The Effects of Methyl Viologen on *Gloeocapsa* sp. LB795 and Their Relationship to the Inhibition of Acetylene Reduction (Nitrogen Fixation) by Oxygen

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(Received 15 August 1978; revised 10 October 1978)

Methyl viologen (10 μM) markedly inhibited acetylene reduction (nitrogen fixation) by old but not young cultures of *Gloeocapsa* sp. LB795, apparently by causing the alga to produce H_2O_2 . H_2O_2 inhibited acetylene reduction when added to cultures at concentrations greater than 10 μM . As catalase (EC 1.11.1.6) is not present in *Gloeocapsa* sp. LB795, H_2O_2 is probably removed by a non-enzymic reaction with ascorbate and also by an enzyme-catalysed reaction with glutathione. Enzymes catalysing the decomposition of H_2O_2 were most active in young cells which were therefore better able than old cells to metabolize H_2O_2 produced in the presence of methyl viologen. The maximum activities of these enzymes coincided with maximum nitrogenase activity during the growth of batch cultures, and may provide a protective mechanism for nitrogenase.

INTRODUCTION

Gloeocapsa sp. UTEX LB795 [redesignated *Gloeotheca* sp. by Stanier & Cohen-Bazire (1977)] is unusual, although not unique among blue-green algae (Singh, 1973; Carpenter & Price, 1976), in that it fixes nitrogen aerobically even though it does not possess heterocysts. These specialized cells characterize most other nitrogen-fixing blue-green algae and are probably the major site of aerobic nitrogen fixation in these organisms (Tel-Or & Stewart, 1977). In the unicellular alga *Gloeocapsa*, however, there are no obviously differentiated cells and so nitrogen fixation must take place in the same cell as photosynthesis. However, photosynthesis produces oxygen whilst the nitrogenase of *Gloeocapsa* sp. is rapidly inactivated by oxygen both *in vitro* (Gallon *et al.*, 1972) and *in vivo* (Gallon *et al.*, 1974). In order for such an oxygen-sensitive enzyme to remain active in a cell which is producing oxygen, some method of protecting the enzyme must exist. In part, the temporal separation of maximum nitrogenase activity from maximum photosynthetic oxygen evolution, described by Gallon *et al.* (1975), may provide this protection. However, an additional mechanism must operate since nitrogenase activity was always present at a low level during the period of maximum oxygen evolution and oxygen was still evolved during the period of maximum nitrogenase activity.

Several mechanisms by which oxygen is toxic to living organisms are known (Halliwell, 1978*a*). Many of these involve the production, from oxygen, of free radicals such as superoxide ($\text{O}_2^{\cdot-}$) and hydroxyl radicals which, in photosynthetic organisms, may arise from the reaction between oxygen and reduced intermediates of the photosynthetic electron transport chain. Since methyl viologen greatly stimulates the production of radicals (Farrington *et al.*, 1973), the effects of this compound on acetylene reduction by *Gloeocapsa* seemed worthy of

study. In particular, we hoped to elucidate the mechanisms by which oxygen inhibits acetylene reduction in this organism and also investigate any systems which might exist to protect nitrogenase from such inhibition.

METHODS

Growth of cultures. *Gloeocapsa* sp. LB795 [University of Texas Culture Collection (UTEX)], *Gloeocapsa* sp. 1430/3 [Culture Centre of Algae and Protozoa (CCAP), Cambridge] and *Anabaena cylindrica* 1403/2a (CCAP) were grown in 15 l of sterile medium free of combined nitrogen, as described by Gallon *et al.* (1978). *Plectonema boryanum* 594 (UTEX) was grown in the same medium except that NaCl was replaced by NaNO₃ (2 mM). *Aphanothece pellida* was a generous gift from Dr P. K. Singh, Central Rice Research Institute, Cuttack, India, and was grown either in the above nitrogen-free medium or in a modified Chu. No. 10 medium, which had the following composition (μ M): CaCl₂, 400; Na₂SiO₃, 200; Na₂CO₃, 200; MgSO₄, 100; K₂HPO₄, 60; tri-sodium citrate, 12; FeCl₃, 12; H₃BO₃, 8; MnSO₄, 0.2; ZnSO₄, 0.2; MoO₃, 0.08; CuSO₄, 0.08.

The contaminating bacteria of *Gloeocapsa* sp. LB795 and *Aphanothece pellida* were grown in the medium described by Gallon *et al.* (1978) supplemented with sodium glutamate (10 mM).

Preparation of extracts of *Gloeocapsa* sp. LB795. All operations were performed at 4 °C. Cells were centrifuged at 10000 *g* for 10 min, resuspended in buffer (composition dependent on the enzyme subsequently assayed) to 1 % (v/v) of the original culture volume and broken in a French press at 138 MPa. The resulting broken-cell preparation, or a cell-free extract obtained by centrifuging the broken-cell preparation at 30000 *g* for 20 min, was used for enzyme assays.

Enzyme assays. Nitrogen fixation by algal cultures (10^5 to 5×10^8 cells ml⁻¹) was measured by the acetylene reduction technique after incubation with or without the addition of other compounds. Unless otherwise stated, assays were performed aerobically at 25 °C under illumination at 2500 lx. Cells were incubated either as described by Tözüm *et al.* (1977) or as follows. Cells were concentrated 20- to 50-fold by settling under gravity and 5 ml samples were transferred to a water-jacketed cylindrical vessel (15 ml) where they were vigorously stirred and, after any preliminary incubation, the vessel was sealed; acetylene was then added to 1 % (v/v) and after a further 15 to 60 min incubation, the ethylene formed was measured by gas-liquid chromatography. The rates of acetylene reduction were linear for up to 60 min. Incubations with H₂ were carried out at pressures exceeding 1 atm because the different diffusion properties of the gases used made it impossible to release excess pressure without altering the composition of the gas phase (Bothe *et al.*, 1977). Controls with added Ar were included in these experiments.

