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Requirement of Low Oxidation-Reduction Potential for Photosynthesis in a Blue-Green Alga (*Phormidium* sp.)

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Abstract. Photosynthesis in a *Phormidium* species which forms dense conical-shaped structures in thermal springs is strongly inhibited by aeration but is stimulated by sulfide and other agents (cysteine, thioglycolate, sulfite) which lower the oxidation-reduction potential. The compact structures which this alga forms in nature may restrict oxygen penetration from the environment so that the anaerobic or microaerophilic conditions necessary for photosynthesis can develop. The alga may be defective in a regulatory mechanism that controls the reoxidation of reduced pyridine nucleotides formed during photosynthesis. It is suggested that other mat-forming and benthic blue-green algae may also prefer anaerobic conditions for growth and photosynthesis.

Key words: Photosynthesis — Blue-Green Alga — *Phormidium* sp. — Oxidation-Reduction Potential — Sulfide — Hot Spring — Stromatolite — Anaerobiosis.

Factors favoring the development of blue-green algae in nature are poorly understood, but anaerobiosis has been suggested as one possible favorable condition (Stewart and Pearson, 1970). Many planktonic blue-green algae develop in the depths of lakes where anaerobic conditions prevail and many benthic blue-green algae from compact mats in which oxygen penetration is restricted so that anaerobic conditions might develop. It is interesting that although dense, compact mats are frequently formed by blue-green algae and photosynthetic bacteria (Brock, 1969; Bauld and Brock, 1973), they are not generally formed by eucaryotic algae. In earlier work, the formation by *Phormidium* species of characteristic conical structures (nodes) resembling Precambrian stromatolites was described (Walter *et al.*, 1972, in press). In studies to determine the reasons why these conical structures develop, we compared photosynthesis in intact nodes with that in nodes dispersed by gentle homogenization. We found that homogenization strongly reduced photosynthesis, but this lost activity could be restored by addition of agents lowering the oxidation-reduction potential. Although bacterial photosynthesis can be stimulated by lowered oxidation-reduction potential, this has not been previously reported for blue-green algae. [Stewart and Pearson (1970) showed only that photosynthesis in two blue-green algae (*Anabaena flos-aquae* and *Phormidium* sp.) was inhibited by high partial pressures of oxygen.] We hypothesize that formation of compact conical structures by the algae restricts oxygen penetration and creates the microaerophilic conditions apparently preferred by this algae, thus permitting effective growth in an oxidizing environment.

Materials and Methods

Collection of Material. Most of the algal material for these experiments was collected at temperatures between 42–47° C from the eastern effluent of Tromp Spring, a small pool in the Midway Geyser Basin of Yellowstone National Park, but a few experiments were done with material collected from several other nearby springs. A large cork borer was used to remove cylindrical cores of material from areas covered with nodes approximately 1–4 mm in height and 0.5–1.5 mm in diameter. The cores were transported in water-filled vials to the dissection site. Micro-scissors were used to remove each node at its base. The dissected material was stored in spring water until homogenization or distribution to 5 ml screw cap vials. Homogenates were prepared by gently dispersing the sample in spring water with a plastic homogenizing tube and Teflon homogenizer. The amounts of sample and water were calculated to give the same amount of material per vial as when intact nodes were used.

Culture Methods. For isolation and growth of axenic cultures, medium D of Castenholz (1969) was used, with pH adjusted to 7.5. Growth under anaerobic conditions was achieved using the Gas Pak technique (Becton, Dickinson Co.). For liquid medium, 16 mm culture tubes were used containing 5 ml medium, and agar medium was prepared in Petri dishes. The tubes and plates were introduced into Gas Pak jars and anaerobic conditions created, then

the jars were incubated in a 37° C walk-in incubator in front of cool-white fluorescent lights so that intensities of about 200 foot-candles were achieved at the level of the cultures.

