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PHOTOOXIDATIVE REACTIONS OF *c*-PHYCOCYANIN

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SUMMARY

1. Aging cultures of blue-green algae, as well as cultures kept under certain adverse physiological conditions, often show bleaching of photosynthetic pigments.

2. Crude extracts containing *c*-phycocyanin of the blue-green algae, *Plectonema boryanum* or *Anacystis nidulans*, bleach when exposed to short wavelengths of visible light. Purified preparations of *c*-phycocyanin are not light sensitive, but can be sensitized by addition of catalytic amounts of FMN. This reaction proceeds only in the presence of oxygen. FMN and light can be replaced by H_2O_2.

3. When phycocyaninless extracts of the algae are exposed to light, peroxides are rapidly built up. There is an inverse relation between concentration of peroxides and *c*-phycocyanin in algal cultures grown at various light intensities.

4. A protective function of *c*-phycocyanin in certain blue-green algae against photooxidative death is suggested.

INTRODUCTION

The blue-green algae are unique among all photosynthetic organisms in possessing a chlorophyll-based, oxygen-evolving photosynthetic apparatus built into a procaryotic cellular organization. Under certain ecological conditions, as yet not well understood, blue-green algae form water blooms[1]. The massive die-off of these blooms in a closed ecosystem, such as a lake, reservoir or fishpond, often results in severe oxygen depletion, leading to fish kills. We have shown with axenic cultures in the laboratory, as well as under certain field conditions, that such massive die-off of blue-green algae may be due to photooxidation[2]. This work revealed that algal suspensions of blue-green algae (*e.g. Anacystis nidulans* and *Synechococcus cedrorum*) undergo photooxidative death when kept in buffer, water or culture media depleted of CO_2, in the light under an atmosphere of pure oxygen. In the culture medium, under these conditions, photooxidative death is accompanied by total and rapid bleaching of *c*-phycocyanin and partial bleaching of chlorophyll *a*. Similar bleaching of *c*-phycocyanin has been observed in *A. nidulans* following CO_2 (ref. 3) or nitrogen starvation[4], and in *Gleocapsa* (strains 6501, 6909) after exposure to low light intensities[5].

To understand the bleaching phenomenon of blue-green algal cells, we studied bleaching of *c*-phycocyanin in preparations and in cells at different physiological states. The relationship of this phenomenon to photooxidative death is discussed.

MATERIALS AND METHODS

Growth of algae

Plectonema boryanum 594 (Gomot) (University of Indiana Culture Collection, Bloomington, Ind.) and *A. nidulans* 6311 (Department of Bacteriology and Immunology, University of California, Berkely, Calif.) were used. *P. boryanum* was grown in the modified Chu No. 10 medium[6] in an atmosphere of air at 26 °C in 250-ml flasks containing 100 ml medium. Illumination was provided by 40 W white fluorescent light (incident light intensity of $3 \cdot 10^3$ ergs/cm^2 per s at the flask). *A. nidulans* was grown in Zehnder and Gorham medium No 11 (ref. 7) with $NaNO_3$ concentration modified to 1.5 g/l in an atmosphere of air at 35 °C, in 250-ml flasks containing 100 ml medium under white fluorescent light (incident light intensity of $1 \cdot 10^4$ ergs/cm^2 per s at the flask).

Preparation of crude extract

Plectonema boryanum culture (500–1000 ml) containing 50–100 mg protein/l (6–12 days of growth) was filtered through a membrane filter of 0.8 μm mean pore size (Millipore Filter, AA type); the cell paste was washed with 100 ml 0.1 M potassium phosphate buffer (pH 7.0), recentrifuged and then suspended in 10 ml buffer. *Anacystis nidulans* cultures (500–1000 ml) containing 50–100 mg protein/l (4–8 days of growth) were centrifuged (20 min, 25000 $\times$ *g*), and the cell pellet was washed with buffer, recentrifuged and suspended in 10 ml buffer.

The algal suspension (10 ml) was mixed with 26 g glass beads (diameter 0.1–0.11 mm) in a 50-ml Braun shaking flask and agitated at 4000 cycles/min for 30 s in a homogenizer (Model MSK, B. Braun Apparatbau Melsungen, W. Germany) at 4 °C.

