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INTERACTIONS AMONG SULFIDE-OXIDIZING BACTERIA

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Introduction

Alternations between aerobic and anaerobic conditions prevail in aquatic environments. Such conditions promote various interactions among bacterium, including synergism, a phenomenon whereby bacterial growth is amplified as a result of proximity to another strain. Another is competition, whereby rivals interfere with each other's growth in a negative fashion (Atlas and Bartha, 1981). The aim of these experiments was to study the responses of different phototrophic bacteria in a competitive experimental system, one in which primary factors such as H_2S or light limited photometabolism. Two different types of bacteria shared one limited source of sulfide under specific conditions of light. The selection of a purple and a green sulfur bacteria and the cyanobacterium was based on their physiological similarity and also on the fact that they occur together in microbial mats. They all share anoxygenic photosynthesis, and are thus probably part of an evolutionary continuum of phototrophic organisms that runs from, strictly anaerobic physiology to the ability of some cyanobacteria to shift between anoxygenic bacterial-style photosynthesis and the oxygenic kind typical of eukaryotes. Hartman (1983) and Trueper (1982) suggest parallelism among such bacterial photosystems.

Chlorobium phaeobacteroides is a strictly anaerobic green sulfur bacterium that uses sulfide as an electron donor for carbon dioxide photoassimilation. In the course of carbon dioxide reduction sulfide is oxidized to sulfur which is stored outside the cells. Some strains further oxidize sulfur to sulfate. Production of sulfur corresponds with the equation:

$$2H_2S+CO_2 \rightarrow 2S+H_2O+(CH_2O)$$ (Kondratieva, 1979)

Oscillatoria limnetica is a facultatively oxygenic cyanobacterium which can use hydrogen sulfide anaerobically in a reaction that oxidizes hydrogen sulfide to S^o. The strain performs anoxygenic photosynthesis, driving electrons from hydrogen sulfide to PS I. *O. limnetica* is capable of anoxygenic photosynthesis at low H_2S concentrations (0.1-0.3 mM). A lag period of 2 hours is required for the shift. Once adaptation is complete, *O. limnetica* can grow photoautotrophically under anaerobic conditions with the same efficiency as it does by oxygenic photosynthesis (Cohen et al., 1975 a,b). Sulfide itself is a weak dibasic acid, with a pH of 6.76 and 11.96 at the ionic strength of the reactions. The concentration of the ionized and undissociated H_2S will drastically change in the pH between 7 and 8. Sulfide may cause deleterious effects on the cells and probably is the reason for the lag period. It is possible that the low redox potential

elicits a process in which reduction of an electron carrier causes it to become sulfide-resistant and thereby functional in the use of sulfide electrons. Addition of sodium thiosulfate to cell suspensions allows elimination of the preincubation period. It also decreases pH, turning most sulfide into H_2S (Belkins, unpublished). Sulfide is oxidized to sulfur according to the equation:

$$2H_2S+CO_2 \rightarrow (CH_2O)+2S+H_2O$$

S^o is expelled from the cells as refractile globules either free in the medium or adhering to the filaments.

Chromatium vinosum, a purple sulfur bacterium, forms S^o inside the cells during anoxygenic photosynthesis. These sulfur globules provide a reservoir of photosynthetic electron donors for CO_2 fixation. CO_2 is fixed as part of an anaerobic, obligately phototrophic metabolism. The final result of H_2S utilization corresponds to the equation:

$$H_2S+2CO_2+2H_2O \rightarrow H_2SO_4+2CH_2O$$

C. vinosum is inhibited by oxygen. Important redox enzymes are probably poorly protected.

MATERIALS AND METHODS

Semi-open system

A semi-open system was used with two of four 210 ml flasks taken from an Ecologen model E-40 (no. 57435, New Brunswick Scientific Co.). Each flask has two rubber-stoppered slits to facilitate sample extraction. The two flasks were connected by plastic covers attached with epoxy glue. Apertures (3.7 cm) for the filter membrane were drilled through the plastic covers. The membrane was glued with epoxy to a plastic and cardboard circular base from both sides to prevent leakage (Fig. II-21).