Enzymes were assayed at 25 °C using 0.5 ml of broken-cell preparation or cell-free extract of *Gloeocapsa* sp. LB795. Spectrophotometric assays were performed using a Pye-Unicam SP1700 spectrophotometer; polarographic measurements were made in a Clark-type oxygen electrode, (Rank Bros, Bottisham, Cambs.). All assays included appropriate substrate blanks and a boiled enzyme control. Unless stated otherwise, enzyme activity is expressed as katal (kat) under the conditions of assay.

Superoxide dismutase (EC 1.15.1.1) was assayed by its effect in decreasing the rate of cytochrome *c* reduction by a xanthine/xanthine oxidase (EC 1.2.3.2) system. The conditions of assay were those of McCord & Fridovich (1969) except that EDTA was omitted from the reaction mixture. One unit of superoxide dismutase activity is defined as that amount which causes a 50 % decrease in the rate of cytochrome *c* reduction under these conditions.

Catalase (EC 1.11.1.6) was assayed polarographically by the production of oxygen from H₂O₂ (Chua, 1971), or the disappearance of H₂O₂ was measured spectrophotometrically at 240 nm (Chance & Maehly, 1955).

Peroxidase (EC 1.11.1.7) was assayed spectrophotometrically by following guaiacol oxidation at 470 nm (Chance & Maehly, 1955).

Glutathione peroxidase (EC 1.11.1.9) was measured as described by Little & O'Brien (1968). An excess of glutathione reductase (NADPH) (EC 1.6.4.2) was used to couple reduced glutathione (GSH) oxidation, in the presence of cumene peroxide, to the oxidation of NADPH, which was monitored by the resulting decrease in absorbance at 340 nm. Because high rates of NADPH oxidation were observed with boiled cell extracts, presumably arising from the presence of oxidized glutathione (GSSG) in the assay mixture, all reported activities have been corrected for this as well as for any NADPH oxidation found in the absence of GSH or cumene peroxide.

Ascorbate peroxidase was assayed by measuring the disappearance of H₂O₂ catalyzed by this enzyme in the presence of ascorbate. Cell-free extract or broken-cell preparation of *Gloeocapsa* sp. (0.5 ml) was incubated with sodium ascorbate (60 μ mol), H₂O₂ (30 μ mol) and KH₂PO₄/K₂HPO₄ buffer, pH 7.0 (30 μ mol), in a total volume of 3 ml. At 30 s intervals, 0.01 ml samples were transferred to an oxygen electrode containing, in

3 ml, catalase (2 μ kat), bovine serum albumin (300 μ g) and $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.0 (150 μ mol). The resulting evolution of oxygen was measured and compared with that produced from standard amounts (0.1 to 1 μ mol) of H_2O_2 under the same conditions.

Glutathione dehydrogenase (ascorbate) (EC 1.8.5.1) was assayed by coupling the oxidation of GSH by dehydroascorbate to NADPH oxidation in the presence of excess glutathione reductase (NADPH). The incubation mixture contained, in a total volume of 3 ml, dehydroascorbate (9 μ mol), GSH (75 μ mol), NADPH (0.36 μ mol), glutathione reductase (NADPH) (0.2 μ kat in 100 μ g bovine serum albumin), cell-free extract or broken-cell preparation of *Gloeocapsa* sp. (0.5 ml) and Tris/HCl buffer, pH 7.0 (300 μ mol) containing EDTA (9 μ mol). The decrease in absorbance at 340 nm was measured. Solutions of dehydroascorbate were prepared in buffer which had been bubbled with nitrogen and were used immediately. All reported activities for glutathione dehydrogenase (ascorbate) have been corrected for the rates of NADPH oxidation observed with boiled cell extracts as well as for any oxidation found in the absence of GSH or dehydroascorbate.

Glutathione reductase (NADPH) (EC 1.6.4.2) was assayed spectrophotometrically at 340 nm (Scott *et al.*, 1963).

Measurement of photosynthesis. Photosynthetic oxygen exchange by cells of *Gloeocapsa* sp. was measured polarographically at 25 °C under illumination at 2500 lx unless stated otherwise. Photosynthetic $^{14}\text{CO}_2$ fixation was measured as described by Gallon *et al.* (1978) except that, after incubation, cells were collected by filtration, washed three times with incubation medium and dried before their radioactivity was determined by scintillation spectrophotometry.

Measurement of cell density of cultures. The number of cells in samples removed from cultures of *Gloeocapsa* sp. was determined by counting in a Hawksley haemocytometer. The values reported are the means of at least three determinations.

Other measurements. Glutathione was assayed as described by Tiebe (1969). Ascorbate was assayed spectrophotometrically as described by Roe (1954). Protein was measured by reference to bovine serum albumin using the method of Lowry *et al.* (1951), as modified by Bailey (1962).