The mixture was centrifuged (10 min at 12000 $\times$ *g*), and the clarified supernatant was collected and stored at —30 °C. This preparation was designated as the crude extract.

Preparation of purified c-phycocyanin

The crude extract was centrifuged at 50000 rev./min (Spinco No. 50 rotor) for 16 h at 4 °C. The clear phycocyaninless supernatant was used for the determination of peroxides. The blue pellet was resuspended in buffer, dialyzed for 24 h against buffer at 4 °C and fractionated with ammonium sulfate. The *c*-phycocyanin, which precipitated with 20–35% saturation, was collected, resuspended in 3 ml buffer, redialyzed for 24 h and passed through a Sephadex G-200 column (2.5 cm $\times$ 35 cm) with buffer. The eluted *c*-phycocyanin was collected, refractionated with ammonium sulfate and dialyzed and stored at —30 °C. The ratio of phycocyanin to total protein content of the preparation (measured by absorbance at 620 nm and 280 nm, respectively) varied from 2.0 to 6.5, depending on the age of the culture from which the pigment was obtained. Only pure *c*-phycocyanin preparations having ratios of 6–6.5 were used in irradiation experiments.

Irradiation experiments

Irradiation experiments of crude extracts or purified *c*-phycocyanin were performed, unless mentioned otherwise, in quartz cuvettes at room temperature, under an atmosphere of air using a high pressure mercury vapour lamp (Phillips

SP 500 W) with a 365-nm interference filter (RD/UV-1, Baird-Atomic, Cambridge, Mass., U.S.A.). The light energy was $2.5 \cdot 10^5$ ergs/cm^2 per s at the front surface of the cuvette, as measured with a Yellow Spring Kettering radiometer (Model 65 with a 6551 probe, Yellow Springs, Ohio, U.S.A.). Other interference filters used were: 313 nm (Baird-Atomic, No. 10-69-4), 437 nm (No. 50002) and 585 nm (No. 6565132) (Jena Glaswerk, Schott and Gen. Mainz, W. Germany). Irradiation experiments using sunlight were performed at an intensity of $6 \cdot 10^5$ ergs/cm^2 per s.

Preparation of urea-treated c-phycocyanin

Urea (2.5 M final concn) was added to a solution of purified *c*-phycocyanin in buffer (absorbance of 1–2 units at 620 nm). The solution was kept in the dark for 12 h at room temperature prior to the experiment.

Bleaching of c-phycocyanin by H_2O_2

H_2O_2 ($3 \cdot 10^{-3}$ M final concn) was added to a solution of purified *c*-phycocyanin in buffer (absorbance of 1–2 units at 620 nm) with or without peroxidase (3 units/ml; Worthington, Freehold, N. J., U.S.A.) at room temperature in the dark.

Analytical methods

Peroxides and peroxidase were determined in the crude extracts and in the phycocyaninless supernatant as described by Worthington[8]. Protein was determined by the method of Lowry *et al.*[9] and chlorophyll *a* was determined by the method of Parsons and Strickland[10]. Spectrophotometric measurements were carried out in a Perkin–Elmer 137 UV spectrophotometer (Perkin–Elmer Corp., Norwalk, Conn., U.S.A.).

RESULTS

Crude extracts containing *c*-phycocyanin, prepared from *P. boryanum* or *A. nidulans*, showed a main absorption peak at 620–625 nm and a broad shoulder at 350–450 nm. When the crude extract of *P. boryanum* was exposed to sunlight, the 620–625 nm peak disappeared and absorbance generally decreased in the visible region (Fig. 1).

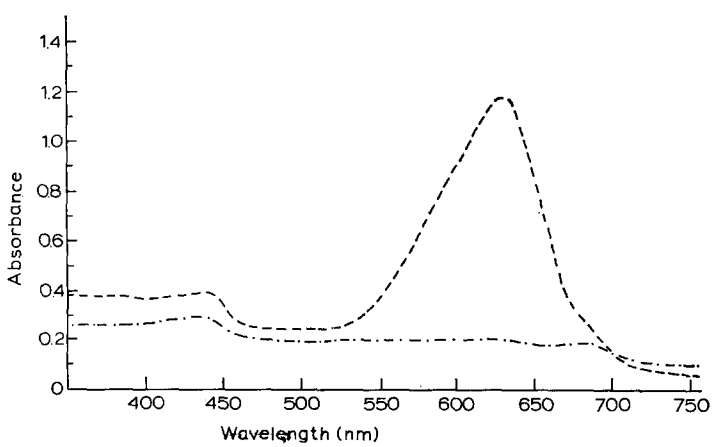

Fig. 1. Absorption spectra of crude extract of *P. boryanum* before (– – – –) and after (–·—·–) illumination by direct sunlight ($6 \cdot 10^5$ ergs/cm^2 per s) at room temperature for 1 h.