Light Measurements

Since light intensity can play an important role in determining the species composition among phototrophic bacteria in natural environments, the light emitted by one or two 60 watt incandescent lamps was measured by a Li Cor quanta meter which defined our light intensities. Under conditions of high light intensity (saturation conditions) the brown *C. phaeobacteroides* and the purple *C. vinosum* have similar generation times, although the latter need more light.

Millipore Membrane

A 3 um Millipore membrane was used between the two cell suspensions. The bacterial linear dimensions were smaller than the membrane pores (1.504 um in length as determined by Coulter Counter, 0.853 by transmission electron microscopy, 0.431 by scanning electron microscopy for *Chromatium vinosum*, and around 0.328, 0.225, and 0.053 um^3 for *Chlorobium* species (Montesinos et al., 1983)). Nonetheless, bacterial passage from one to the other system could not be confirmed by microscopy. Sulfur content per cell seems to determine cell volume in *Chromatium vinosum* (Guerrero et al., 1984). *O. limnetica* forms filaments larger than pore size.

Bacterial Strains

Chromatium vinosum UA 6001 was isolated by H. van Gemerden from Lake Cisc (Banyoles, Spain). *Chlorobium phaeobacteroides* UA 5001 was isolated from Vilar, Ciso, and other Spanish lakes. *Oscillatoria limnetica* was isolated from Solar Lake in the Gulf of Akaba in the Red Sea.

Growth Conditions

Cultures of *Chromatium* and *Chlorobium* were grown in Pfennig's medium under nitrogen atmosphere. Initial inocula were taken from stationary cultures (10 ml tubes) and inoculated into 150 ml bottles in Pfennig and Lippert medium (van Gemerden and Beeftink, 1983) for two to three days before experiments. Cultures were incubated at room temperature under light (20-30 uE m^{-2} s^{-1} before inoculation in the semi-open system. *O. limnetica* was grown in agar tubes and inoculated to CHU11 medium improved by Y. Cohen (Waterbury and Stanier, 1981).

Chemical and Biological Parameters

Hydrogen sulfide was measured by colorimetric assay (Cline, 1969). Elemental sulfur was measured according to Bartlett and Skoog (1961). The difficulty in obtaining a standard sulfur solution was that only relative absorbance measurements are given but even these provide useful qualitative information. Sulfate was determined according to Tabatabai (1974). Protein determinations were carried out according to the method of Bradford (1976).

Experiments were done as follows: 210 ml cell suspension of each bacteria containing 25 mM buffer HEPES/NaOH, pH 7.1, and 100 mM $NaHCO_3$ were illuminated by one or two 60 Watt incandescent lamps which provided 26-30 uE m^{-2} s^{-1} between the bottom and surface of the flasks at 26°C. Inocula were maintained under N_2. The whole system was kept in the dark for fifteen minutes, after which samples for sulfide determination were taken. The system was

stored in the light, and sulfide was added in the initial defined concentrations. Samples were taken every four hours in the dark for H_2, S^o, and SO_4^{2-}. Every 8 hours protein was determined; samples, taken with disposable syringes, were immediately fixed, filtered, or frozen as required.

Chlorobium-Chromatium: Initial sulfide concentrations were 5.18 mM and 2.39 mM, respectively. Light intensity was set at 20-29 $\mu E\ m^{-2}\ s^{-1}$ for both systems.

Oscillatoria-Chlorobium: Initial sulfide concentration was 0.8 and 0.7 mM H_2S respectively and light intensities were set at 26-31 and 1.15-1.19 $\mu E\ m^{-2}\ s^{-1}$, respectively.
Low redox potential in the *Oscillatoria limnetica* system was obtained by the addition of 1.2 mM dithionite, which elicits a one to two hour lag period. pH was adjusted to 7.0 by 25 mM buffer HEPES/NaOH and 25 mM NaOH.

