-NaNO₃, or in nitrogen-free medium sparged with N₂ containing 0·03% (v/v) CO₂.

Acetylene reduction (N₂ fixation). During incubation of cultures of Gloeothece under various gas phases, acetylene reduction was measured as described by Tözüm et al. (1977). The effect of transient exposure to O₂ was measured as follows. Exponentially growing cultures of Gloeothece (10⁹–10⁶ cells ml⁻¹), harvested 6 to 9 d after inoculation, were concentrated five-fold by settling under gravity and 50 ml samples were incubated aerobically, with stirring, at 25 °C, in sealed 150 ml Erlenmeyer flasks. Illumination at 2·5 klx (17 μE m⁻² s⁻¹) was provided by Osram white fluorescent lights. Acetylene was added to 0·01 atm and 0·5 ml samples of the gas phase were removed after various incubation periods for determination of ethylene by gas chromatography. After 20 min incubation, the experimental culture was bubbled with either O₂ or O₂/N₂ (1·1, v/v) for 2 min, followed by 1 min with air. Control cultures were bubbled with air for 3 min. The flasks were then sealed, acetylene added to 0·01 atm and further 0·5 ml samples of the gas phase removed at suitable intervals during the following 2 to 3 h.

Longer-term exposure to O₂ was achieved by bubbling cultures of Gloeothece (2–15 litres) with O₂ or with O₂/N₂ (4·1, v/v) containing 0·03% (v/v) CO₂. Samples (50 ml) were then removed and incubated aerobically for a suitable period after which acetylene reduction was measured as described by Tözüm et al. (1977).

Respiratory O₂ consumption. O₂ consumption by 3 ml samples of exponentially growing cultures of Gloeothece was measured polarographically at 25 °C in the dark, in a Clark-type O₂ electrode (Rank Bros., Bottisham, Camb., UK). The cultures used were either uncentrinated or concentrated threefold by settling under gravity. The electrode was calibrated with air-saturated water and the concentration of dissolved O₂ was adjusted by bubbling with O₂ or N₂ and also by illumination of cultures at 6 klx (66 μE m⁻² s⁻¹) under a tungsten lamp, before measurement of O₂ consumption. Control incubations, with boiled cells, were included throughout and all data are corrected for O₂ exchange in these controls.

O₂ consumption by broken cell preparations of Gloeothece, prepared in 50-mm-HEPES/NaOH (pH 7·5) as described by Tözüm & Gallon (1979), was also measured polarographically. The reaction mixture (3 ml) contained 32 mm-HEPES/NaOH (pH 7·5), 1 mm-MgCl₂ and 0·5 ml of a broken cell preparation of Gloeothece. NADH or NADPH (5 μmol) was added to start the reaction followed, in some cases, 2 to 5 min later by addition of ADP (5 μmol) and KH₂PO₄ (5 μmol).

Intracellular Ca²⁺. To minimize contamination by Ca²⁺, all reagents were of the highest purity available (BDH) and were dissolved in fresh double-distilled water. All solutions were stored in polypropylene containers and polypropylene vessels were used for all experimental manipulations.

Exponentially growing cultures of Gloeothece (100 ml) were harvested 6 to 8 d after inoculation and were incubated in 5 mm-HEPES/NaOH (pH 7·5) for 30 min with or without 1 mm-EDTA. The cells were collected by centrifugation at 10000 g for 10 min. They were then suspended in double-distilled water, washed twice with 0·2 mm-MgCl₂ to saturate the chelating capacity of any EDTA present in solution (Azzi & Chance, 1969), and then twice with double-distilled water and finally resuspended in 2 ml double-distilled water. Cells were broken in a French press at 138 MPa and centrifuged at 32000 g for 20 min. KCl was added to the supernatant to a final concentration of 1·35 M and, after standing for 15 min, the precipitate was removed by centrifugation at 35000 g for 20 min. The Ca²⁺ concentration of a sample (0·1 ml) of the supernatant was measured using the bioluminescent method of Blinks et al. (1978). Each assay contained, in a final volume of 1 ml, 5 mm-PIPES/NaOH (pH 6·5) and 0·15 M-KCl. The reaction was started by addition of 20 μg aequorin (Sigma) dissolved in 2 mm-KCl and passed through a column of Sephadex G-25 immediately before use. Total luminescence during a 10 s integration time was measured at 30 °C using a Biolumat LB 9500 photon counter (Laboratory Impex, Twickenham, Middlesex, UK). Calibration curves were obtained by taking through the extraction procedure standards containing 0·1 pmol to 1 μmol CaCl₂, with and without 1 mm-EDTA.