The efficiency of bleaching depends on the wavelength of radiation; the shorter the wavelength of radiation, the greater the amount of bleaching of *c*-phycocyanin in the range tested (Table I).

c-phycocyanin in the crude extract is not bleached when kept in the dark. Only 20% of the absorbance is lost within 4 h when the crude extract is exposed to sunlight at low temperature (Fig. 2). Purified *c*-phycocyanin is not light sensitive, and is not bleached when irradiated with 365-nm light or with sunlight at room

TABLE I

RELATION OF BLEACHING *c*-PHYCOCYANIN TO WAVELENGTH OF IRRADIATION

Crude *P. boryanum* extract was irradiated using different filters as described in Materials and Methods. Total light energy falling on the cuvette amounted to $3.7 \cdot 10^8$ ergs/cm^2 per s at all the wavelengths. The absorbance of the pigment before and after irradiation was measured at 620 nm; initial absorbance of the extract was 1.5 units.

Wavelength of irradiation (nm)	*Percent of bleaching*
313	87
365	82
435	8
582	0

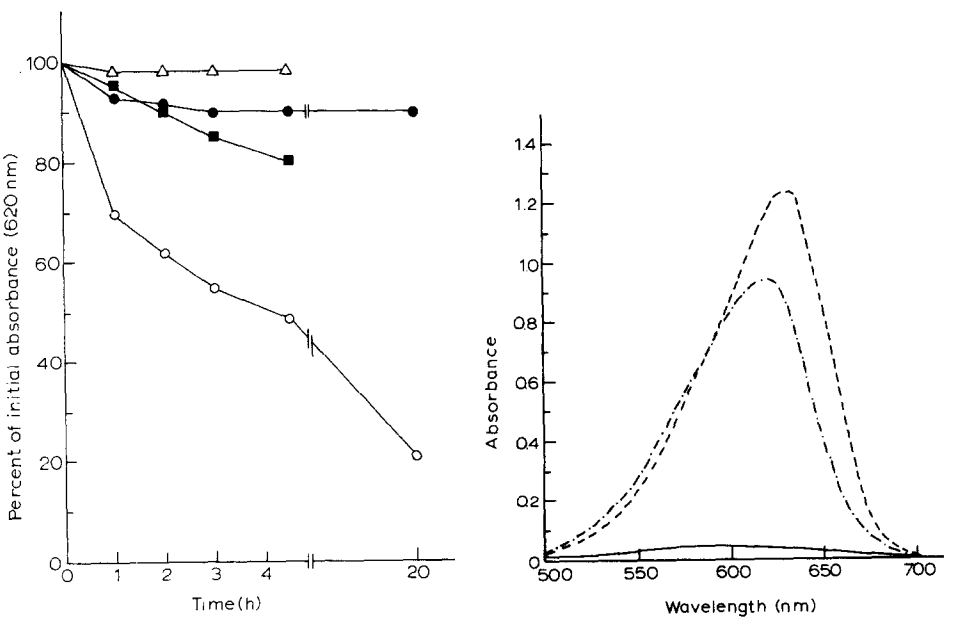

Fig. 2. Effect of temperature and light on photobleaching of *c*-phycocyanin (initial absorbance at 625 nm was 1.5 units) in crude extract. Sunlight ($6 \cdot 10^5$ ergs/cm^2 per s) at room temperature (○—○); sunlight at 5 °C (■—■); dark at room temperature (●—●); dark at 5 °C (△—△).

Fig. 3. Effect of FMN on absorbance of purified *c*-phycocyanin preparation. Concentration of FMN, $5 \cdot 10^{-4}$ M; irradiation, 365 nm. Absorbance of preparation without FMN before and after irradiation (– – –); with FMN after 1 h in the dark (–·—·–); and after 1 h irradiation (———).

temperature. Dialyzed crude extract also is not bleached under these conditions, but the bleaching effect can be restored by adding the dialyzate to the dialyzed extract. The dialyzable factor necessary for the bleaching is thermostable (after 10-min boiling).

