N85-32738

ENRICHMENTS FOR PHOTOTROPHIC BACTERIA AND CHARACTERIZATION BY MORPHOLOGY AND PIGMENT ANALYSIS

D. Brune

Introduction

The purpose of this investigation was to examine several sulfide-containing environments for the presence of phototrophic bacteria and to obtain enriched cultures of some of the bacteria present. The field sites were Alum Rock State Park, the Palo Alto salt marsh, the bay area salt ponds (see map 1), and Big Soda Lake (near Fallon, Nevada). Bacteria from these sites were characterized by microscopic examination, measurement of *in vitro* absorption spectra, and analysis of carotenoid pigments.

Field observations at one of the bay area salt ponds, in which the salt concentration was saturating (about 30 percent NaCl) and the sediments along the shore of the pond were covered with a gypsum crust, revealed a layer of purple photosynthetic bacteria under a green layer in the gypsum crust. Samples of this gypsum crust were taken to the laboratory to measure light transmission through the crust and to try to identify the purple photosynthetic bacteria present in this extremely saline environment.

Materials and Methods

Water samples from Alum Rock State Park and from the Palo Alto salt marsh were collected in small screw-cap bottles or tubes. Pieces of the gypsum crust from the salt pond were placed in plastic bags. Water samples taken from different depths in Big Soda Lake using a sampling bottle were transferred to 150 ml glass bottles and to 500 ml and 1 liter plastic bottles that were completely filled and kept on ice during transportation to the laboratory.

In order to stop the movement of motile bacteria and to compress the bacterial cells being examined into one focal plane, agar slides were used for microscopic examination. The agar slides were prepared as follows (Dr. D. Caldwell, personal communication):

1. Difco bacto agar was dissoved in boiling H_2O to form a 0.75 percent solution.

2. The 0.75 percent agar was poured into 250 ml flasks to a depth of about 1 cm and allowed to solidify.

3. Salts were dialysed from the agar by washing with 3 changes of distilled H_2O over a 24 hour period while swirling on an orbital shaker.

4. A layer of clean microscope slides was placed on a clean, wet surface. (Water under the slides and on the edges of touching, adjacent slides inhibits seepage of agar around and under the slides.)

5. The agar was melted by autoclaving and poured over the slides to a depth of about 3 mm.

6. A glass plate was placed a few inches above the slides to protect them from gathering dust and the slides were allowed to dry over night.

7. The slides were then separated and stored in a box at room temperature for future use.

Water samples from the study areas were used to inoculate a culture medium for green and purple sulfur bacteria prepared as described by van Gemerden and Beeftink (1983). Pure bacterial cultures provided by Dr. R. Guerrero were also grown in this medium. NaCl (3 percent) was added to the medium for bacteria from the Palo Alto salt marsh. Samples of gypsum crust containing a distinct purple layer were placed in the same culture medium modified by including autoclaved water from the salt pond (500 ml/l of medium), thus giving a final salt concentration of about 15 percent. To discourage the growth of algae and cyanobacteria in this medium the inoculum was illuminated through a filter made from 2 layers of red and 2 layers of blue cellulose acetate. This filter absorbs visible light required for algal and cyanobacterial growth but transmits infrared light used in bacterial photosynthesis. The transmission spectrum of this filter is shown in Figure II-1.

Absorption and transmission spectra were obtained using a Varian Techtron model 635 UV-Vis spectrophotometer equipped with an X-Y recorder. Two methods were examined for obtaining *in vivo* spectra of photosynthetic sulfur bacteria. In the simplest method, cells in aqueous suspension were placed in the sample cuvette and water in the reference cuvette. The cuvettes were oriented so that the measuring beam of the spectrophotometer passed through the frosted rather than the clear glass faces of the cuvettes. This minimized the effect of light scattering by the bacterial cells by imposing a larger and nearly identical

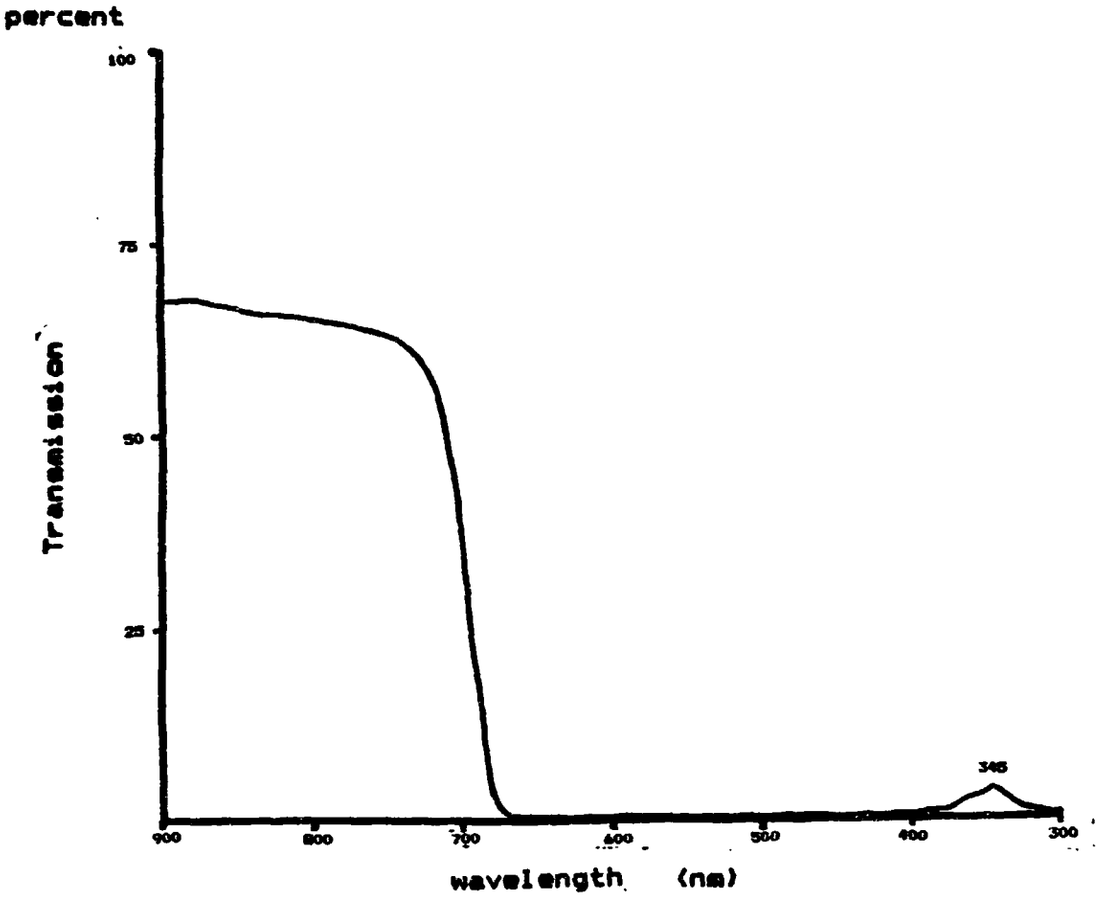

Figure II-1. Transmission spectrum of cellophane filter.