RESULTS AND DISCUSSION

Inhibition of nitrogen fixation by methyl viologen

Methyl viologen at 10 μM caused little inhibition of acetylene reduction (nitrogen fixation) when added to *Gloeocapsa* sp. UTEX LB795 from a 4 d-old culture, but its inhibitory effect steadily increased as the culture aged (Fig. 1). Similar results were obtained with 10 μM -benzyl viologen. The degree of inhibition of acetylene reduction depended on the length of exposure of the algal cells to methyl viologen. For example, acetylene reduction by 10 d-old cells was only slightly inhibited by 10 μM -methyl viologen after 10 min, but was completely inhibited after 2 h. The inhibition could be reversed, at least in part, by removing methyl viologen within 30 min of addition, but was essentially irreversible after 60 min exposure to the inhibitor. An axenic strain of this alga, *Gloeocapsa* sp. CCAP 1430/3, showed a similar response when exposed to methyl viologen. However, acetylene reduction by another unicellular blue-green alga, *Aphanothece pellida* (Singh, 1973), was only slightly inhibited by 10 μM -methyl viologen at any stage of growth in either of the culture media used for this organism. Furthermore, acetylene reduction by young or old cultures of the heterocystous blue-green alga *Anabaena cylindrica* CCAP 1403/2a was not inhibited by methyl viologen.

When old cells of *Gloeocapsa* sp. LB795 were incubated in the dark or in the presence of 10 μM -3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), the inhibition of acetylene reduction caused by methyl viologen was relieved, markedly under aerobic conditions and completely under anaerobic conditions (Table 1). Anaerobic conditions alone had very little effect on the extent of inhibition. Since DCMU and darkness both inhibit photosynthetic non-cyclic electron transport and its associated oxygen evolution, this process was apparently required for methyl viologen to inhibit acetylene reduction. Furthermore, because complete reversal of this inhibition was found only when photosynthesis was prevented under anaerobic conditions, atmospheric oxygen also appeared to be necessary for the effect of methyl viologen on acetylene reduction.

Fig. 1. Variation with age of culture of the effect of methyl viologen on acetylene reduction by *Gloeocapsa* sp. LB795. Cells were collected from batch cultures after 4 to 15 d incubation and 50 ml samples were incubated aerobically in the presence or absence of 10 μ M-methyl viologen in 150 ml Erlenmeyer flasks for 60 min at 25 °C with illumination at 2500 lx. The flasks were then sealed, acetylene was added to 1% (v/v) and, after a further 60 min incubation, the ethylene produced was measured by gas-liquid chromatography. Each value for acetylene reduction is expressed as a percentage of the acetylene reduced in the absence of methyl viologen and is the mean of at least eight determinations.

Table 1. *Effect of methyl viologen on acetylene reduction by Gloeocapsa* sp. LB795

Cells were collected after 10 to 15 d growth and 50 ml samples were incubated in 150 ml Erlenmeyer flasks for 60 min at 25 °C under the conditions indicated; where indicated, illumination was at 2500 lx. All flasks, except those incubated aerobically were sealed during this incubation. Acetylene reduction was then measured as described in Fig. 1. The protein content of the cells was 0.27 mg (10^6 cells) $^{-1}$.

Because the effect of methyl viologen varied according to the age of the algal culture, the results shown are from a single experiment using 12 d-old cells; they are, however, typical. The values in parentheses show the rates as a percentage of those in the absence of methyl viologen under the same assay conditions.

Addition	Assay conditions		Acetylene reduction [pmol min $^{-1}$ (ml cells) $^{-1}$]	
	Illumination	Gas phase		
None	Light	Air	57.3	(100)
10 μ M-Methyl viologen	Light	Air	0	(0)
10 μ M-DCMU	Light	Air	64.7	(100)
10 μ M-Methyl viologen + 10 μ M-DCMU	Light	Air	38.8	(60)
None	Light	N $_2$	72.2	(100)
10 μ M-Methyl viologen	Light	N $_2$	2.9	(4)
10 μ M-DCMU	Light	N $_2$	83.0	(100)
10 μ M-Methyl viologen + 10 μ M-DCMU	Light	N $_2$	82.7	(100)
None	Dark	Air	38.9	(100)
10 μ M-Methyl viologen	Dark	Air	29.2	(75)
None	Dark	N $_2$	14.9	(100)
10 μ M-Methyl viologen	Dark	N $_2$	14.7	(99)
Catalase (2.5 μ kat ml $^{-1}$)	Light	Air	49.8	(100)
10 μ M-Methyl viologen + Catalase (2.5 μ kat ml $^{-1}$)	Light	Air	52.8	(106)
5 mM-Sodium ascorbate	Light	Air	52.6	(100)
10 μ M-Methyl viologen + 5 mM-Sodium ascorbate	Light	Air	39.4	(75)

(a) Production of superoxide ($O_2^{\cdot-}$) (b) Production of H_2O_2 (c) Removal of H_2O_2

Fig. 2. Production of superoxide and hydrogen peroxide in *Gloeocapsa* sp. LB795 and possible mechanisms for their removal. MV_{red} and MV_{ox} signify reduced and oxidized methyl viologen, respectively. Enzymes: 1.6.4.2, glutathione reductase (NADPH); 1.8.5.1, glutathione dehydrogenase (ascorbate); 1.11.1.9, glutathione peroxidase; 1.15.1.1, superoxide dismutase.

Methyl viologen is highly toxic to plants. It is reduced by photosynthetic electron transport and then autoxidizes, producing the free radical ion superoxide ($O_2^{\cdot-}$), as shown in Fig. 2(a) (Farrington *et al.*, 1973). Superoxide, in turn, may give rise to powerful oxidants such as hydroxyl radicals and singlet oxygen which will destroy many biologically important molecules including the polyunsaturated fatty acids of biological membranes (Halliwell, 1978b).