Other measurements. H₂ evolution was measured as described by Bothe et al. (1977) using exponentially growing cultures of Gloeothece, concentrated 20-fold by settling under gravity and incubated in sealed 25 ml bottles for 6 to 18 h.

Lipoxygenase (EC 1.13.11.12) was assayed polarographically as described by Wallace & Wheeler (1979). The assay mixture (3 ml) contained 50 mm-sodium phosphate buffer, pH 6·8 and 12 mm-sodium linoleate. The reaction was started by addition of 0·5 ml of a broken cell preparation of Gloeothece.

The purity of cyanobacterial cultures was routinely monitored by streaking cultures onto nutrient agar or onto 1% (w/v) agar containing growth medium supplemented with 10 mm-glucose and 0·02% (w/v) Casamino acids (Rippka et al., 1979).

Cell numbers were measured in a haemocytometer (Tözüm & Gallon, 1979).

RESULTS AND DISCUSSION

O₂ and acetylene reduction

Illuminated cultures of Gloeothece sp. ATCC 27152, growing exponentially under aerobic conditions, exhibited maximum rates of acetylene reduction (N₂ fixation) under 0·1 atm O₂.
Oxygen and acetylene reduction in Gloeothecae

As the concentration of O₂ was increased, the rate of acetylene reduction decreased, reaching zero above 0.7 atm O₂. Batch cultures of Gloeothecae in the stationary phase of growth (10 to 14 d after inoculation) were rather more sensitive to elevated concentrations of O₂. For example, whilst exponentially growing cultures consistently reduced acetylene 1 h after transfer to 0.6 atm O₂ (Fig. 1), acetylene reduction by stationary phase cultures was completely inhibited under these conditions (data not shown).

The rate of acetylene reduction by illuminated cultures also decreased as the concentration of O₂ in the gas phase fell below 0.1 atm O₂ (Fig. 1). It appears, therefore, that photosynthesizing cultures of Gloeothecae need atmospheric O₂ in order to sustain maximum rates of acetylene reduction. Possibly, some respiratory activity is needed to supply reduced ferredoxin, ATP or carbon skeletons for N₂ fixation. In this context, it may be significant that there is no unequivocal evidence that photosynthesis directly provides reductant for N₂ fixation in Gloeothecae (Gallon, 1980).

Maximum rates of acetylene reduction also occurred under 0.1 atm O₂ when exponentially growing cultures of Gloeothecae were incubated in the dark. It appears, therefore, that atmospheric O₂ is needed to sustain maximum rates of acetylene reduction in the dark, as in the light. However, because cultures of Gloeothecae were able to fix N₂ anaerobically in the dark (Fig. 1), O₂ is not an essential requirement for this process.