scattering on the light beams passing through the reference and sample cuvettes.

In the other method (Trueper and Yentsch, 1967), the bacterial cell suspension was loaded into a syringe. The bacteria were then collected by forcing the suspension through a Swinney filtration device with a fiberglass or millipore filter. Pieces of aluminum foil with rectangular slits about 15 mm x 4 mm were placed over the windows separating the spectrophotometer sample compartment from the photomultiplier compartment, with the slits aligned so that the reference and measuring light beams passed through them. The filter with the collected bacterial cells was taped over the slit on the sample side while an identical filter moistened with distilled water was taped over the slit on the reference side of the spectrophotometer.

Pieces of the gypsum crust from the salt pond sediment were prepared for light absorption measurement as follows. Loose particulate matter on top of the crust and black mud below the crust were removed by washing with tap water. Sections of crust about 1 cm x 2 to 2.5 cm were cut out using a hack saw blade. Four distinct layers seen in cross section through the gypsum crust could be observed. The uppermost layer was tan and about 2 mm thick. Under this was a green layer of similar thickness. Below the green layer was a thinner, somewhat irregular purple layer, and below this a very irregular, rather crumbly black layer. Using sand paper on the 1 cm x 2-2.5 cm blocks, it was possible to remove layers from the lower surface selectively and thus to obtain slabs consisting of the tan and green layers and the tan layer only. An attempt to obtain the purple layer only by removing the layers above was unsuccessful, although it was possible to obtain discontinuous patches of the purple layer attached to a green layer backing. Spectra of these layers were obtained by placing them in the sample cuvette holder of the spectrophotometer or taping them over a slit on a piece of aluminum foil taped over the window of the spectrophotometer sample compartment. (A piece of white tissue paper was placed over the window to the photomultiplier on the reference side of the sample compartment to partly offset light scattering by the gypsum crust.)

Carotenoids were extracted from bacterial cells and separated by thin layer chromatography as described by Montesinos et al (1983) with minor modifications. Chromatography was performed on both neutral alumina (Merck Type E, 60 F 254) and silica gel (Merck Kieselgel 60) plates without activation by heating prior to use. Colored spots were scraped from the chromatography plates and extracted with about 1 ml of acetone. After centrifugation to sediment the powdered silica gel or alumina, the liquid was decanted and placed in a microcuvette (1 cm pathlength, 0.6 ml volume) for measurement of the absorption spectrum.

Cells from Big Soda Lake used for carotenoid determination were collected by centrifugation from 1 liter of water collected from a depth of 20 m. The extract from the purple layer of the gypsum crust was made using a culture somewhat enriched in purple sulfur bacteria by growth under infrared illumination. This was not a pure culture, however, and also contained some filamentous cyanobacteria.

Carotenoids from these natural samples were chromatographed simultaneously with extracts of known carotenoid composition from cells of *Thiocapsa roseopersicina*, *Rhodopseudomonas capsulata*, *Chromatium vinosum*, and *Thiocystis gelatinosa* for comparison. These extracts were provided by Dr. R. Guerrero.

Results and Discussion

Figures II-2 through II-5 show the *in vivo* absorption spectra of 4 species of purple sulfur bacteria. These spectra were obtained using the first of the methods described, although similar spectra could be obtained using the second method (cells collected on filters). The first method is generally preferable with pure cultures because of its simplicity. The second method is useful, however, for concentrating cells from large dilute samples (e.g., the bacterial plate from Big Soda Lake) or samples in which settling of bacterial cells from an aqueous suspension prevents using the first method.

Figures II-2 through II-5 demonstrate that the absorption spectra of the pure cultures of purple sulfur bacteria differ significantly in wavelength, which ranges between 800 and 900 nm and between 430 and 550 nm. Light absorption between 800 and 900 nm is due to bacteriochlorophyl (Bchl a). Differences in absorption in this part of the spectrum are due to different interactions of Bchl a molecules with each other and with proteins (Thornber et al., 1978). Differences in absorption from 430 and 550 nm is due to the presence of different carotenoids. Although the absorption spectrum of a bacterial species depends on culture conditions, particularly light intensity (Thornber et al., 1978), Figure II-5 demonstrates, that absorption spectra may be characteristic enough to distinguish bacterial species.

The absorption of an enriched culture of a green sulfur bacterium obtained from a Palo Alto salt marsh water sample is shown in Figure II-6. The absorption maxima at 756 and 457 nm are characteristic of Bchl c (Pfennig, 1978). The cells failed to grow without NaCl added to the culture medium. Microscopic examination showed spherical cells about 1 um in diameter. This green bacterial species was identified as *Prosthecochloris aestuarii*.