Because methyl viologen apparently requires photosynthesis and oxygen in order to produce maximum inhibition of acetylene reduction by *Gloeocapsa* sp., it probably exerts its effect by the production of superoxide. Furthermore, because neither darkness nor DCMU completely relieved the effect of methyl viologen under aerobic conditions (Table 1), there may be a non-photosynthetic mechanism for reducing methyl viologen and generating superoxide in *Gloeocapsa* sp. LB795 or in its contaminating bacteria. However, since *Gloeocapsa* sp. 1430/3 showed a similar response under the conditions indicated in Table 1, it is apparently the alga itself which can reduce methyl viologen non-photosynthetically.

All aerobic organisms so far examined contain superoxide dismutase (EC 1.15.1.1) which converts superoxide to oxygen and H_2O_2 (Fig. 2b). Alternatively, in the chloroplasts

of higher plants, superoxide may also react non-enzymically with the ascorbate which is often present in these organelles, again producing H_2O_2 (Fig. 2*b*). In the presence of catalase (EC 1.11.1.6), the H_2O_2 generated by these reactions would be removed according to the equation: $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$.

Cell-free extracts of *Gloeocapsa* sp. LB795 contained superoxide dismutase activity [2 to 9 units (mg protein)⁻¹] at levels similar to those reported in extracts of *Anabaena cylindrica* (Henry *et al.*, 1978). However, the catalase activity of the *Gloeocapsa* sp. extracts was very low or undetectable, either polarographically or spectrophotometrically. The maximum catalase activity found was 0.5 pkat (mg protein)⁻¹, much less than that found by Chua (1971) in any extract of the five species of blue-green algae which he investigated. Catalase activity [1.58 nkat (mg protein)⁻¹] was detected polarographically in a cell-free extract of *Plectonema boryanum* UTEX 594; although this activity was lower than that reported in this organism by Chua, it was nevertheless sufficient to demonstrate that the lack of activity in *Gloeocapsa* sp. extracts was not due to any problem with the assay.

In *Gloeocapsa* sp. LB795, therefore, the addition of methyl viologen may lead to production of H_2O_2 by the reactions in Fig. 2(*a, b*), but this H_2O_2 apparently is not removed by catalase.

Methyl viologen (10 μM) almost completely inhibited the photosynthetic assimilation of $^{14}\text{CO}_2$ by *Gloeocapsa* sp. LB795. This inhibition may also be related to the production of superoxide and H_2O_2 by the inhibitor, or may reflect the diversion of photosynthetic electron transport away from NADP^+ . The former explanation is more probable since it is unlikely that 10 μM -methyl viologen would completely prevent the reduction of NADP^+ . Chua (1971) showed that the maximum effect of methyl viologen on photosynthesis by *Phormidium luridum* was obtained only at concentrations of 2 mM or greater.

Within 2 min of adding 0.1 mM-methyl viologen to *Gloeocapsa* sp. LB795, photosynthetic oxygen evolution in the light ceased and oxygen uptake began (Fig. 3*a*). A similar effect was observed using 10 μM -methyl viologen, but the response was much slower (5 to 10 min). The effect of 0.1 mM-methyl viologen on photosynthetic oxygen exchange was the same for cells of different ages; the rate of oxygen uptake was, on average, 52 % of the rate of previous oxygen evolution, after correction for dark oxygen consumption. This uptake of oxygen in the presence of methyl viologen may be explained by the conversion of oxygen to superoxide and H_2O_2 referred to above, though the stoichiometry of the gas exchange may not be significant because the concentration of methyl viologen was probably not sufficient to saturate these reactions.

In chloroplast preparations, it is necessary to inhibit catalase (which often contaminates such preparations) with KCN or NaN_3 before a light-dependent oxygen uptake is observed with methyl viologen. Chua (1971) found that 1 mM-KCN markedly stimulated the methyl viologen-requiring oxygen uptake by all of the blue-green algae he investigated except for *Anabaena flos-aquae*. Neither 1 mM-KCN nor 1 mM- NaN_3 altered the rate of oxygen uptake by *Gloeocapsa* sp. LB795 in the presence of methyl viologen either in the light or in the dark, thereby supporting the enzymic data suggesting that *Gloeocapsa* sp. LB795 lacks catalase.

In addition to its effect in the light, 0.1 mM-methyl viologen stimulated the rate of oxygen uptake by *Gloeocapsa* sp. LB795 in the dark by 23 %. This is consistent with the idea that methyl viologen may also be reduced non-photosynthetically in this alga.

When catalase was added to *Gloeocapsa* sp. LB795 which had been treated with methyl viologen in the light, there was an immediate evolution of oxygen, after which oxygen consumption continued at a reduced rate (Fig. 3*b*). Since it is highly unlikely that added catalase could enter the algal cells, it appeared that in the presence of methyl viologen *Gloeocapsa* sp. excreted H_2O_2 .

Catalase also completely reversed the effect of methyl viologen on acetylene reduction by *Gloeocapsa* sp. (Table 1). It is possible, therefore, that methyl viologen inhibits acetylene reduction by causing the alga to produce H_2O_2 in amounts which it cannot metabolize and which it therefore excretes.