Initial isolations were done by placing small amounts of algal mats on the surface of agar plates and observing for migration of filaments. Filaments which had migrated furthest from the inoculum were picked with sterile insect pins and transferred to liquid medium. When good growth had occurred in liquid medium, agar plates were reinoculated and migrating filaments again picked to liquid. Eventually 15 axenic cultures were obtained, using material taken from a variety of springs. Growth could be obtained during aerobic incubations, but only from large inocula and the cultures were paler green and appeared less healthy. Use of the Gas Pak system resulted in much more reproducible culture and isolation of this *Phormidium* species.

For photosynthesis experiments on axenic cultures, tubes of liquid medium were inoculated and incubated as described above and after 4—5 days the clumps of algal material which had developed were removed and handled in a manner similar to the natural material (see below).

Anaerobic Incubations. For experiments involving stimulation by sulfide or other reducing agents, all manipulations of material were done anaerobically. Immediately after dissection, the material was placed in spring water and continuously bubbled with nitrogen gas to remove and exclude oxygen. Nitrogen was introduced through a long hypodermic needle inserted through the serum stopper below liquid level and vented through a shorter hydrodermic needle above liquid level. The nodes were then transferred to 5 ml serum bottles and gassing was continued for 10 min with nitrogen. Homogenization was accomplished by transferring dissected material to a plastic tube, bubbling with nitrogen for 10 min, and homogenizing under flowing nitrogen. Subsamples of anaerobic homogenates were introduced by injection through the stoppers of vials previously gassed for 10 min with nitrogen. Any reagents added were prepared in nitrogen-gassed water and injected through the serum stopper.

Aerobic controls were prepared from the anaerobic materials by exposing open vials to atmospheric oxygen for 10—15 min during which the vials were frequently shaken to insure thorough aeration.

Measurement of Photosynthesis. For field studies, following the dissection and preparation of vials (which took between 2 and 5 hrs for each experiment), reagents were added, the vials were filled to a known volume with spring water and then were placed in a hot spring at the temperature of collection. Each vial was oriented to take advantage of the full sunlight that was present for all experiments. After a 10 min preincubation, a measured volume of $NaH\,^{14}CO_3$ (New England Nuclear Corp.; specific activity 20 μCi/200 μg, 20 μCi/ml) was injected into each vial. Dark controls were wrapped with aluminum foil immediately after the addition of isotope. At the end of the desired incubation period, 0.5 ml of 38 % formalin was added to each vial to stop further isotope uptake. Some vials received 0.1 ml of 5×10^{-5} M DCMU [3- (3,4-dichlorophenyl)-1,1-dimethylurea] an inhibitor of algal but not bacterial photosynthesis, to give a final concentration of 1×10^{-6} M in the incubation vial.

For laboratory studies, similar procedures were used except that suspensions were prepared in culture medium which had previously been rendered anaerobic by bubbling with N_2 gas. A heated copper oxide scrubber was used to remove any residual O_2 from the prepurified N_2. All solutions were prepared in N_2-bubbled water and all manipulations were done under an atmosphere of N_2. After the anaerobic algal suspension had been prepared by homogenization, a subsample was placed in an Erlenmeyer flask and aerated using a Burrill wrist-action shaker for 60 min at room temperature, to provide the aerobic control. Incubations were carried out in front of fluorescent light bulbs in the 37° C walk-in incubator room. Sufficient time was allowed for temperature equilibration before the radioisotope was injected. No DCMU controls were necessary in these experiments.

Analysis of each set of samples was completed within 2 days of incubation. Intact materials were homogenized to give an even suspension of cells like that in the field-homogenized samples. Radioactivity was measured by filtering 1.0 ml of each suspension through a Millipore filter, rinsing 3 times with deionized water to remove unincorporated $NaH\,^{14}CO_3$, drying, and counting with a Nuclear Chicago Model gas flow counter. In some experiments, counts were performed with a Beckman β-mate II liquid scintillation counter and in the culture experiments a Packard Tri-Carb scintillation counter was used.

Chlorophyll *a* was determined for each vial by adding a known volume of acetone to the pellet remaining after centrifuging a known volume of suspension. Extraction was allowed to proceed in the cold for 16—24 hrs, during which the mixture was thoroughly agitated 3—4 times. Following a second centrifugation, the optical density at 663 nm was determined using a Beckman DB-G grating spectrophotometer. The concentration of chlorophyll *a* was then calculated by standard procedures (Brock and Brock, 1967). The radioactivity per vial and the chlorophyll *a* per vial were calculated and used to compute the photosynthetic activity expressed as cpm/μg chlorophyll *a*. Values for dark controls were subtracted, so that the final value represents light-stimulated uptake of $^{14}CO_2$.