The dialyzable factor responsible for the light-dependent bleaching can be replaced by adding catalytic amounts of FMN ($5 \cdot 10^{-4}$ M) to *c*-phycocyanin solution before irradiation (Fig. 3). Bleaching of the pure extract in the presence of FMN is oxygen dependent (Fig. 4). No more than 25 % of the initial absorbance at 620 nm is lost by irradiation at 365 nm under an atmosphere of nitrogen. Under a nitrogen atmosphere, the FMN remains reduced and the *c*-phycocyanin is only partially bleached. Under atmospheres of air (Fig. 3) or pure oxygen (Fig. 4) 75 %–100 % of the *c*-phycocyanin was bleached, indicating that $FMNH_2$ is readily oxidized and can continuously react with the *c*-phycocyanin, or that oxidation of FMN produces peroxide which could in turn react with the pigment.

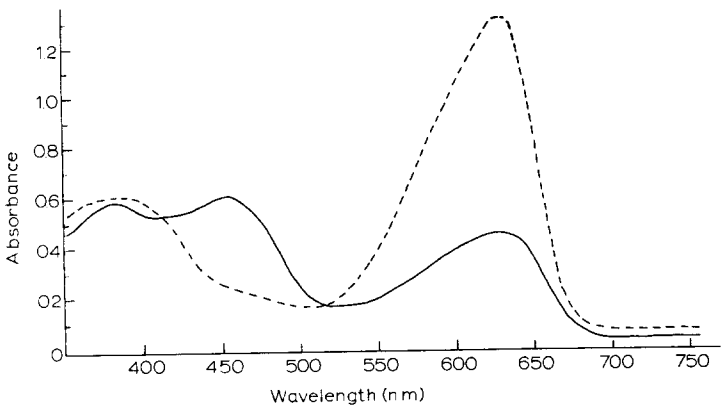

Fig. 4. Photobleaching with 365 nm light at intensity of $2.5 \cdot 10^5$ ergs/cm^2 per s at surface of tube of purified *c*-phycocyanin preparation in the presence of FMN under an atmosphere of nitrogen (– – –) or oxygen (———). Initial absorbance at 625 nm of *c*-phycocyanin preparation, 1.7 units; illumination time, 30 min.

Bleaching of the crude extract and the FMN-dependent bleaching of *c*-phycocyanin can both be inhibited by adding glutathione (10^{-2} M) to the reaction mixture prior to irradiation. However, glutathione does not restore the initial absorbance of the *c*-phycocyanin after its photosensitized oxidation with FMN.

Purified *c*-phycocyanin can be sensitized to light by treatment with urea (Fig. 5). Urea-treated *c*-phycocyanin is totally bleached within 90 min when irradiated without addition of FMN. In the dark the urea-treated *c*-phycocyanin retains most of its absorbance.

The bleaching effect of light and FMN on pure *c*-phycocyanin can be replaced by adding H_2O_2 to *c*-phycocyanin in the dark (Fig. 6). This effect is enhanced by addition of peroxidase.

Crude extracts prepared from *P. boryanum* or *A. nidulans* show no peroxidase activity. The phycocyaninless supernatant obtained from the centrifugation of the crude extract rapidly forms stable peroxides when irradiated (Fig. 7). However, traces of *c*-phycocyanin in the supernatant prevent the accumulation of peroxides

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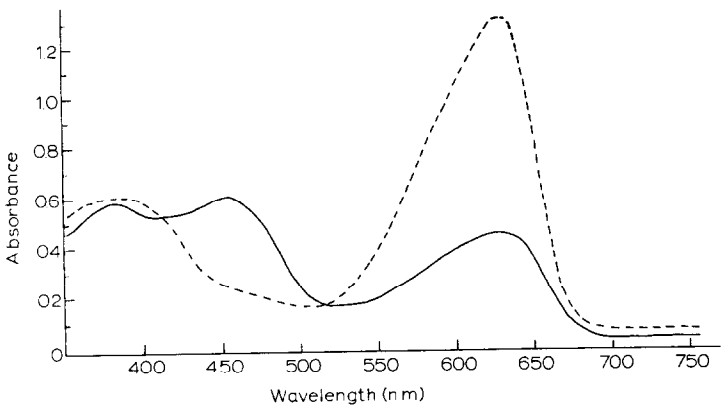