O. limnetica-C. vinosum: Initial sulfide concentration was established at 1.5 mM for both bacteria and light intensity was 26-31 $\mu E\ m^{-2}\ s^{-1}$ for both systems. As in the *Oscillatoria limnetica-Chlorobium* interaction, low redox potential was obtained by 1.2 mM dithionite and pH 7.0 was fixed by 25 mM buffer HEPES/NaOH.

Results and Discussion

Dissimilatory sulfide oxidation performed by two sulfur bacteria present together in aquatic habitats was examined experimentally to demonstrate H_2S oxidation to S^o and SO_4. In the *Chlorobium-Chromatium* experiment hydrogen sulfide was oxidized rapidly to S^o during the first 12 hours. *Chlorobium* more efficiently oxidized sulfide than *Chromatium* (0.33 mM $Na_2S\ h^{-1}$ and 0.23 mM $H_2S\ h^{-1}$ respectively.) Nevertheless, the rate of sulfide oxidation was higher in *Chromatium*. Apparently elemental sulfur was produced first by *Chlorobium* but only *Chromatium* oxidized S^o to sulfate. (Hydrogen sulfide at pH 7 represents 25 percent of the total sulfide). After 12 hours *C. vinosum* had left only about 0.115 mM H_2S free in the medium while increasing amounts of elemental sulfur produced by its counterpart were probably passively diffusing and used as an energy source. The use of either H_2S or elemental sulfur by *C. vinosum* to form H_2SO_4, the high light intensity, and the temperature were the main factors which inhibited sulfate formation by *C. phaeobacteroides*. *Chlorobium* may not have had high affinity for the elemental sulfur, which was used by its competitor.

Sulfate concentration only increased after 16 hours. Its rate of production was almost linear through the next 16 hours in the *Chromatium* system while *Chlorobium* did not oxidize sulfur.

Protein was synthesized at a high rate during the first 16 hours in *Chromatium vinosum* with a doubling time of 8 hours. The initial rate was 0.15 $\mu g\ ml^{-1}\ h^{-1}$, followed by a slower rate during the next 20 hours (0.004 $\mu g\ ml^{-1}\ h^{-1}$). *Chlorobium* failed to grow: it showed a negative rate of protein synthesis. This can be explained by the partial dilution of the cell suspension when sample volumes are replaced by fresh medium.

The changes in chemical and biological parameters are shown in Figures II-22 and II-23. As a preliminary conclusion, *Chlorobium* may have a higher affinity for sulfide than for S^o, but other factors such as light, high temperature, and the presence of possible toxic end metabolites produced by its counterpart did not permit more than a maintenance metabolism.

Oscillatoria-Chlorobium: Competitive interaction for H_2S was carried out in similar conditions as in the experiment above with *Chromatium* and *Chlorobium*. To prevent light saturation damage, intensities were lowered.

Both bacteria are capable of using H_2 as an electron donor. Sodium sulfide concentration decreased rapidly during the first 4 hours (Fig. II-24). *Chlorobium* used H_2S more efficiently than the cyanobacterium. Sulfide oxidation rates were 175 mM $Na_2S\ h^{-1}$ and 87 mM $Na_2S\ h^{-1}$, respectively. Both bacteria produced and expelled elemental sulfur which remained free in the medium. Only *Chromatium* further oxidized S^o to sulfate, providing itself another energy source besides H_2S. When *Chlorobium* grows under sulfide limitation the ability to use S^o can be observed.

Chlorobium probably uses the passively diffused S^o yielded by *Oscillatoria*. During 24 hours S^o increases in both systems, thus there is no evidence about the source of S^o used as electron donor by *Chlorobium*. However, sulfate increased after 4 hours with a rate of 45.8 μg sulfate $l^{-1}\ h^{-1}$ in the *Chlorobium* system (Fig. II-24). Sulfate is present in large amounts in CHU11 medium, thus some sulfate probably diffused to the *Chlorobium* system. Nevertheless, the rate of sulfate production decreased when sulfide was added (not shown).