The absorption spectrum of a green sulfur bacterial culture obtained from Alum Rock State Park is shown in Figure II-7. Bchl

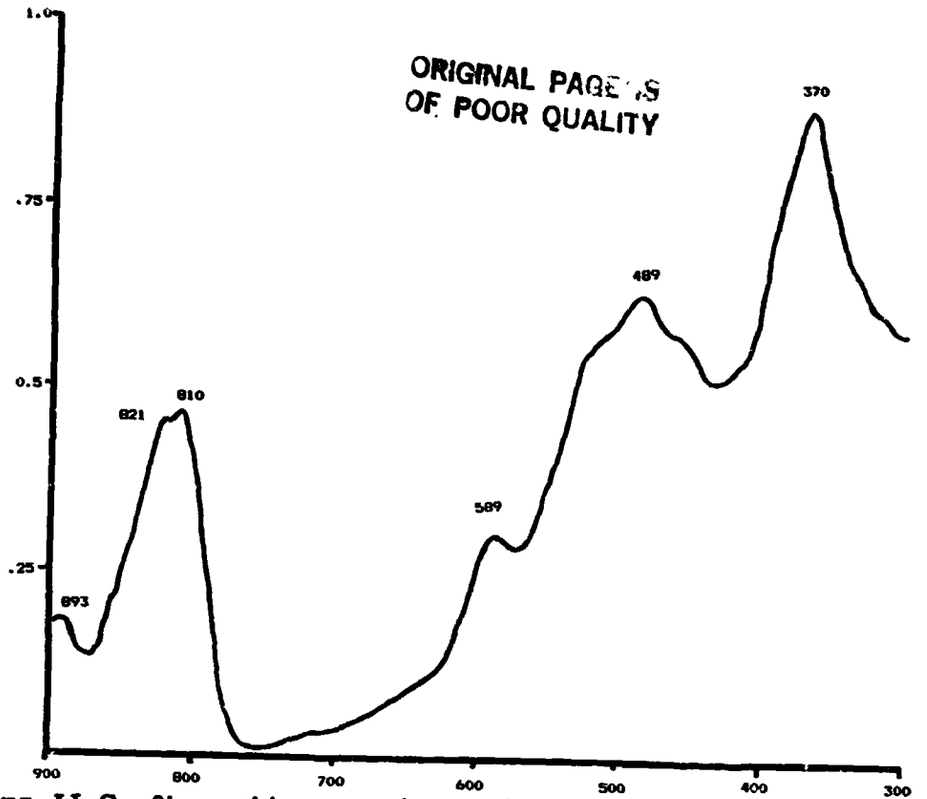

Figure II-2. Absorption spectrum of *Chromatium vinosum*.

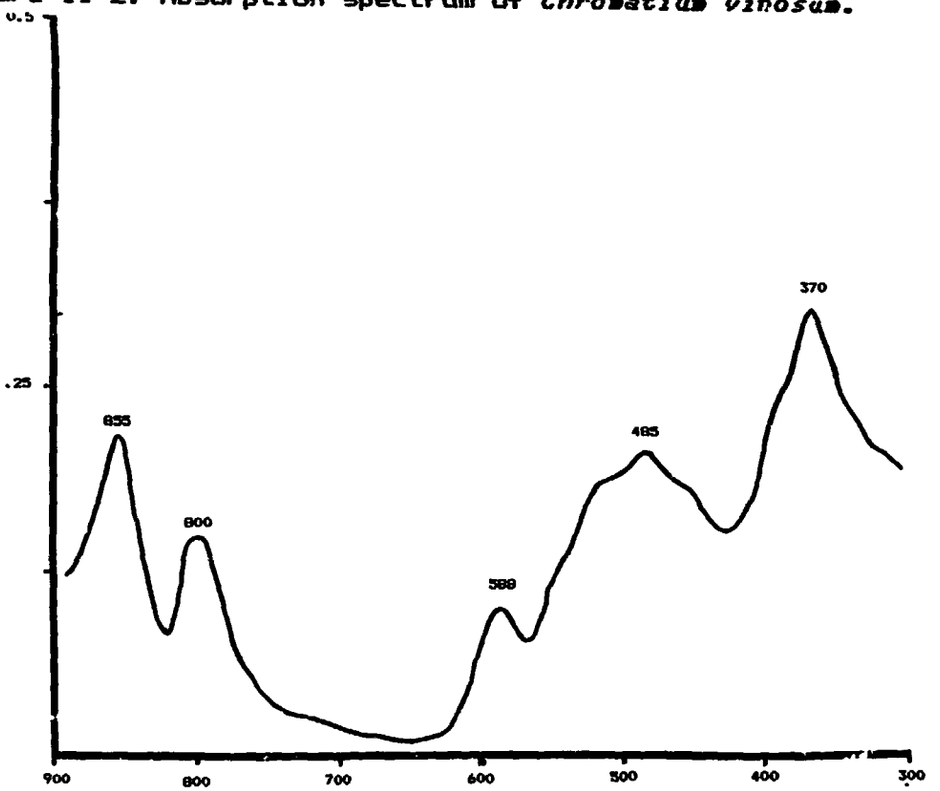

Figure II-3. Absorption spectrum of *Chromatium minutissimum*.

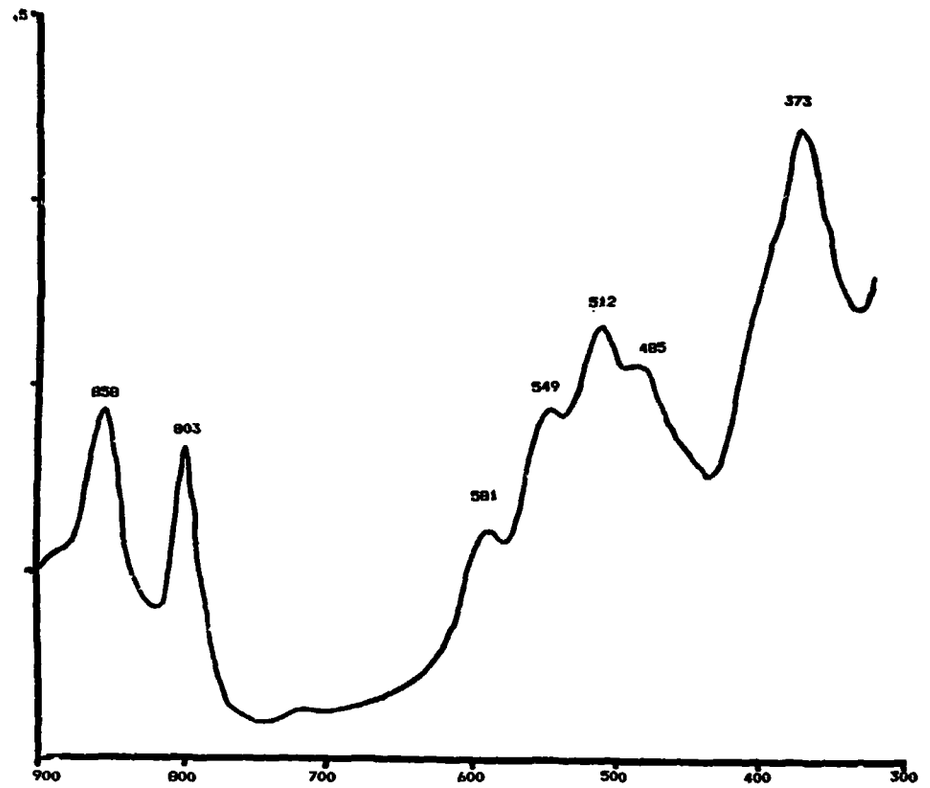

Figure II-4. Absorption spectrum of *Thiocapsa roseopersicina*.