Fig. 3. Effect of methyl viologen on photosynthetic oxygen evolution by *Gloeocapsa* sp. LB795. Cells were concentrated 20- to 100-fold by settling under gravity, and 3 ml samples were incubated in an oxygen electrode at 25 °C. Illumination at 2500 lx commenced at points L and ceased at point D. Methyl viologen (MV; 0.1 mM) and catalase (1.25 μ kat ml⁻¹) were added where indicated. An upward deflection of the trace indicates oxygen evolution, a downward deflection indicates oxygen uptake. The traces are from a single experiment with 12 d-old cultures (8.5×10^8 cells ml⁻¹) and are typical for cells of this age.

Inhibition of nitrogen fixation by added H₂O₂

Acetylene reduction by *Gloeocapsa* sp. LB795 was inhibited by H₂O₂ at concentrations greater than 10 μ M; above 0.1 mM, inhibition was virtually complete within 1 min of the addition of H₂O₂. The inhibition of acetylene reduction could be completely reversed by removing H₂O₂ (initially at 0.1 mM) within 10 min of its addition, but the extent of this reversal decreased as the period of exposure to H₂O₂ was increased until, after 60 min exposure, the inhibition was irreversible. After 60 min, 0.1 mM-H₂O₂ was equally inhibitory in the light, in the dark and under an atmosphere of nitrogen; its effect did not appear to vary with the age of the algal cultures. Furthermore, H₂O₂ had no significant effect on photosynthetic oxygen evolution by *Gloeocapsa* sp.

Acetylene reduction by *Aphanothece pellida* and *Anabaena cylindrica* was also inhibited by 0.1 mM-H₂O₂, although to a lesser extent than with *Gloeocapsa* sp. However, whilst 10 μ M-methyl viologen inhibited acetylene reduction by old cells of *Gloeocapsa* sp., it had little effect on the other two algae. In the case of *Aphanothece pellida*, this is probably because methyl viologen caused no apparent excretion of H₂O₂ even though this alga, like *Gloeocapsa* sp., consumed oxygen when treated with the inhibitor. *Anabaena cylindrica*, however, both consumed oxygen and produced H₂O₂ when exposed to 0.1 mM-methyl viologen. On the other hand, this organism did not show a constant rate of oxygen uptake until at least 10 min after the addition of the inhibitor. This slower response may explain why acetylene reduction by *Anabaena cylindrica* was unaffected by methyl viologen, since the H₂O₂ produced may not reach a high enough concentration to inhibit nitrogenase during the period of exposure to methyl viologen.

The excretion of H₂O₂ by blue-green algae is not confined to cells treated with methyl viologen. Patterson & Myers (1973) observed H₂O₂ excretion by intact, unpoisoned cells of several, but not all, of the blue-green algae which they examined. The mechanism of H₂O₂ production under these conditions is unclear but it may be the result of a reaction between oxygen and a reduced intermediate of the photosynthetic electron transport chain (possibly reduced ferredoxin) in a manner analogous to the reaction between oxygen and reduced methyl viologen (Fig. 2a). Superoxide produced in this way could be converted to

H_2O_2 by the action of superoxide dismutase, by reaction with ascorbate or by further reaction with reduced ferredoxin (Halliwell, 1978*b*) (Fig. 2*b*).

Gloeocapsa sp. LB795 may excrete H_2O_2 in the absence of methyl viologen when exposed to high light intensities (16000 lx), since the rate of photosynthetic oxygen evolution was stimulated by as much as 25 % by the addition of $1.25 \mu\text{kat catalase ml}^{-1}$. However, this effect was not always observed so it is not possible to say with certainty that unpoisoned cells of *Gloeocapsa* sp. excrete H_2O_2 . In contrast, there seems little doubt that *Gloeocapsa* sp. excretes H_2O_2 on treatment with methyl viologen and that H_2O_2 inhibits acetylene reduction by the alga.

Reactions leading to the removal of H_2O_2

Since *Gloeocapsa* sp. may produce H_2O_2 under normal conditions of growth, even if this H_2O_2 is not excreted, the question arises, how, in the absence of catalase, is this H_2O_2 removed? Any H_2O_2 produced is presumably removed, otherwise nitrogenase would be inhibited. Higher plant chloroplasts produce H_2O_2 , but like *Gloeocapsa* sp., they have little or no catalase activity. H_2O_2 either diffuses to the peroxisomes where it is broken down, or it may be removed within the chloroplasts themselves by reaction with endogenous ascorbate or GSH (Halliwell, 1978*b*).

As shown in Table 1, ascorbate reversed the effect of methyl viologen on acetylene reduction by *Gloeocapsa* sp. possibly by reacting non-enzymically with the H_2O_2 produced. However, Groden & Beck (1977) have recently reported an ascorbate-specific peroxidase in spinach chloroplasts which may function to remove H_2O_2 from these organelles.

GSH (1 mM in 5 mM-HEPES/NaOH buffer, pH 7.5) did not reverse the effect of methyl viologen on acetylene reduction so may not react non-enzymically with H_2O_2 . Glutathione peroxidase (EC 1.11.1.9) is known in animal but not in plant tissue (Flohe & Menzel, 1971); the latter contains high activities of peroxidases (EC 1.11.1.7) with artificial substrates such as guaiacol, though the identity of their natural substrate is unknown.

The regeneration of GSH and ascorbate from their oxidized products (formed by reaction with H_2O_2) is accomplished, in chloroplasts, by the enzymes glutathione dehydrogenase (ascorbate) (EC 1.8.5.1) and glutathione reductase (NADPH) (EC 1.6.4.2) (Mapson, 1958; Foyer & Halliwell, 1976). By these reactions, ascorbate and GSH are made available for further reaction with H_2O_2 (Fig. 2*c*).