Light Reduction Experiment. In the light reduction experiment, vials were placed in nylon mesh bags of various thicknesses, providing the following percentages of light transmission: 70 %, 44 %, 14 %, and 7 %. The construction, calibration, and adsorption spectra of these bags have been described by Brock and Brock (1969). Aluminum foil was used to achieve complete darkness. Light intensities in the field were measured with a Gossen Super Pilot light meter equipped with an incident light attachment.

Reducing Solutions. Solutions of various reducing agents were prepared to the appropriate concentrations and adjusted with NaOH or H_2SO_4 to pH 8.0. After pH adjustment, each solution was transferred to a serum bottle, tightly stoppered, and gassed with nitrogen for 10 min to remove oxygen. All reducing solutions were made fresh before the experiment.

Fresh sulfide solutions were prepared for each experiment by dissolving washed crystals of $Na_2S\cdot 9\,H_2O$ in anaerobic deionized water and continuously bubbling with nitrogen during pH adjustment. However, bubbling was stopped after acidification to pH 8 to avoid driving off H_2S, and the sulfide solutions were immediately transferred to tightly stoppered bottles. The actual sulfide concentration in each solution was measured by transferring a sample to zinc acetate solution and assaying for sulfide by the method of Pachmayr (1960) as described by Brock *et al.* (1971).

Sulfide Assay on Algal Mats. Conical structures for sulfide analysis were dissected at poolside and immediately placed in acidified zinc acetate to trap sulfide as ZnS. These samples were homogenized in the laboratory, after which one subsample was used to determine the dry weight of the sample for normalization of the sulfide content. The sulfide from another subsample was distilled under N_2 after acidifying with 25 ml of 1:1 HCl containing 80 g/l $Sn Cl_2$ (Bloomfield, 1969) and the H_2S was trapped in acidified zinc acetate. The sulfide in these distillates was then assayed.

Measurement of Radioactivity Released during Homogenization. ^{14}C labeled intact nodes were acidified and bubbled with air to drive off unincorporated $^{14}CO_2$, then neutralized with 1 N NaOH to pH 8.9. A 1.0 ml sample of the liquid over the nodes was taken for radioactive counting. The remainder was then thoroughly homogenized, and a 1.0 ml sample of the suspension was filtered to remove cells and cell fragments. The two 10 ml samples and the filter were each placed in scintillation vials along with 10 ml Aquasol (Nuclear-Chicago) and counted on a Beckman β-mate II liquid scintillation counter.

Results

The Alga

Typical conically shaped structures (nodes) of the kind formed by this alga are shown in Fig. 1. Although detailed taxonomic studies have not been done, preliminary study suggests that these structures are formed primarily by the species *Phormidium tenue* var. *granuliferum* (Walter *et al.*, in press). However, because of the questionable characterization of all species of *Phormidium*, we refer to this organism throughout the rest of this paper simply by the genus name.

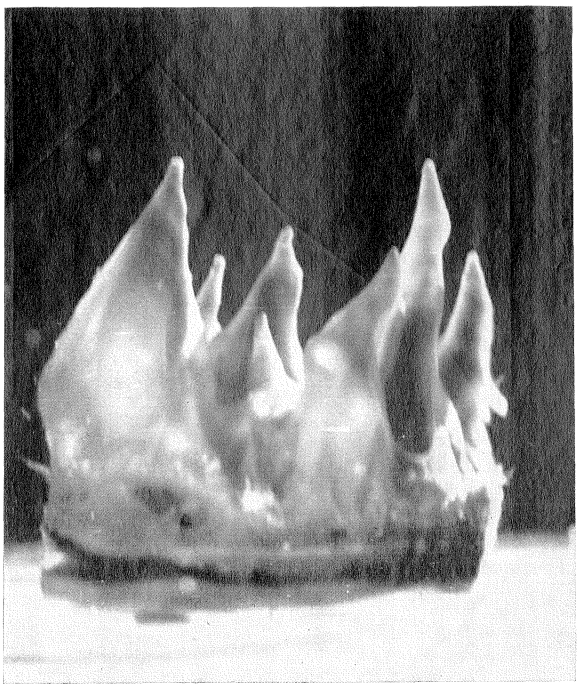

Fig. 1. Photomacrograph of conical structures of *Phormidium* about 1 cm high. Except for the larger size, these structures are similar to those used in the photosynthesis experiments