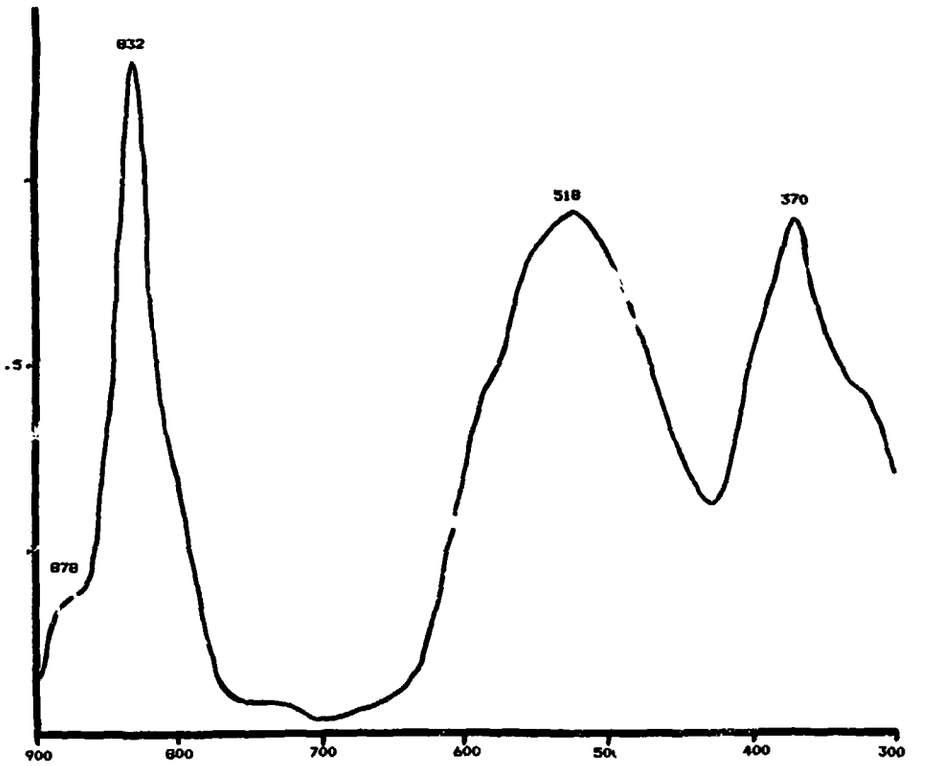

Figure II-5. Absorption spectrum of *Thiocystis gelatinosa*.

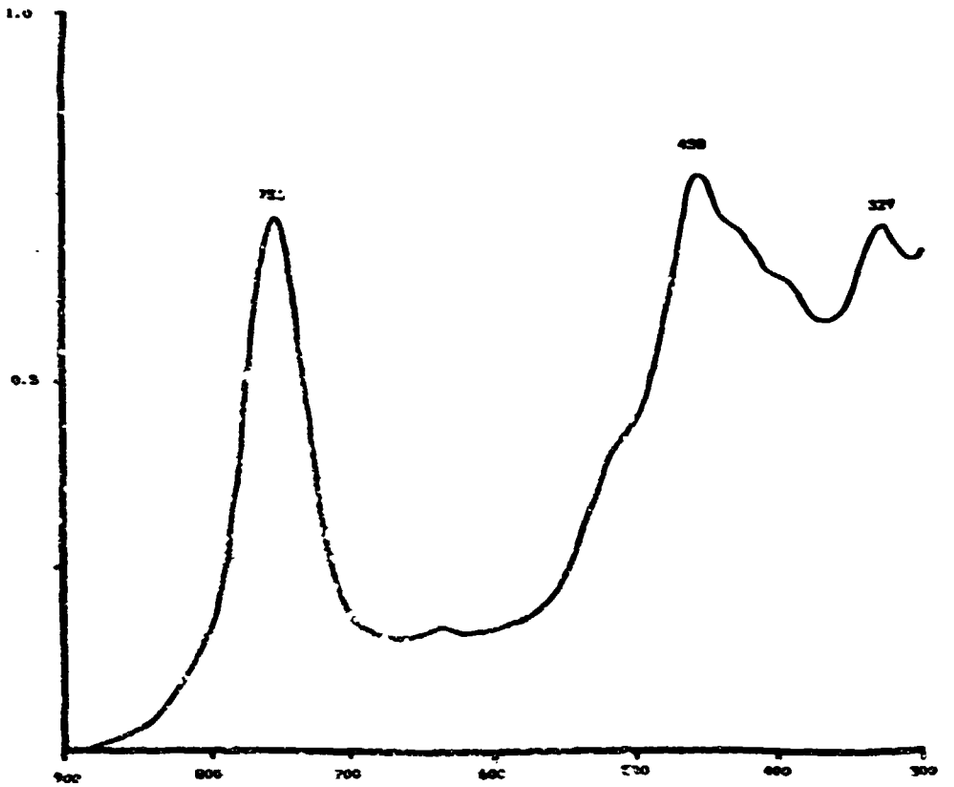

Figure II-6. Absorption spectrum of *Prosthecochloris aestearii*.