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since the peroxides formed react with *c*-phycocyanin. Evidence of this reaction is the bleaching in the dark of pure *c*-phycocyanin by the phycocyaninless supernatant immediately after the latter is irradiated. In a typical experiment, 10 ml phycocyaninless supernatant prepared from a culture of *P. boryanum* containing a total of 60 mg protein and irradiated for 30 min, bleached a *c*-phycocyanin solution (initial absorbance at 620 nm of 1.5 units) by 30 % in the dark.

Crude extracts obtained from *P. boryanum* cultures grown at different light intensities for 20 days were tested to determine whether there is any relation between the *c*-phycocyanin concentration and peroxide production in the living cell. Fig. 8 shows that the concentration of peroxides is inversely related to the presence of *c*-phycocyanin in the cells. At high light intensities, high concentrations of peroxides accumulate and *c*-phycocyanin is bleached. The peroxides found in the cells are produced only in the light, since a 2-h dark incubation of the cells grown in intermediate illumination ($2.6 \cdot 10^3$ ergs/cm^2 per s) reduced the intracellular peroxide content by 70%.

The production of *c*-phycocyanin and chlorophyll *a* in relation to protein production in growing cultures of *P. boryanum* is shown in Fig. 9. During the early growth phase (0–4 days) total protein, *c*-phycocyanin and chlorophyll *a* content all increase at the same rate. Later (4–8 days), the ratio of *c*-phycocyanin to total protein

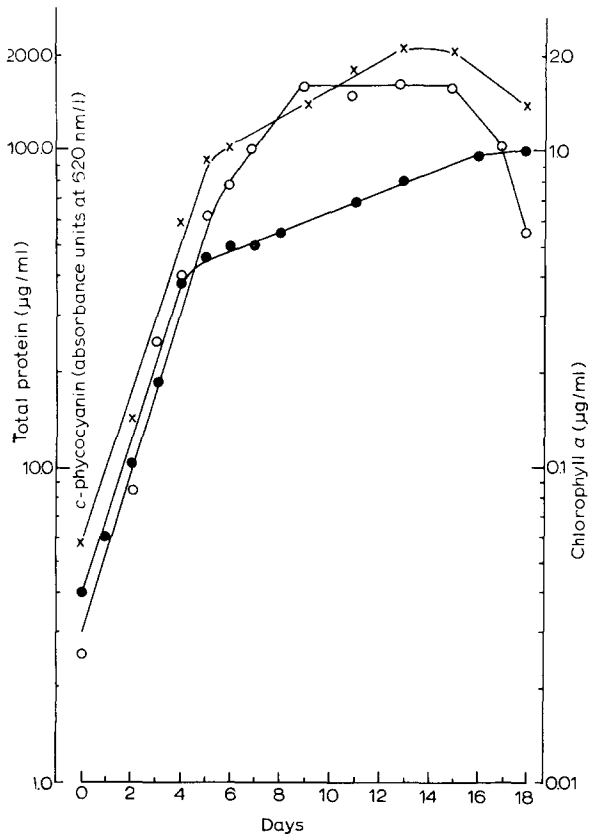

Fig. 9. Growth curve of *P. boryanum* culture. Total protein (●—●); *c*-phycocyanin absorbance units (○—○); chlorophyll *a* (×—×). Culture conditions described in Materials and Methods.

increases, while towards the stationary growth phase (after 15 days) *c*-phycocyanin content rapidly decreases. These results are similar to those obtained by us with *A. nidulans* (except that the doubling time for *A. nidulans* was 15 h at 35 °C) as compared to a doubling time of 26 h for *P. boryanum* at 26 °C.