Photosynthesis in Intact and Dispersed Nodes

In initial studies to determine why this alga forms nodes during growth, experiments were performed comparing photosynthesis in intact and dispersed material. As seen in Fig. 2, photosynthesis (normalized to chlorophyll content) was completely inhibited in dispersed suspensions as compared to intact nodes. Although the dispersion process was gentle, and microscopic examination did not reveal detectable cell breakage, experiments were set up to see if dispersion might induce cell leakage. Only an insignificant amount of incorporated radioactivity was released upon dispersion of intact nodes, using the procedure described in "Materials and Methods". Also, supernatants of centrifuged dispersed material did not show detectable chlorophyll, which might have been released during cell breakage. We concluded that the dispersion process itself was not damaging the organism.

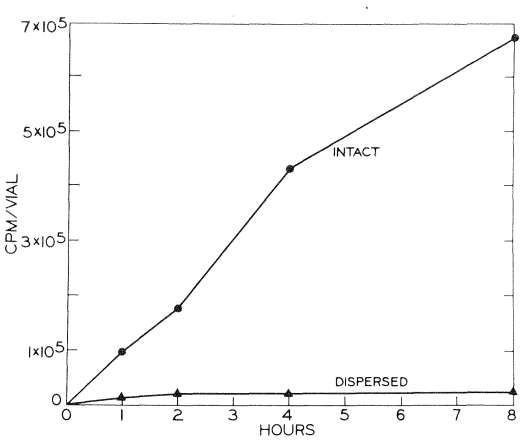

Fig. 2. Rate of light-dependent uptake of $^{14}CO_2$ by intact and dispersed nodes. Each vial contained about 1.8 μg/ml chlorophyll *a* and 6 μCi/ml NaH $^{14}CO_3$. Counted with a liquid scintillation counter. All incubations in air

Another consequence of dispersion was that algal filaments from the interior of the structure, grown at a low light intensity due to selfshading, would be exposed to bright light when evenly dispersed in the suspension, so that light inhibition of photosynthesis might occur. An experiment was therefore performed in which the rate of photosynthesis was measured

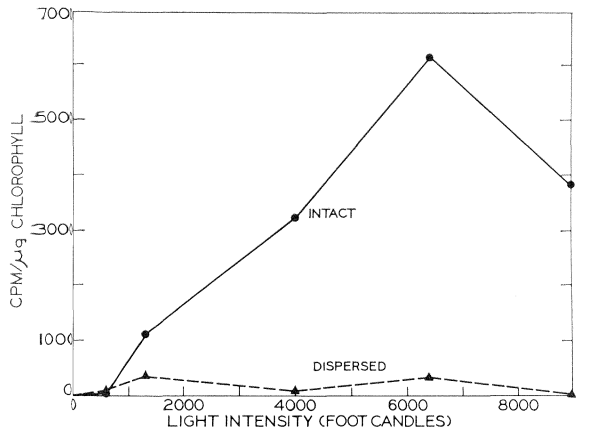

Fig. 3. Effect of light intensity on photosynthesis by intact and dispersed nodes. Each vial contained about 1.5 μg/ml chlorophyll *a* and 0.6 μCi/ml $NaH\ ^{14}CO_3$. Incubation time = 1 hr. Counted with gas-flow counter. Light intensity was reduced from full sunlight as described in "Materials and Methods". All incubations in air

at various light intensities. As seen in Fig. 3, intact material showed significantly higher photosynthesis at all light intensities than did dispersed material, suggesting that the main inhibitory effect of dispersion was not exposure to bright light.