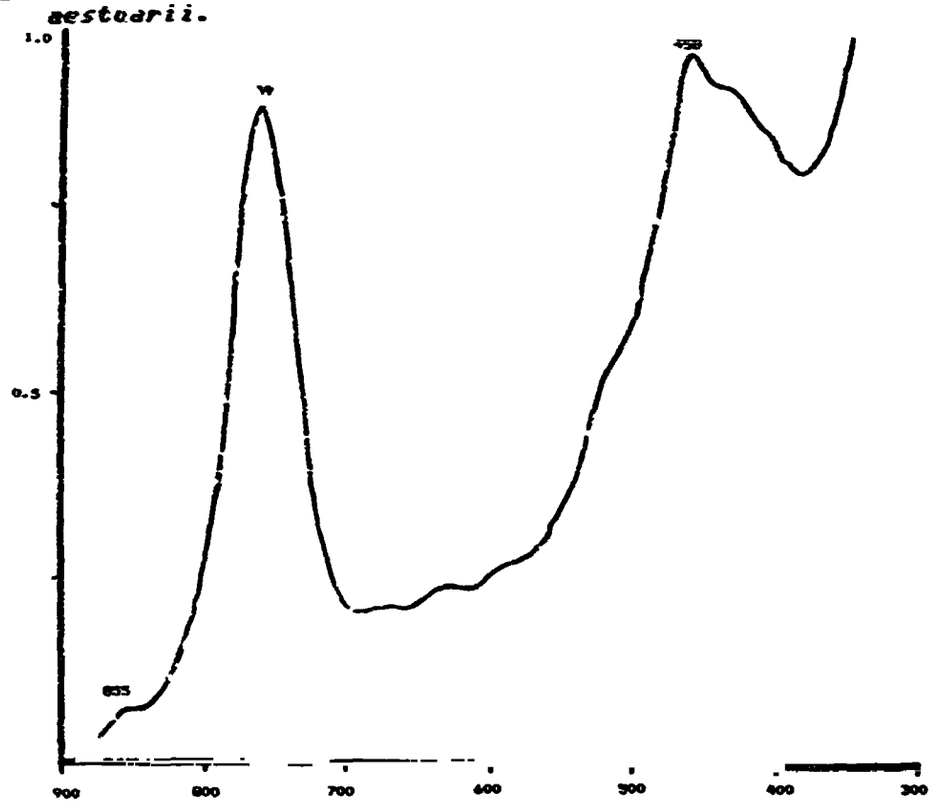

Figure II-7. Absorption spectrum of *Chlorobium limicola*.

c is the principle photosynthetic pigment. (An absorption band at 855 nm is due to the presence of unidentified purple photosynthetic bacteria in the culture.) No added salt was required for growth. The cells were short rods, often joined end to end to form chains. On this basis these bacteria were identified as *Chlorobium limicola*.

The absorption spectra of the tan upper layer of the gypsum crust and of this layer together with the adjacent green layer are shown in Figures II-8 and II-9. The absorption spectrum of the green layer alone was obtained from another piece of the crust: it was practically identical to the spectrum in Figure II-9. The gypsum crust itself absorbs short wavelength light very strongly and becomes increasingly transparent with increasing wavelength (Figure II-8). This crust is sufficiently transparent to red light to permit growth of cyanobacteria, which contribute a prominent absorption peak (about 675 nm) in the red part of the spectrum. Both filamentous and coccoid cyanobacteria were observed during microscopic examination of scrapings from the gypsum crust. The filamentous form grew profusely in bacterial culture medium with about 15 percent NaCl. The tan and green layers together absorb most of the incident light at wavelengths below about 700 nm but are transparent to longer wavelength (infrared) radiation. The ability of Bchl to absorb infrared light clearly is important for the growth of purple sulfur bacteria in the gypsum crust.

The relatively high transmission of infrared light by the gypsum crust makes this natural light filter rather similar to the artificial filter constructed from red and blue cellophane (Fig. II-1). Bacterial cultures grown by illumination through this artificial filter were dominated by purple sulfur bacteria while a control culture grown in unfiltered light was completely overgrown by cyanobacteria. Thus the artificial filter mimics the natural filter in providing a light environment which selectively encourages the growth of purple sulfur bacteria in the presence of cyanobacterial competitors.

Attempts to obtain a continous piece of the purple bacterial layer large enough to obtain an absorption spectrum were unsuccessful. However, the absorption spectrum of a portion of the green layer with discontinuous purple patches adhering to it is shown in Fig. II-10. Infrared absorption bands at about 850 and 795 nm, typical of Bchl a in purple photosynthetic bacteria, are present in this spectrum.

Figure II-11 shows the absorption spectrum of photosythetic bacterial cells taken from a plate located 20 m below the surface of Big Soda Lake. To obtain this spectrum cells from 1 liter of lake water were concentrated to a volume of about 2.5 ml by centrifuging and resuspending. This spectrum strongly resembles the absorption spectrum of a pure culture of *Thiocystis gelatinosa* (Fig. II-5), except for the presence of a small absorption band at about 675 nm. This absorption peaks at 675

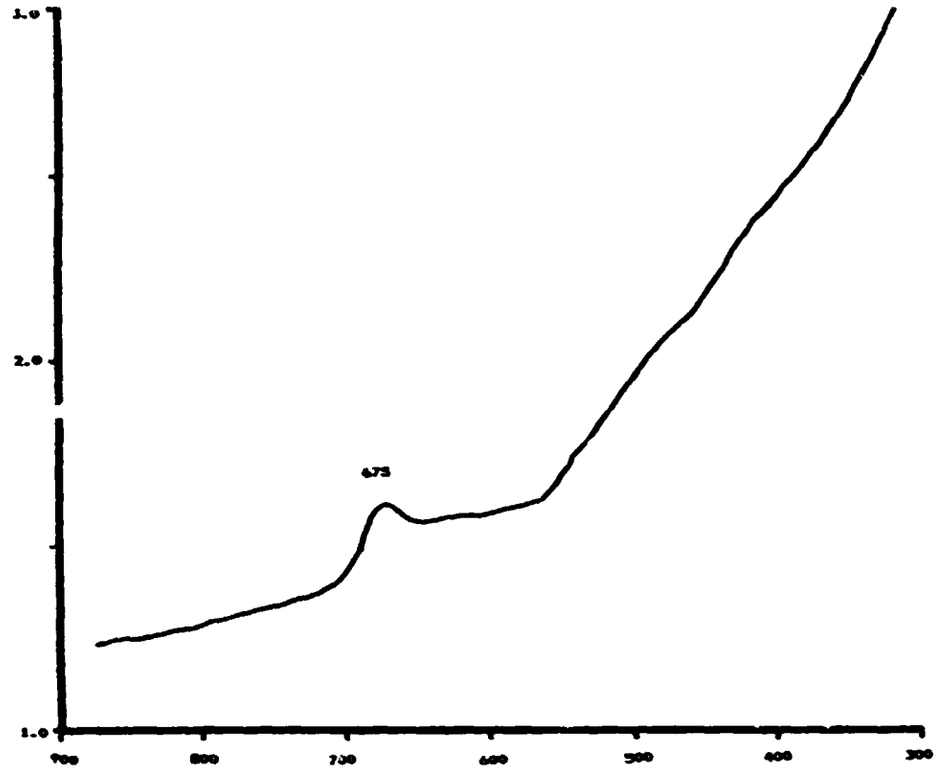

Figure II-8. Absorption spectrum of the tan layer of gypsum crust.