Acetylene reduction by cultures of Gloeothecae incubated under 0.2 to 0.6 atm O₂ was stimulated by addition of 5 mM-sodium ascorbate (Fig. 1). Sodium ascorbate also, to some extent, prevented methyl viologen from inhibiting acetylene reduction, and it may destroy toxic radicals produced by reduction of O₂ (Tözüm & Gallon, 1979). It is possible, therefore, that elevated concentrations of O₂ may, in part, inhibit nitrogenase as a result of the production of toxic oxygen radicals.
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Fig. 3. Effect of 12 h exposure to 1 atm O₂ on acetylene reduction by Gloeothecce. Cultures (2 litres) were bubbled with O₂ for 12 h. Samples (50 ml) were then removed and, after aerobic incubation for the period indicated, were assayed for acetylene reduction. To these cultures was added 5 mM-HEPES/NaOH, pH 7.5 (●), along with 0.1 mg chloramphenicol ml⁻¹ (▼), or 2 mM-NH₄Cl (■). Although the data shown are from a single experiment they are typical of 12 experiments involving 1 to 12 h exposure to O₂.

Fig. 4. Effect of 14 d exposure to 0.8 atm O₂ on acetylene reduction by Gloeothecce. Cultures (15 litres) were bubbled with O₂:N₂ (4:1, v/v) containing 0.03% (v/v) CO₂ for 14 d, after which 50 ml samples were removed. To these was added HEPES/NaOH (5 mM), pH 7.5 (●), along with 2 mM-NH₄Cl ( ■). Acetylene reduction was then measured after aerobic incubation for the period indicated. The data shown are from a single experiment but are typical of six experiments involving 1 to 14 d exposure to 0.8 atm O₂.

Illuminated cultures of Gloeothecce, exposed to an inhibitory concentration (1 atm) of O₂ for 2 min, resumed acetylene reduction after a lag period of about 30 min following return to air. However, within 2 h the rate of acetylene reduction in treated cultures was comparable to that in control cultures which had not been exposed to O₂ (Fig. 2). Exposure for 2 min to 0.5 atm O₂ had a similar effect although, in this case, acetylene reduction did not cease completely and recovered to its rate in control cultures within 1 h (results not shown). Recovery of acetylene reduction, following 2 min exposure to either 1 atm O₂ (Fig. 2) or 0.5 atm O₂, was completely prevented by addition of 0.1 mg chloramphenicol ml⁻¹, implying that recovery was the result of de novo protein synthesis.

The length of the lag period that preceded reappearance of nitrogenase activity may be related to the length of exposure to 1 atm O₂. For example, whilst cultures of Gloeothecce, exposed to 1 atm O₂ for between 2 min and 12 h recovered the ability to reduce acetylene within 30 to 60 min (Fig. 2, Fig. 3), cultures exposed to 1 atm O₂ for 24 h recommenced acetylene reduction only after a lag period of about 7 h. Following 36 h exposure to 1 atm O₂, the lag period was 13 h and following 100 h exposure, a lag of 23 h preceded reappearance of acetylene reduction (data not shown). On the other hand, when cultures were exposed to 0.8 atm O₂ for between 24 h and 14 d, the ability to reduce acetylene consistently returned within 1 h of transfer to air (Fig. 4). The pattern of recovery of nitrogenase activity following exposure of Gloeothecce to elevated concentrations of O₂ therefore appears to vary with respect to the concentration of O₂ used, as well as with respect to the length of exposure to O₂.

Nitrogenase synthesis appears to continue for up to 4 h in cultures of Gloeothecce incubated under 1 atm O₂ (Mullineaux et al., 1983). This is in contrast to the repressive effect of O₂ in some other N₂-fixing organisms (Eady, 1981). The relatively short lag period before nitrogenase activity reappears following up to 12 h exposure to 1 atm O₂ or up to 14 d exposure to 0.8 atm O₂ suggests that synthesis of the enzyme might continue for periods longer than 4 h under elevated
concentrations of O\(_2\). In contrast to the effect of O\(_2\), 12 h exposure of immobilized cultures of *Gloeothece* to 0-2 mM-NH\(_4\)Cl, which represses nitrogenase synthesis, resulted in no reappearance of nitrogenase activity for 7 to 12 h after removal of NH\(_4\)Cl (Gallon et al., 1984). However, nitrogenase synthesis may not continue indefinitely under 1 atm O\(_2\) because a lag period of several hours preceded reappearance of nitrogenase activity following 24 to 100 h exposure to O\(_2\). Possibly, the carbon and/or nitrogen reserves of the cell became depleted during prolonged incubation under 1 atm O\(_2\), thereby inhibiting nitrogenase synthesis.