Variation of enzyme activities during growth of algal cultures

Broken-cell preparations and cell-free extracts of *Gloeocapsa* sp. LB795 from cultures of different ages were examined for the activities of ascorbate peroxidase, guaiacol-dependent peroxidase, glutathione dehydrogenase (ascorbate) and glutathione reductase (NADPH). No ascorbate peroxidase was detected, though high non-enzymic rates of H_2O_2 decomposition were observed in the presence of ascorbate. The results for the other enzymes, along with those for nitrogenase and superoxide dismutase, are shown in Fig. 4.

Nitrogenase activity was maximal between 6 and 8 d after inoculation of batch cultures. Maximum activities of superoxide dismutase, glutathione dehydrogenase (ascorbate) and glutathione reductase (NADPH) were also observed at this stage of growth, though the variation in activity was less dramatic with superoxide dismutase than with the other enzymes. Glutathione peroxidase activity was maximal between days 4 and 6 which corresponded fairly well with the other enzymes. However, *in vivo*, the activity of this enzyme may extend over a longer period during the growth of algal cultures than is suggested by the results in Fig. 4. The reason for this is that the instability of glutathione peroxidase made it a difficult enzyme to assay; freshly prepared extracts and assay solutions were necessary for its detection. In contrast to the other enzymes, guaiacol-dependent peroxidase activity did not appear until the cultures were 9 d-old and was maximal between 10 and 12 d after inoculation. It is possible therefore, that, with the exception of this peroxidase, these

Fig. 4. Variation with age of culture of the activities of various enzymes in *Gloeocapsa* sp. LB795. Acetylene reduction was measured using intact organisms as described in Fig. 1. Other enzymes were assayed using cell-free extracts (for details see Methods). The cultures were inoculated with approximately 10^5 cells ml^{-1} . Growth was exponential, usually until 11 d after inoculation, with a doubling time of 46 to 58 h. The results show the means of at least three experiments: superoxide dismutase activities are expressed as units $(10^6 \text{ cells})^{-1}$; all other activities are expressed as pkat $(10^6 \text{ cells})^{-1}$.

enzymes may have a role in the protection of nitrogenase from damage by superoxide and H_2O_2 produced from oxygen.

Extracts of *Gloeocapsa* sp. LB795 contained both glutathione [$0.24 \mu\text{g} (10^6 \text{ cells})^{-1}$] and ascorbate [$0.29 \mu\text{g} (10^6 \text{ cells})^{-1}$] so all the reactions shown in Fig. 2 could occur *in vivo*, though enzymes have only been identified for those reactions indicated. The removal of superoxide and H_2O_2 in this way might serve to protect nitrogenase under normal growth

conditions, but would be particularly important in cells exposed to conditions which stimulated superoxide and H_2O_2 formation. Such conditions would include, for example, high light intensities, atmospheres containing elevated concentrations of oxygen or the presence of methyl viologen. However, as shown in Fig. 2, the removal of superoxide and H_2O_2 depends on a supply of NADPH to regenerate GSH from GSSG. Normally, photosynthesis would provide this NADPH but this may not be so in the presence of methyl viologen, which would itself be reduced photosynthetically in preference to NADP^+ . However, as mentioned above, $10\ \mu\text{M}$ -methyl viologen almost certainly does not completely inhibit the photosynthetic production of NADPH. Therefore, under the conditions used to study the effect of methyl viologen on acetylene reduction, the scheme in Fig. 2 would probably operate to remove superoxide and H_2O_2 principally at the expense of NADPH supplied by photosynthesis, though other sources could also exist. Acetylene reduction was completely inhibited by $1\ \text{mM}$ -methyl viologen, even in young cells of *Gloeocapsa* sp. LB795. Presumably, under these conditions, NADPH production was insufficient to support the reactions removing superoxide and H_2O_2 .

Because the process of photosynthetic oxygen evolution and therefore electron transport is relatively less active in young cells of *Gloeocapsa* sp. (Gallon *et al.*, 1974), the amount of methyl viologen reduced by young cells would be less than that reduced by old cells. Furthermore, any superoxide or H_2O_2 produced in response to $10\ \mu\text{M}$ -methyl viologen would be more rapidly destroyed in young cells, because the appropriate enzymes are more active at this stage of growth. In young cells, therefore, there should be little net accumulation of superoxide and H_2O_2 when exposed to methyl viologen, so this inhibitor would have little effect on acetylene reduction. However, in older cells, where the reduction of methyl viologen would be greater and the removal of superoxide and H_2O_2 would be less efficient, there would be a marked inhibition of acetylene reduction. Guaiacol-dependent peroxidase activity, although high in older cells, apparently cannot remove H_2O_2 *in vivo*, possibly because there is little or no endogenous substrate for the enzyme in the algal cells.

When $0.1\ \text{mM}$ -methyl viologen was added to illuminated *Gloeocapsa* sp. the rate of oxygen consumption, relative to the previous rate of oxygen evolution, was constant regardless of the age of the algal cells. However, the amount of H_2O_2 produced per mol oxygen consumed in the presence of methyl viologen was less in 6 to 11 d-old cells, which contained relatively high activities of the enzymes associated with H_2O_2 breakdown, than in older cells, where these activities were lower (Fig. 5). This supports the idea that the reactions which remove H_2O_2 are saturated by the amount of H_2O_2 produced by old cells but can cope with the effects of methyl viologen, at least in part, in young cells.