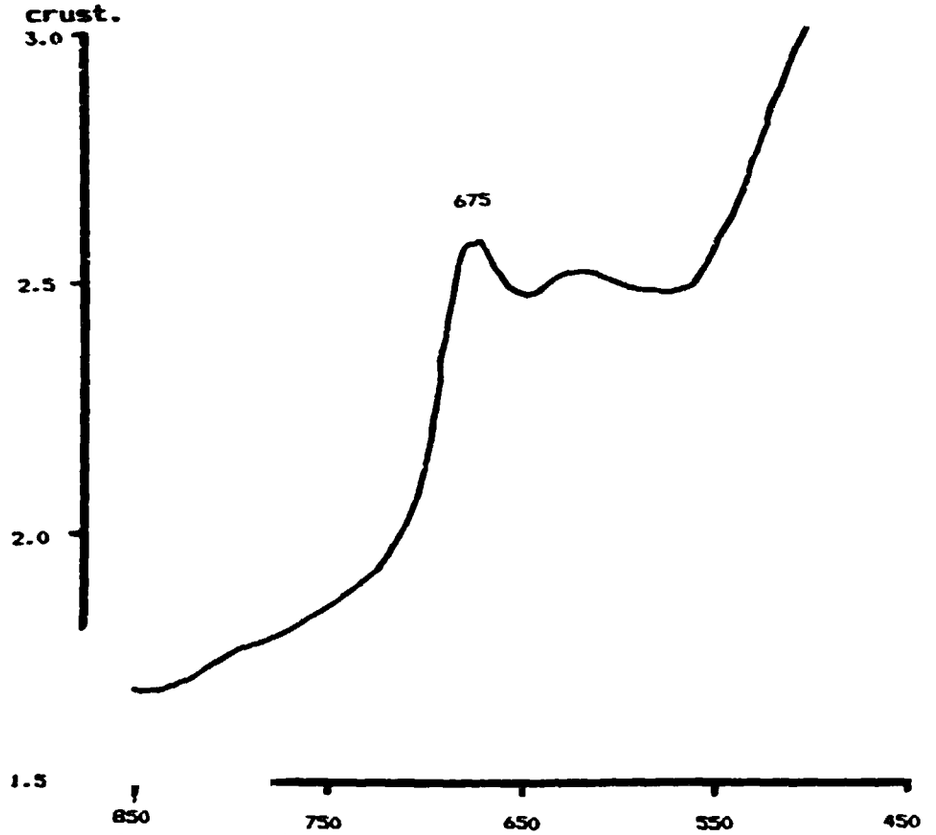

Figure II-9. Absorption spectrum of tan and green layers of gypsum crust.

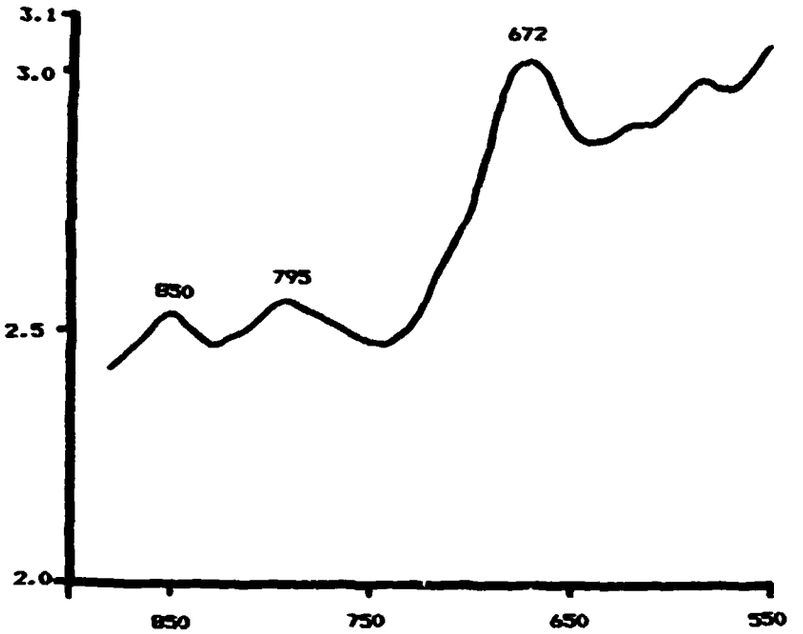

Figure II-10. Absorption spectrum of photosynthetic bacterial layer and part of the overlying layer of gypsum crust.