Although *Gloeothece* may continue to synthesize nitrogenase during exposure to elevated concentrations of O\(_2\), acetylene reduction was not observed at any time during 100 h exposure to 1 atm O\(_2\) or during 14 d exposure to O\(_2\)/N\(_2\) (4:1, v/v) containing 0-03\% (v/v) CO\(_2\). In the absence of N\(_2\)-fixation, these cultures would become nitrogen-deficient during exposure to either 1 atm or 0-8 atm O\(_2\). In view of this, it is more likely that depletion of cellular carbon, rather than cellular nitrogen, interrupted nitrogenase synthesis during 24 to 100 h exposure to 1 atm O\(_2\).

Pienkos et al. (1983) have reported that cultures of *Anabaena* sp. CA, a heterocystous cyanobacterium, recover the ability to reduce acetylene within 30 to 60 min whilst still exposed to 1 atm O\(_2\). In this organism, as in *Gloeothece*, nitrogenase synthesis apparently continues under elevated concentrations of O\(_2\), although the two organisms differ in their ability to maintain an active nitrogenase under these conditions. However, in both *Gloeothece* (Fig. 2, Fig. 3) and *Anabaena* CA (Pienkos et al., 1983) brief exposure to O\(_2\), followed by return to air, results in only a short lag period before acetylene reduction reappears. In contrast, in *Anabaena flos-aquae*, recovery of nitrogenase activity was not complete until 8 to 10 h after exposure for 10 min to 1 atm O\(_2\) (Bone, 1971). In some species of cyanobacteria, therefore, transient exposure to O\(_2\) may repress nitrogenase synthesis.

Addition of either 0-1 mg chloramphenicol ml\(^{-1}\) or 2 mM-NH\(_4\)Cl completely abolished recovery of nitrogenase activity following transfer to air of cultures of *Gloeothece*, previously exposed to 1 atm O\(_2\) for 12 h (Fig. 3). The involvement of protein synthesis in this recovery may therefore be for de novo synthesis of nitrogenase itself. However, although addition of NH\(_4\)Cl to cultures exposed to 0-8 atm O\(_2\) for 14 d markedly decreased the recovery of acetylene reduction, it did not prevent it completely (Fig. 4). In this latter case, as in *Anabaena* CA (Pienkos et al., 1983), it is possible that only part of the nitrogenase activity that reappears is the result of de novo synthesis of the enzyme. Some of the inactivation of nitrogenase by 0-8 atm O\(_2\) may therefore be freely reversible.

The N\(_2\)-fixing aerobe *Azotobacter* exhibits a reversible conformational protection of nitrogenase during transient exposure to elevated concentrations of O\(_2\) (Robson & Postgate, 1980). A similar mechanism may exist in *Gloeothece* but there is little evidence for this (Hamadi & Gallon, 1981). On the other hand, in cultures of *Gloeothece* exposed to 1 atm O\(_2\) for 12 h, and in cultures in which O\(_2\) inactivation of nitrogenase had been caused by addition of 1 mM-EDTA (Hamadi & Gallon, 1981; Gallon, 1980), reappearance of nitrogenase activity may be entirely a consequence of synthesis of new enzyme. In these cases, inactivation of nitrogenase by O\(_2\) may have been irreversible.

It appears, therefore, that in *Gloeothece* synthesis of nitrogenase continues for several hours under 1 atm O\(_2\) and for several days under 0-8 atm O\(_2\). Nevertheless, the enzyme is rapidly and, largely, irreversibly inactivated by exposure to O\(_2\) at these concentrations. Although continued synthesis of nitrogenase during O\(_2\) stress appears wasteful it may be advantageous in allowing rapid recovery of nitrogenase activity following return to less extreme conditions.