Stimulation of Photosynthesis by Sulfide

Because of the compact nature of the *Phormidium* nodes, it seemed possible that oxygen diffusion might be inhibited, allowing partial anaerobic conditions to develop within the structures. Consequently, a series of experiments was performed to measure photosynthesis under conditions of lowered redox potential. Because the photosynthetic bacterium *Chloroflexus* was also present in these structures (Bauld, 1973; Walter *et al.*, in press), and because this organism shows a significant stimulation of photosynthesis by sulfide (Bauld and Brock, 1973; Castenholz, 1973) it was essential to measure differentially bacterial and algal photosynthesis. This was done by adding DCMU to some of the vials in each experiment to inhibit algal photosynthesis without inhibiting bacterial photosynthesis. Control experiments had shown that at a concentration of 1×10^{-6} M, DCMU inhibited greater than 90 % of algal photosynthesis, without detectably affecting bacterial photosynthesis. It was thus possible to calculate algal photosynthesis by subtracting the photosynthetic rate measured in the presence of DCMU from that measured in its absence.

Table 1. Effect of anaerobic conditions and sulfide on algal photosynthesis

Intact nodes	cpm/μg chlorophyll *a*
Air	220
N_2	473
$N_2 + S^=$	2449
Dispersed nodes	**cpm/μg chlorophyll *a***
Air	0 [a]
N_2	70
$N_2 + S^=$	585

Bacterial photosynthesis (DCMU resistant uptake) has been deducted. Sulfide concentration, 0.67 μg/ml (20 μM).

[a] Uptake of radioactivity in light was the same as uptake in the dark.

As seen in Table 1, algal photosynthesis was stimulated significantly by anaerobic conditions (N_2 atmosphere) and even more strikingly by sulfide. Although the intact nodes also showed stimulation by N_2 and sulfide, stimulation was considerably greater in the dispersed material. Addition of N_2 and sulfide restored photosynthetic ability of the dispersed material to levels higher than those of intact nodes in air, confirming that the dispersion process was not damaging the algal filaments. Thus, the principal effect of dispersion is probably to aerate the material and a probable secondary effect is to dilute the endogenous sulfide levels in the intact nodes, since sulfide is present in the natural nodes (see below).

To further investigate the sulfide stimulation of photosynthesis in this blue-green alga, an experiment with a series of sulfide concentrations was performed. As seen in Fig. 4, sulfide stimulation is concentration dependent, with an optimum at about 2.3 μg/ml sulfide (72 μM). A higher concentration was inhibitory, but significant stimulation occurred even at quite low concentrations (around 0.1 μg/ml). Although not shown in the figure, bacterial photosynthesis was also stimulated by sulfide, with an optimum around 1 μg/ml, but inhibition did not occur at the high sulfide concentration.

Effect of Lowered Oxidation-Reduction Potential on Photosynthesis

In order to determine if the stimulation of algal photosynthesis by sulfide was due to the ability of this agent to lower the redox potential, several other substances which lower redox potential were also tested.

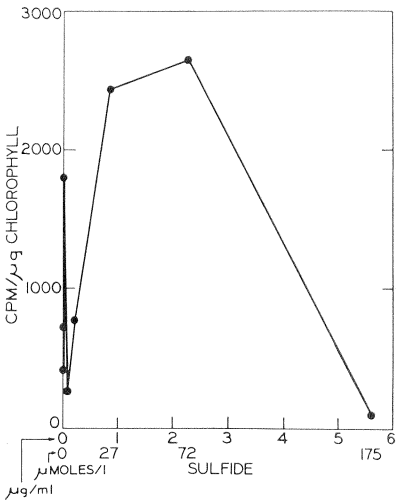

Fig. 4. Stimulation of algal photosynthesis by sulfide. All vials contained dispersed material. Each vial contained about 1.6 μg/ml chlorophyll *a* and 0.4 μCi/ml NaH $^{14}CO_3$. Incubation time = 1.5 hrs. All preparations and incubations were done in a nitrogen atmosphere. Sulfide concentrations are those actually measured by chemical assay at the time sulfide additions were made. Bacterial photosynthesis (DCMU-resistant uptake) has been deducted. In the same experiment, uptake of ^{14}C by the alga in air instead of N_2 was lower (158 cpm/μg chlorophyll *a*)