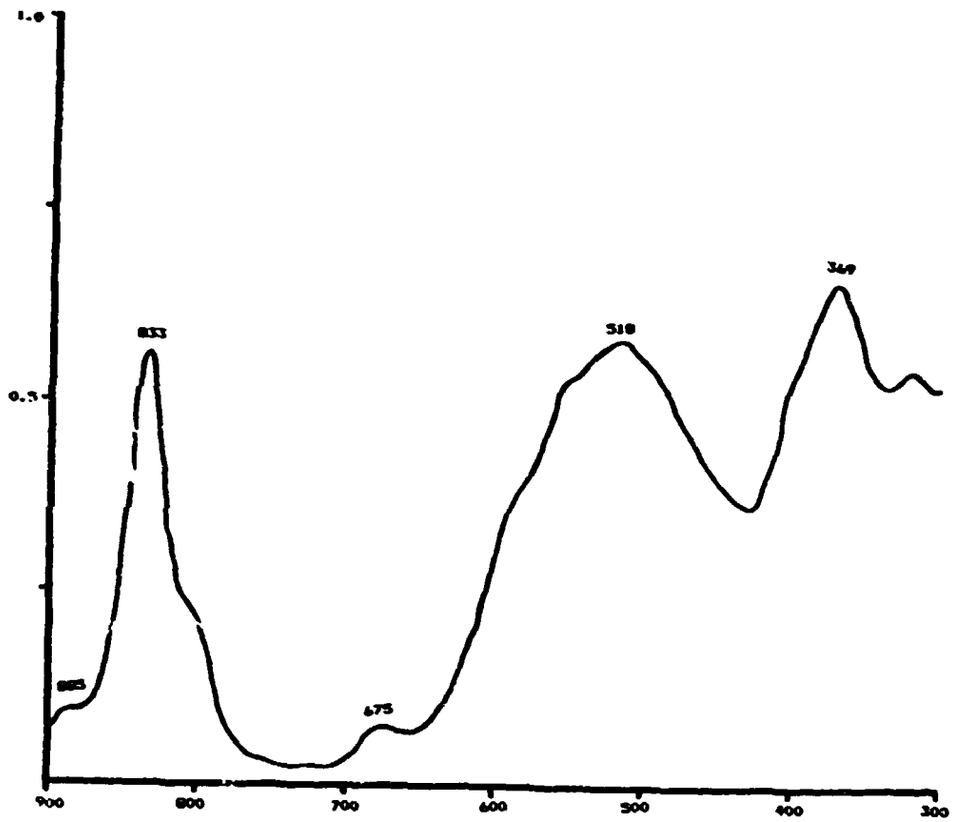

Figure II-11. Absorption spectrum of the bacterial plate sample from Big Soda Lake.

nm, apparently due to the green algae observed on microscopic examination of the bacterial sample from Big Soda Lake. These algae, clusters of cells enclosed in a thick gelatinous sheath, were tentatively identified as *Oocystis* sp. When a portion of the bacterial sample was centrifuged on a percoll density gradient, a heavy band of purple bacterial cells collect at a density of 1.030 g/cm^3, and a lighter unidentified purple band at 1.020 g/cm^3. The green algae separated into a still lighter band having a buoyant density of 1.017 g/cm^2.

Although the similarity of the absorption spectrum of the bacterial cells from Big Soda Lake to that of a pure culture of *Thiocystis gelatinosa* suggests that the dominant organism in the bacterial plate is *T. gelatinosa*, this evidence is not conclusive. A definitive identification of the dominant bacteria species in the plate at the time of sampling is of particular interest, since a previous study (Cloern et al., 1983) reported that *Ectothiorhodospira vacuolata* was the dominant organism in the bacterial plate two years ago. To further characterize this bacterial sample, carotenoids were extracted, separated by thin layer chromatography, and identified on the basis of their Rf values and absorption spectra.

These results and the results of similar experiments of chromatographic separation on silica gel and alumina were performed using purple sulfur bacteria that grew out of the gypsum crust and are shown in Tables I and II. Carotenoids on the plates were identified from their absorption spectra after extraction into acetone or petroleum ether. These spectra were compared with published spectral data (Good, 1973) and with spectra of okenone, rhodopin, and spirilloxanthin in petroleum ether provided by Dr. R. Guerrero.

The sample from Big Soda Lake contained only a single carotenoid identified as okenone. The spectrum of okenone extracted from this sample is shown in Figure II-12. An identical spectrum was obtained for okenone from the *Thiocystis gelatinosa* extract. The Rf values for okenone from the Big Soda Lake sample and from the *Thiocystis gelatinosa* extract were different on alumina. (An excessive amount of okenone was probably applied in the *T. gelatinosa* extract so that the solvent was unable to dissolve and transport all of it simultaneously).

The extract from the bacteria obtained from the gypsum crust contained spirilloxanthin and two other major carotenoids. The absorption spectrum of spirilloxanthin from *Thiocapsa roseopersicina* is shown in Figure II-13. The spectrum of spirilloxanthin from the bacteria under the gypsum crust was identical. One of the other major carotenoids appeared as a yellow spot which ran slightly ahead of spirilloxanthin on the chromatograms. (This carotenoid was not completely separated from spirilloxanthin on alumina.) The other major carotenoid was

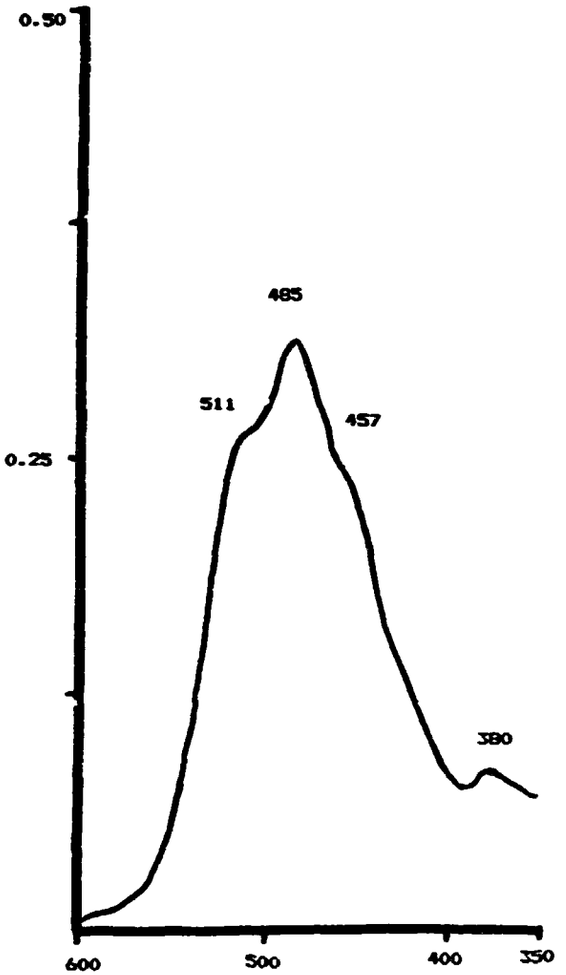

Figure II-12. Okenone (from *Thiocystis gelatinosa*) in acetone.