*Plectonema boryanum* is a non-heterocystous, filamentous cyanobacterium capable of N\(_2\) fixation under microaerobic, but not aerobic, conditions. However, Wang et al. (1982) have reported that, when incubated with extracts of *Gloeothece*, cultures of *P. boryanum* can fix N\(_2\) anaerobically. Extracts of *Gloeothece* contain ascorbate and other reducing agents (Tözüm & Gallon, 1979) and, in view of its stimulation of acetylene reduction in *Gloeothece* (Fig. 1), it is possible that ascorbate is responsible for the effect on *P. boryanum* of extracts of *Gloeothece*. In support of this, addition of 5 mM-sodium ascorbate to cultures of *P. boryanum* UTEX 594 resulted in low, but consistent, rates of acetylene reduction during aerobic incubation. However,
addition of sodium ascorbate did not allow aerobic growth of *P. boryanum* in nitrogen-free medium.

**Respiration in Gloeoeethece**

The rate of O$_2$ consumption by cultures of *Gloeoeethece*, incubated in the dark, increased as the dissolved O$_2$ concentration increased (Fig. 5). In this respect, *Gloeoeethece* resembles *Azotobacter*, where respiratory O$_2$ consumption increased by a factor of five when the concentration of O$_2$ was increased from 0.05 to 0.55 atm (Dalton, 1980). In *Gloeoeethece* the equivalent increase was 2.7-fold (Fig. 5). In *Azotobacter* respiration may function as an O$_2$-scavenging mechanism which thereby protects nitrogenase from O$_2$ (Yates & Eady, 1980). Natural populations of *Gloeoeethece* probably fix most of their N$_2$ in the dark (Mullineaux et al., 1981), and under these conditions respiration may have a major role in protecting nitrogenase from inactivation by O$_2$.

The greatest stimulation of respiratory O$_2$ consumption in response to increasing concentrations of O$_2$ took place between 0 and 0.4 atm O$_2$ (Fig. 5). Between 0.4 and 0.8 atm O$_2$, little further stimulation occurred and it is unlikely that respiratory O$_2$ consumption would be very effective in protecting *Gloeoeethece* nitrogenase above 0.4 atm O$_2$. Consistent with this, exposure of *Gloeoeethece* to more than 0.7 atm O$_2$ led rapidly to complete cessation of acetylene reduction (Figs 1–4). The stimulatory effect of O$_2$ on respiratory O$_2$ consumption (Fig. 5) was measured within 15 min following exposure of cultures to the new concentration of O$_2$. It is not known for how long these rates of respiration would be maintained, but it is unlikely to be for more than 1 h. In support of this, preliminary data suggest that cultures of *Gloeoeethece* incubated in the dark for 1 h under 1 atm O$_2$ exhibit rates of CO$_2$ evolution and O$_2$ consumption markedly lower than those in control cultures. It cannot therefore be assumed that respiratory O$_2$ consumption could protect nitrogenase during prolonged exposure of cultures of *Gloeoeethece* to O$_2$.

In *Azotobacter*, the respiratory chain is branched (Haddock & Jones, 1977). The different branches have different sensitivities to various inhibitors, including CN$^-$ (Jones & Redfearn, 1967). As a result of this branched electron transport chain, at high concentrations of O$_2$ respiration in *Azotobacter* is poorly coupled to ATP synthesis and apparently functions primarily to consume O$_2$.

Addition of 0.1 mM- or 1 mM-KCN (Fig. 5) to cultures of *Gloeoeethece* partially inhibited respiratory O$_2$ consumption. However, there was always residual O$_2$ uptake of about 35% of the
Oxygen and acetylene reduction in Gloeothece

Table 1. Effect of various additions on \(O_2\) uptake by cultures of Gloeothece

\(O_2\) uptake was measured polarographically in air using cultures (3 ml) concentrated threefold under gravity. All incubations also contained 5 mM-HEPES/NaOH, pH 7.5. The rates of oxygen consumption are typical.