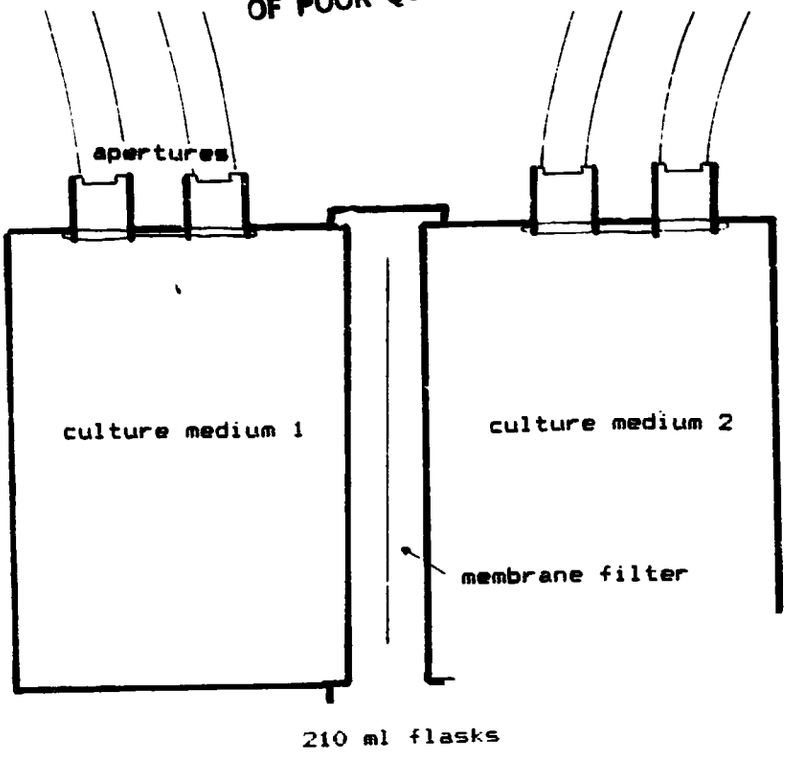

Figure II-21. Diagram of two culture media.

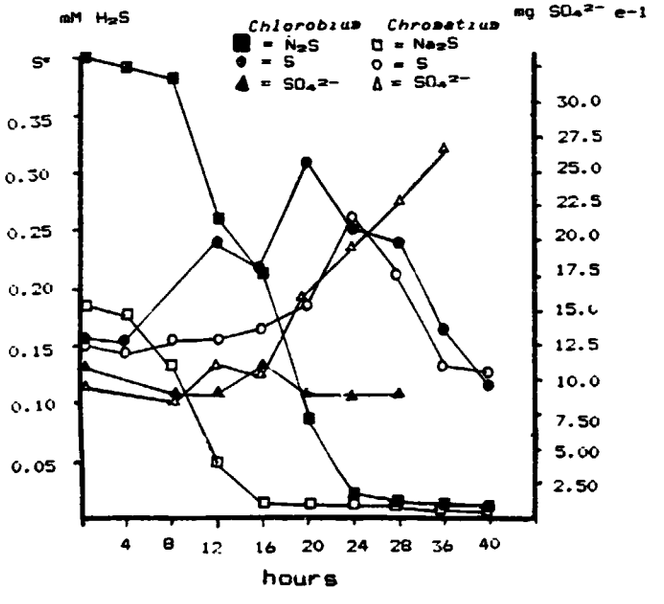

Figure II-22. Production and removal of sulfides and sulfates in *Chlorobium-Chromatium* cells grown in a semi-open system, illuminated by 60 Watt incandescent lamps, at 28o C. $S^{=}$ served as electron donor only to *Ch. vinosum*, which performed full oxidation to SO_4^{2-}.