Protection of Gloeocapsa sp. LB795 nitrogenase from inactivation by oxygen

One explanation for the inactivation of *Gloeocapsa* sp. LB795 nitrogenase by oxygen is that it is caused by H_2O_2 produced as a consequence of the reaction between oxygen and a reduced component of the photosynthetic electron transport chain. The results presented in this paper suggest that such an inhibition may occur *in vivo*, at least in old cells exposed to methyl viologen. On the other hand, acetylene reduction by *Gloeocapsa* sp. LB795 was inhibited by $0.5\ \text{atm}$ oxygen to a greater extent in the dark than in the light (A. F. Hamadi & J. R. Gallon, unpublished observation), in contrast to the inhibition caused by methyl viologen (Table 1). There may, therefore, be a separate mechanism for each of these inhibitions. For example, $0.5\ \text{atm}$ oxygen may stimulate respiration which, in the dark, could significantly decrease the amount of reductant available for nitrogen fixation. However, because GSH and ascorbate may react with molecular oxygen as well as with superoxide and H_2O_2 , the protective mechanisms described above could be equally applicable to such an oxygen inhibition not involving H_2O_2 . *Gloeocapsa* sp. contains both ascorbate and glutathione and the activities of the enzymes associated with the reactions described in

Fig. 5. Molar ratio of H_2O_2 production to oxygen uptake in cells of *Gloeocapsa* sp. LB795 of various ages exposed to methyl viologen. Cells were concentrated and incubated in the oxygen electrode as described in Fig. 3. After measuring the rate of oxygen evolution under illumination at 2500 lx, 0.1 mM-methyl viologen was added to the algal cells and about 2 min after the establishment of a constant rate of oxygen consumption, catalase ($1.25 \mu\text{kat ml}^{-1}$) was added. (For a typical trace, see Fig. 3*b*.) The resulting immediate evolution of oxygen was used to calculate H_2O_2 production by reference to the evolution of oxygen caused by standard amounts of H_2O_2 (see Methods for details). H_2O_2 production was then compared with the amount of oxygen consumed during the period between the additions of methyl viologen and catalase. For this, it was assumed that, in the absence of methyl viologen, photosynthetic oxygen evolution would have continued at a constant rate. Each value is the mean of at least three determinations.

Fig. 2 largely parallel the activity of nitrogenase in cultures of different age. These would provide one mechanism by which nitrogenase could be protected from inactivation by oxygen.

However, other protective systems may exist alongside the above scheme. The nitrogenase of *Gloeocapsa* sp. may be protected from oxygen by the thick slime capsule limiting the diffusion of atmospheric oxygen into these cells. However, since the photosynthetic production of oxygen by intact cells can readily be measured, this slime capsule does not appear in practice to be a great barrier to oxygen. In *Gloeocapsa* sp., therefore, as in *Klebsiella pneumoniae* (Wilcockson, 1977), the production of slime appears to have little effect in protecting nitrogenase from damage by oxygen.

Another possibility is that the nitrogenase of *Gloeocapsa* sp. might be confined to specialized cells analogous to heterocysts but which do not appear different from ordinary vegetative cells on microscopic examination. The patterns of $^{15}\text{N}[\text{N}_2]$ and $^{15}\text{NH}_4^+$ incorporation by *Gloeocapsa* (Meeks *et al.*, 1978) support this suggestion, as does the observation that a heterocyst-type glycolipid can be found in extracts of the alga (Lorch & Wolk, 1974). However, since this glycolipid is not always apparent (Gallon *et al.*, 1978) and is more obvious when the alga is subjected to inhibitors such as fluoroacetate than when it is fixing nitrogen maximally, it is difficult to demonstrate a direct relationship between this glycolipid and nitrogenase.

Alternatively, *Gloeocapsa* sp. nitrogenase may be located in a subcellular region to which oxygen cannot readily penetrate. The nitrogenase activity of crude preparations of *Gloeocapsa* sp. is associated with subcellular particles (Gallon *et al.*, 1972) possibly fragments of the thylakoid membranes of the alga. If nitrogenase were deeply embedded in the lipids of these membranes, oxygen would perhaps not be able to inactivate the enzyme. This seems not the case, however, since particulate preparations of *Gloeocapsa* sp. nitrogenase were inactivated by exposure to oxygen (Gallon *et al.*, 1972). Furthermore, since the thylakoid membranes are also the site of photosynthetic oxygen evolution in blue-green

algae, such a location for *Gloeocapsa* sp. nitrogenase may increase the need for alternative protective mechanisms.

Another possible protective mechanism is that the respiration of the bacteria which contaminate the slime capsule of *Gloeocapsa* sp. LB795 may maintain a low oxygen concentration within the algal cells. Again, this does not appear very likely, since the bacteria do not seem to make a major metabolic contribution in extracts of *Gloeocapsa* sp. LB795. For example, although the bacteria possess catalase activity and also presumably possess 2-oxoglutarate dehydrogenase (EC 1.2.4.2), since they grow well on medium containing glutamate as sole carbon and nitrogen source, neither of these enzymes could be detected in extracts of *Gloeocapsa* sp. LB795. On the other hand, *Gloeocapsa* sp. 1430/3, which lacks these contaminating bacteria, grows more slowly in nitrogen-free medium, and shows lower nitrogenase activity than *Gloeocapsa* sp. LB795. The possibility exists, therefore, that the bacteria contribute something to algal nitrogen fixation, though whether it is related to the protection of nitrogenase from inactivation by oxygen is not clear.