<table>
<thead>
<tr>
<th>Addition</th>
<th>(O_2) consumption [nmol min(^{-1}) (10(^6) cells(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.00</td>
</tr>
<tr>
<td>0.1 mM-KCN</td>
<td>0.37</td>
</tr>
<tr>
<td>1 mM-KCN</td>
<td>0.32</td>
</tr>
<tr>
<td>1 mM-Salicylhydroxamate</td>
<td>1.00</td>
</tr>
<tr>
<td>15 (\mu)M-Disulfiram</td>
<td>1.00</td>
</tr>
<tr>
<td>1 mM-EDTA</td>
<td>0.60</td>
</tr>
<tr>
<td>5 mM-CaCl(_2)</td>
<td>1.04</td>
</tr>
<tr>
<td>5 mM-MgCl(_2)</td>
<td>0.81</td>
</tr>
<tr>
<td>1 mM-EDTA + 5 mM-CaCl(_2)</td>
<td>0.91</td>
</tr>
<tr>
<td>1 mM-EDTA + 5 mM-MgCl(_2)</td>
<td>0.63</td>
</tr>
<tr>
<td>1 mM-EDTA + 1 mM-KCN</td>
<td>0.12</td>
</tr>
</tbody>
</table>

uninhibited rate (Table 1). In higher plants, \(CN^-\)-insensitive respiration has been equated with a branched electron transport chain (Lance, 1981), although recently this has been questioned on the grounds that lipoxygenase (EC 1.13.11.12) activity could explain \(CN^-\)-resistant \(O_2\) uptake (Kelly, 1982). However, unlike \(CN^-\)-resistant respiration in the mitochondria of higher plants, \(O_2\) uptake in *Gloeothece* was not inhibited by either salicylhydroxamate, an inhibitor of lipoxygenase (Parish & Leopold, 1978), or disulfiram (Table 1). Furthermore, no lipoxygenase activity has been detected in extracts of *Gloeothece*.

The rate of \(CN^-\)-resistant respiration in cultures of *Gloeothece* increased with increasing \(O_2\) concentration (Fig. 5). Between 0.1 atm and 0.2 atm \(O_2\), the increase in the rate of \(CN^-\)-resistant respiration could entirely account for the increase in respiration observed in the absence of \(CN^-\). However, above 0.2 atm \(O_2\), although \(CN^-\)-resistant respiration continued to increase, the increase was insufficient to explain the increase in \(O_2\) consumption by uninhibited cultures of *Gloeothece*. Presumably, \(CN^-\)-sensitive respiration was also stimulated over this range of \(O_2\) concentrations.

It is therefore possible that *Gloeothece*, like *Azotobacter*, possesses a branched electron transport chain and that electron flow down a \(CN^-\)-resistant branch is mainly, though not exclusively, stimulated under elevated concentrations of \(O_2\). By analogy with *Azotobacter*, this \(CN^-\)-resistant branch may be poorly coupled to ATP synthesis and may function mainly to consume \(O_2\). Cultures of *Gloeothece* may, therefore, exhibit respiratory protection of nitrogenase.