C-3

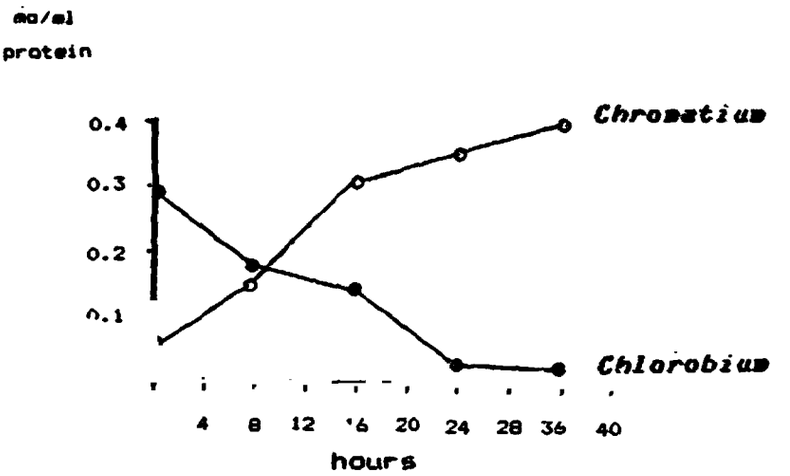

Figure II-23. Protein synthesis. Effective growth is performed only by *C. vinosum*. Descending *Chlorobium* line represents effect of semicontinuous dilution and/or death of the culture.

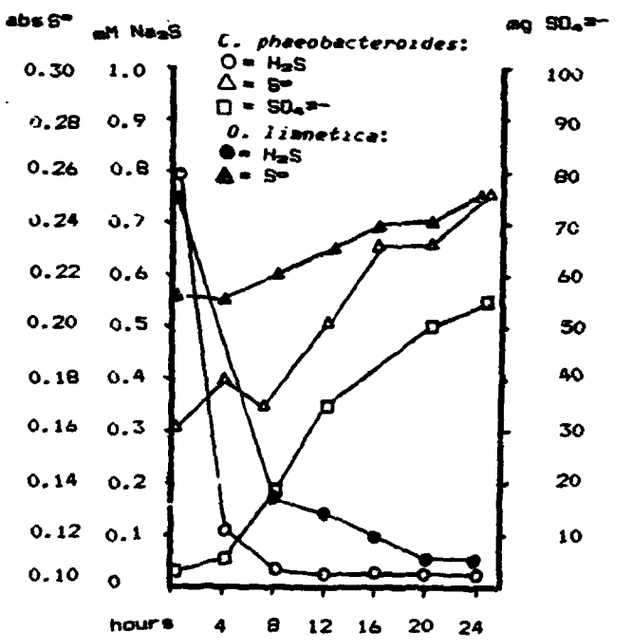

Figure II-24. Changes in chemical parameters during sulfide oxidation to sulfur and sulfate

The absence of sulfide available as electron donor for *O. limnetica* after 8-16 hours probably caused the shift to oxygenic photosynthesis, yielding oxygen toxic to the green sulfur bacterium. Protein synthesis increased during the first 8 hours, and some other growth occurred between 8 and 16 hours and then decreased rapidly after 16 hours (Fig. II-26).

Chlorobium phaeobacteroides' requirements for large amounts of sulfide, low light intensity, and low temperatures place this green sulfur bacterium in an ecological niche with no competitors. In aquatic habitats the uppermost limit of the green bacteria growth layer must be confined to a level of permanent sulfide production. *O. limnetica*, however, is substantially independent of H_2S, and it can shift between photosynthetic systems as environmental conditions require.

Oscillatoria-Chromatium were cultured together in the semi-open system to test competition for sulfide as it probably occurs in nature. Most *Chromatium* dependent upon sulfide are inhibited by oxygen. They lack an assimilatory sulfate reduction metabolism. *C. vinosum* can grow under low sulfide concentrations and high light intensities. Competition for sulfide in a semi-open system with the cyanobacterium grants an advantage to *C. vinosum* with respect to the use of S^o free in the medium, the sulfur being a product of hydrogen sulfide oxidation. Sulfuric acid is produced by photometabolism of *C. vinosum*.

Hydrogen sulfide was utilized by both bacteria as an electron donor. No net growth occurred during oxidation of S^o to sulfate as indicated by protein determination, perhaps due to damage caused by oxygen production by *Oscillatoria*. Some growth or maintenance metabolism possibly occurred since sulfate increased during the first 8 hours. This could not be determined accurately with our techniques (Fig. II-27).