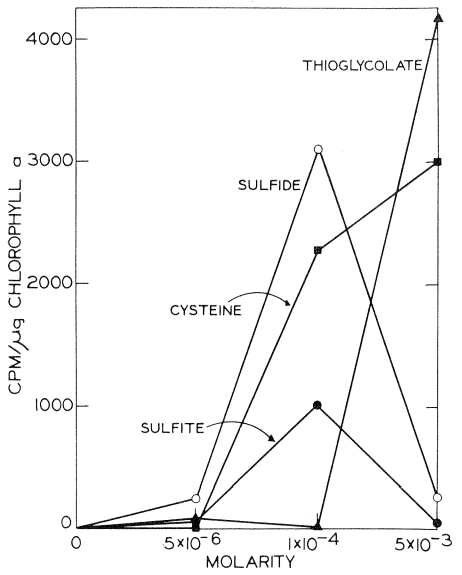

Fig. 5. Stimulation of algal photosynthesis by reducing agents. All vials contained dispersed material. Each vial contained about 1 μg/ml chlorophyll *a* and 0.4 μCi/ml NaH $^{14}CO_3$. Incubation time = 2 hrs. All preparations and incubations were done in a nitrogen atmosphere. Bacterial photosynthesis has been deducted. In the same experiment, uptake of ^{14}C by the alga in air instead of N_2 was 18 cpm/μg chlorophyll *a*

As seen in Fig. 5, stimulation was observed with all of these agents, although the concentration which gave best stimulation varied among the different agents. Both sulfide and sulfite were toxic at the highest concentration, whereas the two organic reducing agents, thioglycolate and cysteine, were non-toxic but less effective since significant stimulation required considerably higher concentrations. Two other reducing agents, ascorbic acid and thiosulfate, did not show significant stimulation at any of the concentrations tested. Bacterial photosynthesis was not stimulated significantly by any of the agents tested except sulfide (data not shown).

Redox potential measurements with an Orion redox electrode of the agents used above showed that all of the agents had potentials which were either negative or near zero at concentrations which stimulated algal photosynthesis. Because of the range of compounds which stimulated photosynthesis, it seems likely that the effect is due to the lowering of oxidation-reduction potential rather than to a specific effect of these compounds on algal photosynthesis.

Sulfide in the Natural Mats

In considering sources of reducing agents in the natural environment, attention was directed to the nodes themselves. Although the water of the springs studied is low in sulfide, it seemed possible that sulfide formed by sulfate-reducing bacteria might be present in the nodes. Consequently, sulfide assays were performed on nodes of different sizes to obtain some idea of the range of sulfide concentrations present. Concentrations of sulfide were found in these nodes which were similar to those stimulating photosynthesis in dispersed material.

Stimulation of Photosynthesis in Axenic Cultures

Experiments were performed to determine if axenic cultures of the *Phormidium* would also show stimulation of photosynthesis by lowered oxidation-reduction potential. Preliminary experiments with 3 of the 15 cultures isolated showed stimulation by anaerobic conditions and by sulfide similar to those seen in the natural material. More detailed studies were performed with a single culture, strain 52—12. The results of this experiment are shown in Table 2. As seen, aeration greatly inhibits photosynthesis, and both sulfide and cysteine strongly stimulate photosynthesis above the levels seen with N_2 alone. Thioglycolate, the other reducing agent tested, did not stimulate photosynthesis with this culture, although further studies at other concentrations were not per-

Table 2. Stimulation of photosynthetic $^{14}CO_2$ uptake in a pure culture of *Phormidium* (strain 52-12) by anaerobic conditions and reducing agents