In some species of cyanobacteria, \(H_2\) donates electrons via an 'uptake' hydrogenase to an electron transport chain with \(O_2\) as terminal acceptor (Bothe *et al.*, 1980). The resulting \(O_2\) consumption may contribute to the protection of nitrogenase from damage by \(O_2\), particularly under conditions of carbon deficiency. Cultures of *Gloeothece* evolved little, if any, \(H_2\) either in the light or, aerobically or anaerobically, in the dark. However, in the presence of 12.4 mM-C\(_3\)H\(_2\) and 0.124 mM-CO (Bothe *et al.*, 1977), \(H_2\) evolution could be measured at a rate of 24.6 pmol h\(^{-1}\) (10\(^6\) cells\(^{-1}\)) in the light and of 4.8 pmol h\(^{-1}\) (10\(^6\) cells\(^{-1}\)) in the dark. This suggests that *Gloeothece* possesses an 'uptake' hydrogenase and may, therefore, recycle \(H_2\) evolved by the action of nitrogenase, possibly in an \(O_2\)-consuming system. However, there is no direct evidence that \(H_2\) has a role in protecting *Gloeothece* nitrogenase from inactivation by \(O_2\) (Tözüm & Gallon, 1979).

\(Ca^{2+}\) and acetylene reduction

Addition of EDTA to cultures of *Gloeothece* inhibits acetylene reduction by disruption of a \(Ca^{2+}\)-dependent process by which nitrogenase is protected from \(O_2\) (Hamadi & Gallon, 1981). EDTA acts by depleting the cyanobacterial cells of \(Ca^{2+}\). The intracellular concentration of
Ca²⁺ in cultures of *Gloeothecae* was 62 pmol (10⁶ cells)⁻¹ under normal growth conditions (equivalent, approximately, to 0.6 mM), but fell to 12 pmol (10⁶ cells)⁻¹ following addition of 1 mM-EDTA.

Addition of 5 mM-CaCl₂ to N₂-fixing cultures decreased the inhibitory effect on acetylene reduction of 0.2 to 0.5 atm O₂, but was without effect at O₂ concentrations outside these limits (Fig. 1).

EDTA (1 mM) inhibited respiratory O₂ uptake (Table 1). This inhibition was prevented by simultaneous addition of 5 mM-CaCl₂ but not of 5 mM-MgCl₂. In this respect, the effect of EDTA on respiration resembles its effect on acetylene reduction (Gallon, 1978). However, EDTA did not permanently affect the intracellular concentration of ATP (Hamadi & Gallon, 1981). It is possible, therefore, that EDTA inhibits a relatively uncoupled respiratory flow.

In broken cell preparations of *Gloeothecae*, addition of 5 mM-CaCl₂ stimulated by 50% both NADH- and NADPH-dependent O₂ uptake. Unstimulated rates were 10⁻⁹ and 14⁻⁸ nmol min⁻¹ (mg protein)⁻¹ respectively. Stimulation was observed whether or not ADP and inorganic phosphate were also present. Furthermore, although oligomycin, an inhibitor of oxidative phosphorylation, at a concentration of 10 μg ml⁻¹, decreased the rate of NADH- and NADPH-dependent O₂ consumption to about 45% of their uninhibited rate, this residual O₂ uptake was still stimulated by addition of 5 mM-CaCl₂. The effect of CaCl₂ on respiratory O₂ consumption does not, therefore, depend upon simultaneous ATP synthesis and may be the result of stimulation of uncoupled electron flow.

CaCl₂ (5 mM) did not greatly affect the recovery of nitrogenase activity following treatment of cultures of *Gloeothecae* with 0.8 atm O₂ for 2 to 7 d. It is unlikely, therefore, that Ca²⁺ ions are specifically involved in this recovery.

In conclusion, therefore, the role of Ca²⁺ in N₂ fixation by *Gloeothecae* may be linked to respiratory protection of nitrogenase from O₂. The mechanism by which Ca²⁺ ions affect respiration is not clear but may be related to membrane integrity. In support of this, Ca²⁺ prevented surface-active compounds from inhibiting acetylene reduction by *Gloeothecae* (Hamadi & Gallon, 1981).

The authors thank the Royal Society for the award of a Scientific Investigations Grant. They also thank Mrs J. H. Thomas for skilled technical assistance and Dr A. E. Chaplin for valuable discussions. In addition, the contributions to this work of Mr S. M. Batchelor and Miss T. J. Fairless are gratefully acknowledged.

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