Chlorobium cells are non-motile, strictly anaerobic, and sulfide-dependent. These cells will be found in the microbial community with no other phototrophic bacteria, i.e, over a sulfide-rich layer where they can absorb light energy from above. Yet purple sulfur bacteria, due to their motility and their ability to store S^o inside their cells, can adjust their sulfide environment and ambient light. Competition at low sulfide concentrations and at high light intensities favors *Chromatium vinosum*, which can efficiently utilize either hydrogen sulfide or sulfur. *O. limnetica*, occupying the surface layer in microbial communities, has another strategy since it can use either H_2S or water as an electron donor. Other strains, such as *Oscillatoria c-mill*, isolated from Wilborg Spring in California, always perform oxygenic photosynthesis and have a high resistance to sulfide presence (Cohen, et al., unpublished data). When sulfide concentration is not limiting, phototrophic bacteria that seem to have a higher affinity and efficiency for sulfide are naturally selected over

Figure II-25

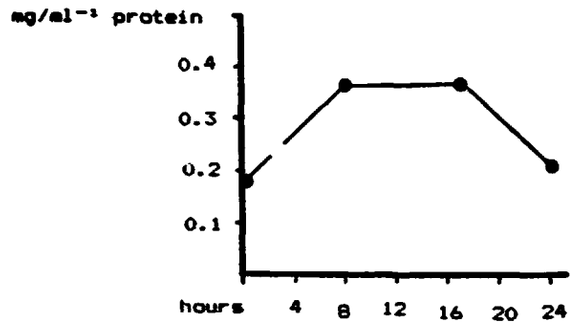

Figure II-27

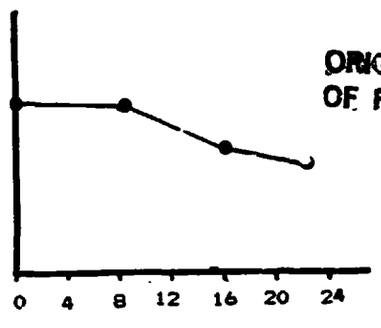

Figure II-25. Protein in *Chlorobium phaeobacteroides* during the first sixteen hours of experiment.

Figure II-27. Lack of growth of *C. vinosum* may have been caused by increasing toxic levels of O_2 due to shift back to oxygenic photosynthesis in *O. limnetica*.

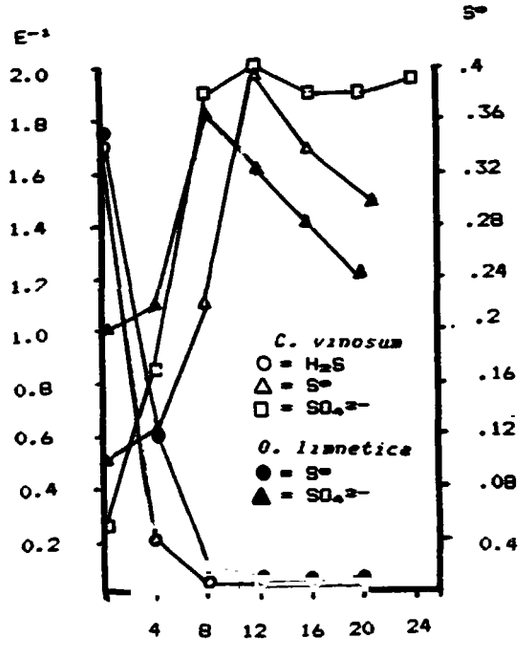

Figure II-26. Sulfur transformations in *Chlorobium-Oscillatoria* cultures.

cyanobacteria. Ecological niches for anaerobic phototrophs are provided in aqueous habitats, where metabolic end products are the primary compounds for their phototrophic metabolism. Maximum efficiency is accomplished by physiological stratification in the microbial populations. Stratification results from competition when primary elements such as H_2S and light become limiting factors.

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