Recently, Bothe *et al.* (1977) have suggested that, in *Anabaena cylindrica*, hydrogenase-catalysed uptake of hydrogen may protect nitrogenase from damage by oxygen. They demonstrated that electrons removed from hydrogen passed down an electron transport chain with oxygen as terminal electron acceptor thereby causing an oxygen uptake. Could a similar mechanism function in *Gloeocapsa*?

Hydrogen (0.2 atm) caused a 35 % stimulation of acetylene reduction by *Gloeocapsa* sp. LB795 under aerobic conditions. This stimulation was also observed under nitrogen but not under Ar/O₂ (4:1, by vol.) so it is apparently not related to oxygen uptake in the presence of hydrogen. The relationships between hydrogen and nitrogen fixation are complex and this makes it difficult to interpret results using intact organisms. However, the effect of hydrogen at 0.2 atm on acetylene reduction at various concentrations of acetylene suggested that, in *Gloeocapsa* sp., hydrogen may exert its effect by removing some competitive inhibitor of acetylene reduction. This inhibitor may be nitrogen. In the absence of added hydrogen, nitrogen inhibits acetylene reduction by *Gloeocapsa* sp. This inhibition is competitive, with an apparent K_1 (N₂) of 0.38 atm $\pm$ 0.06 (S.E.M.). With acetylene at 0.01 atm and oxygen at 0.2 atm, the presence of 0.8 atm nitrogen caused a 50 % inhibition of acetylene reduction. The K_1 value reported here may not be very meaningful since it was obtained from studies with an intact organism rather than with enzyme preparations, but it is nevertheless in agreement with the K_1 (N₂) of 0.4 atm for acetylene reduction by the nitrogenase of *Azotobacter vinelandii* (Rivera-Ortiz & Burris, 1975). After correction for the inhibition caused by nitrogen, the K_m (acetylene) for acetylene reduction by *Gloeocapsa* sp. LB795 in air was 0.016 atm $\pm$ 0.003 (S.E.M.) which also compares well with that of 0.012 atm for *Azotobacter vinelandii* nitrogenase (Rivera-Ortiz & Burris, 1975).

In *Gloeocapsa* sp., therefore, kinetic studies and the lack of stimulation of acetylene reduction under Ar/O₂ (4:1, by vol.) suggest that hydrogen might act by removing the inhibition of acetylene reduction caused by nitrogen. Thus, because added hydrogen inhibits nitrogen reduction but not acetylene reduction by nitrogenase, there would no longer be any competition between these substrates. Consequently, under atmospheres containing nitrogen, acetylene reduction might be stimulated in the presence of hydrogen. However, the stimulatory effect of hydrogen on acetylene reduction by *Gloeocapsa* sp. under aerobic conditions is greater than expected if it were simply the result of the relief of the inhibition caused by nitrogen, unless the K_1 (H₂) for nitrogen reduction by *Gloeocapsa* sp. nitrogenase is considerably lower than that reported for other organisms. An alternative explanation for the effect of hydrogen on acetylene reduction remains possible, therefore.

Hydrogen did not reverse the inhibition of acetylene reduction caused by methyl viologen, though this may not be significant because methyl viologen may interfere with any electron transport chain between hydrogen and oxygen. Indeed, it may be a flow of electrons from hydrogen to methyl viologen which reduces this inhibitor in the dark. Peterson & Burris

(1978) demonstrated a methyl viologen-linked uptake of hydrogen in isolated heterocysts of *Anabaena* 7120, though this was small compared with that supported by oxygen.

When *Gloeocapsa* sp. LB795 was illuminated at 16000 lx, acetylene reduction decreased to 64 % of its rate at 2500 lx. Hydrogen (0.2 atm) reversed this fall in activity by about 10 %, after correction for the stimulation reported above. Furthermore, hydrogen did not reverse the inhibition of acetylene reduction when the alga was exposed to 0.3 or 0.4 atm oxygen. There is no direct evidence, therefore, of a role for hydrogen in the protection of *Gloeocapsa* sp. nitrogenase from inactivation by oxygen, though such a role cannot definitely be discounted.

The addition of catalase also reversed the inhibition of acetylene reduction at 16000 lx by only 10 % and had no effect on the inhibition observed when *Gloeocapsa* sp. was exposed to atmospheres containing an elevated concentration of oxygen. By the same argument, therefore, there is no direct evidence that H_2O_2 is involved in those inhibitions, though it is involved in the inhibition caused by methyl viologen.

In conclusion, no mechanism has been demonstrated which, on its own, protects the nitrogenase of *Gloeocapsa* sp. from inactivation by oxygen, though a combination of two or more of the above mechanisms could achieve this end. Alternatively there may be no protection of nitrogenase from inactivation by oxygen in *Gloeocapsa* sp. Constant nitrogenase activity may be maintained in cells of the alga only by a constant synthesis of new enzyme to replace that inactivated by oxygen. This is supported by the observation that chloramphenicol (0.1 mg ml⁻¹), an inhibitor of protein synthesis, caused a rapid decrease in nitrogenase activity in cells of *Gloeocapsa* sp. under aerobic conditions, but caused a much smaller decrease under nitrogen (Tözüm *et al.*, 1977). On the other hand, this observation could be equally well explained if protein synthesis was necessary to maintain the integrity of some oxygen-protecting mechanism rather than for the synthesis of nitrogenase itself.

The authors gratefully acknowledge the skilled technical assistance of Miss J. H. Aston.

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