Treatment	Counts per min per vial
Air	1,767
N_2	7,045
N_2 0.04 μg/ml $S^=$	9,021
N_2 0.08 μg/ml $S^=$	6,372
N_2 0.16 μg/ml $S^=$	63,069
N_2 0.33 μg/ml $S^=$	67,519
N_2 0.66 μg/ml $S^=$	42,909
N_2 1.3 μg/ml $S^=$	34,824
N_2 3.3 μg/ml $S^=$	21,727
N_2 5×10^{-4} M cysteine	3,375
N_2 5×10^{-3} M cysteine	56,951
N_2 5×10^{-4} M thioglycolate	4,983
N_2 5×10^{-3} M thioglycolate	6,444

Incubations in 10 ml volumes containing about 1 μg chlorophyll *a* per vial. All values corrected for isotope incorporation in dark controls incubated under N_2 (1340 cpm). Sulfide concentrations given are those actually measured by chemical assay at the time the experiment was performed.

formed. The concentrations of sulfide and cysteine which stimulated photosynthesis with the pure culture were similar to those stimulating photosynthesis in the natural material.

Discussion

The striking dependence of photosynthesis on reducing conditions is apparently unprecedented. Stewart and Pearson (1970) demonstrated that several blue-green algae could grow readily in the presence of fairly high levels of sulfide and observed an inhibition of growth at partial pressures of O_2 greater than atmospheric, but did not report stimulation of either growth or photosynthesis under anaerobic conditions. It does, however, seem to be well-documented that blue-green algae are able to grow under anaerobic conditions (Brock, 1973), and Stanier *et al.* (1971) routinely cultivated their unicellular blue-green algae in a nitrogen atmosphere. However, tolerance for anaerobic conditions is quite different from dependence on such conditions. It is possible that the alga under study in the present work is unique, but the phenomenon may be of more general significance.

Cultures of this *Phormidium* grow rapidly and profusely under anaerobic conditions, although anaerobic conditions are not required for growth. However, a constant observation during the isolation and culture of these strains has revealed the tendency of this organism to grow as compact nodes or clumps on agar or in liquid culture. On agar, single hormogonia may glide away from a clump, but soon form tight whorls or knots which eventually develop into small nodes. In liquid culture, these nodes can grow to quite a large size. It seems reasonable that in these nodes oxygen diffusion is restricted and reducing conditions may develop even though the alga would produce O_2 during photosynthesis. Since sulfate-reducing bacteria are not present in these pure cultures, the alga itself may form some other reducing agent which can accumulate within the nodes.

It is possible that we are dealing here with two separate phenomena, one the inhibition of photosynthesis by O_2, and the other the stimulation of photosynthesis by reducing conditions. The former appears to be a general phenomenon in photosynthetic organisms, and has been related through studies on photorespiration to a competition between O_2 and CO_2 for the enolate form of ribulose-diphosphate (Lorimer and Andrews, 1973). It is well established that CO_2 fixation in higher plants (and presumably also in algae) is stimulated by lowered partial pressures of O_2 (Bjorkman, 1971), so that the inhibition of CO_2 fixation by O_2 which we report here is not a unique phenomenon. However, the differential in our experiments between O_2 and N_2 is rather minor compared to that between N_2 alone and N_2 plus reducing agents. Thus it seems likely that the great stimulation of CO_2 fixation by reducing agents reported here is an additional phenomenon, not related directly to photorespiratory events.

Although the results presented here could be interpreted as indicating that the blue-green alga is using sulfide or other reducing agent instead of water as a source of reducing power for photosynthesis, this seems unlikely since CO_2 fixation is inhibited by DCMU, a specific inhibitor of photosystem II (involved in the photolysis of water). Another possible interpretation of our results is that the alga is unable to prevent spontaneous reoxidation of reduced pyridine nucleotides formed during photoreactions so that reducing conditions are required to prevent such reoxidation. Conceivably, the alga is defective in a regulatory mechanism which in other blue green algae prevents reoxidation of reducing power.

The phenomenon we have observed may be present in other blue-green algae and could even be a physiological characteristic of some taxonomic value. Because it is quite easy to screen cultures or natural material for stimulation of photosynthesis by sulfide, a variety of other algae should be checked. Finally, the phenomenon reported here may be of some importance in understanding the mechanism by which low redox potential agents are formed during photosynthesis, and this alga might be a useful tool in analyzing this aspect of the photosynthetic process.

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