DISCUSSION

Death of blue-green algae, such as *A. nidulans*, occurs when cells are incubated at physiological temperature in the light under a pure oxygen atmoshpere in the absence of CO_2 in culture medium[2]; this photooxidative death is accompanied by the bleaching of the intracellular *c*-phycocyanin. Depletion of CO_2 inhibits Photosystem II in the intact algae[5], probably leaving the photosynthetic electron-carrier chain in a reduced state. This may lead to the direct reduction of oxygen to peroxides, as shown by Mehler[11]. In this connection, it is important to note that although *P. boryanum*, investigated here, lacks both peroxidase and catalase, it does not die under the above photooxidative conditions (unpublished data) and remains viable in spite of the build-up of intracellular peroxides when illuminated.

The bleaching of *c*-phycocyanin preparations from *P. boryanum* and *A. nidulans* is light-dependent and occurs in the presence of an oxidizing agent, such as FMN. The oxidative nature of this bleaching of *c*-phycocyanin is supported by the fact that light and FMN can be replaced by H_2O_2 in the dark. This effect of H_2O_2 in the dark is not sufficient proof that peroxide is the sole bleaching agent operating in the light and other mechanisms, such as oxygen singlets in photobleaching of pigments as described by Foote and Denny[26], cannot be ruled out. The utilization of *c*-phycocyanin as hydrogen donor for peroxide reduction may explain the enhancement of *c*-phycocyanin bleaching in the presence of peroxidase (*cf.* Fig. 6). The light-dependent accumulation of peroxides (*cf.* Fig. 7) and their bleaching effect on *c*-phycocyanin could explain the inverse relation observed between intracellular concentrations of *c*-phycocyanin and peroxides (*cf.* Fig. 8). Although peroxides are continually being produced in the light, as shown here and by other investigators[12,13] bleaching of intracellular *c*-phycocyanin is observed only at high light intensities (in growth medium) or in aging cultures. Thus, the bleaching may be related to some disturbance in regular *c*-phycocyanin synthesis, caused by CO_2 depletion or nitrogen starvation which affect the normal rate of CO_2 fixation. Under balanced growth conditions, *c*-phycocyanin is probably being continuously produced (*cf.* Fig. 9) and compensates for the bleaching effect of peroxides produced in the cells.

Pretreatment of *c*-phycocyanin with urea before irradiation eliminates the requirement for an external oxidizing agent for photobleaching. This suggests that the unfolding of the *c*-phycocyanin molecule by urea may expose an oxidizing site(s) in the molecule capable of bleaching the chromophore upon illumination. A similar situation could explain the requirement of catalytic amounts of FMN as an external oxidizing agent for bleaching the intact *c*-phycocyanin molecule. In such a case FMN might serve as a mediator between the reducing and oxidizing sites of the molecule.

c-phycocyanin is ubiquitous in the blue-green algae; it may reach concentrations as high as 24 % of total dry weight[14]. However, there is no general agreement regarding its function in the cell. For example, Thomas and DeRover[15] claimed that *c*-phycocyanin improves the efficiency of the Hill reaction, but only at low light intensities; on the other hand, other workers[16–18] maintain that *c*-phycocyanin is

not necessary for the *in vitro* Hill reaction. The only generally accepted measurable function of *c*-phycocyanin is light-energy harvesting and transfer of the captured energy to the active photosynthetic site[19–22]. However, this function can also be dispensed with since a phycocyaninless mutant of the red alga *Cyanidium caldarium*[23] has the same photosynthetic efficiency as its wild type, when illuminated with white light.

The ubiquitous presence of *c*-phycocyanin and the general lack of peroxidase and catalase, except some low activity described in certain cases by Chua[24], distinguish the blue-green algae from other aerobic photosynthetic oxygen-evolving organisms. This may hint at the possibility that the pigment functions in some way to protect the cell from the peroxides evolved during photosynthesis. Petrack and Lipmann[25] have shown that photophosphorylation mediated by extracts of blue-green algae is enhanced by *c*-phycocyanin only under aerobic conditions. This may indicate a protective role of this pigment against oxidative damage in the photophosphorylation system.

The participation of *c*-phycocyanin in oxidative and photooxidative reactions described in this paper (reduction of FMN, reactions with peroxides) further supports the hypothesis that *c*-phycocyanin could act in protecting the cell against oxidizing agents produced during normal metabolism. Under extremely limiting conditions, such as CO_2 depletion, the protective mechanism breaks down, as indicated by photobleaching, and the blue-green algal cells (*e.g. A. nidulans* and *C. cedrorun*) succumb to photooxidation.

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