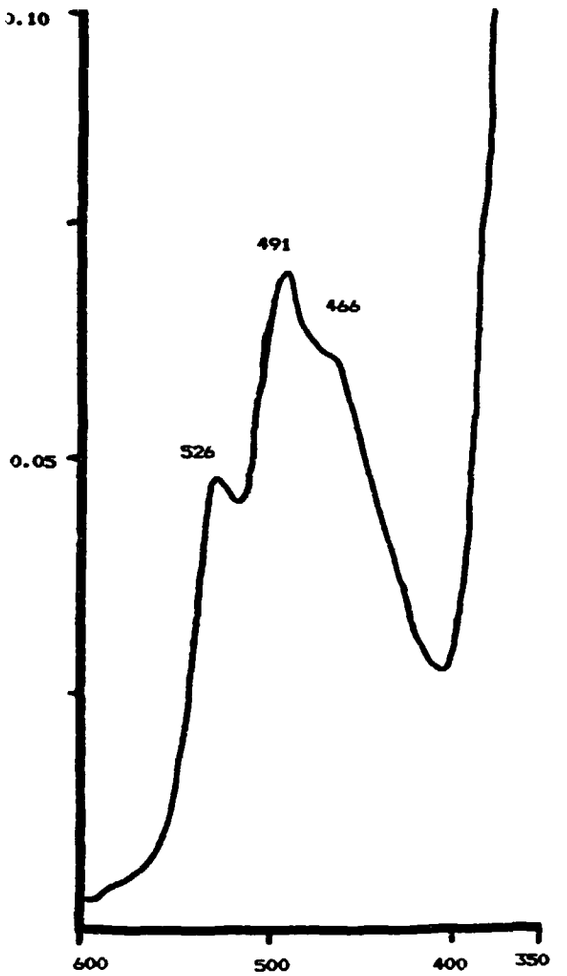

Figure II-13. Absorption spectrum of spirilloxanthin (from *Thiocapsa roseopersicina*) in acetone.

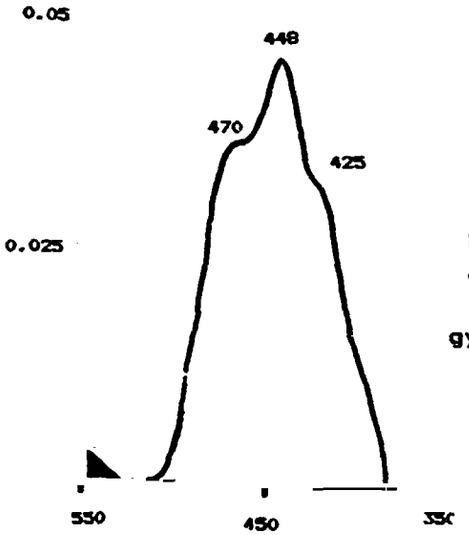

Figure II-14. Absorption spectrum of yellow carotenoid (Rf = 0.73 on silica) from bacteria in gypsum crust in petroleum ether.

a very tightly adsorbed (Rf=0) orange pigment. The absorption spectrum of this orange carotenoid is shown in Figure II-15. Neither the yellow nor the orange carotenoid was identified. It is possible that one or both of these are cyanobacterial pigments, since the culture from which the extract was made still contained cyanobacteria and the initial extract in 90 percent acetone contained about 40 percent as much Chl a as Bchl a, judging from the absorption spectrum of the extract.

Although an extract from *Chromatium vinosum* was used as the standard for rhodopin, the spot for spirilloxanthin in the chromatograms of the *C. vinosum* extract was slightly larger than the spot for rhodopin. An absorption spectrum obtained from the spot identified as rhodopin is shown in Figure II-16.

To complete the identification of the bacterial cells obtained from Big Soda Lake, they were examined microscopically and are described as follows: Individual cells that contain intracellular sulfur globules were spherical and about 2-3 *um* in diameter. Slime capsules surrounded the cells that occur as diplococci, tetrads, and larger clumps. Gas vacuoles were absent; motility was not observed. Pigments were Bchl a and okenone. Except for the lack of motility, these characteristics describe *Thiocystis gelatinosa*, which may be immobile in natural samples (Pfennig and Trueper, 1974). These bacteria were therefore identified as *Thiocystis gelatinosa*.

The bacteria grown from the gypsum crust were also examined microsocpically and may be described as follows: Cells, containing intracellular sulfur globules, were thick rods (3-4 *um* wide and 6-9 *um* long; dividing cells sometimes more than 10 *um* long). Individual cells were colorless; clumps were pink-violet. Cells formed large aggregates, but individual cells were motile, especially when the aggregates were disrupted. Slime capsules and gas vacuoles were absent. The cells occur in highly saline environments and were grown in medium with 15 percent NaCl. Bchl a, spirilloxanthin, and 2 unidentified carotenoids were found in an extract from an enriched culture. On the basis of these characteristics, these bacteria were probably *Chromatium buderi*, although the carotenoid present in described strains of *Chromatium buderi* is rhodopinal rather than spirilloxanthin (Pfennig and Trueper, 1974; Trueper and Pfennig, 1978). Rhodopin has a single absorption maximum at 498 nm (Good, 1973) and therefore cannot be one of the unidentified carotenoids.

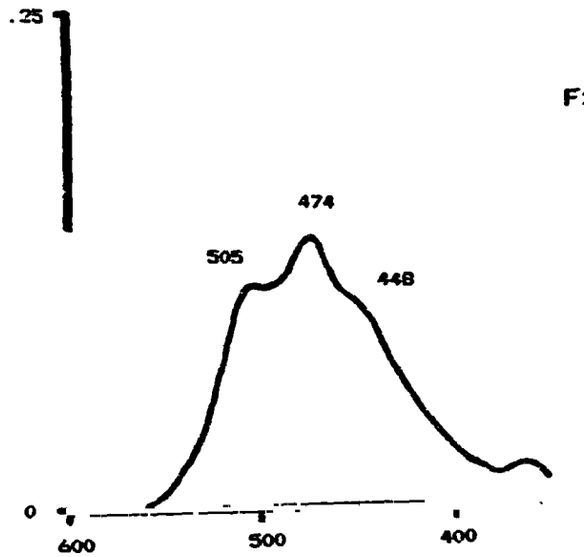

Figure II-15. Absorption spectrum of orange carotenoid (Rf = 0) from bacteria in gypsum crust (in 90% acetone).

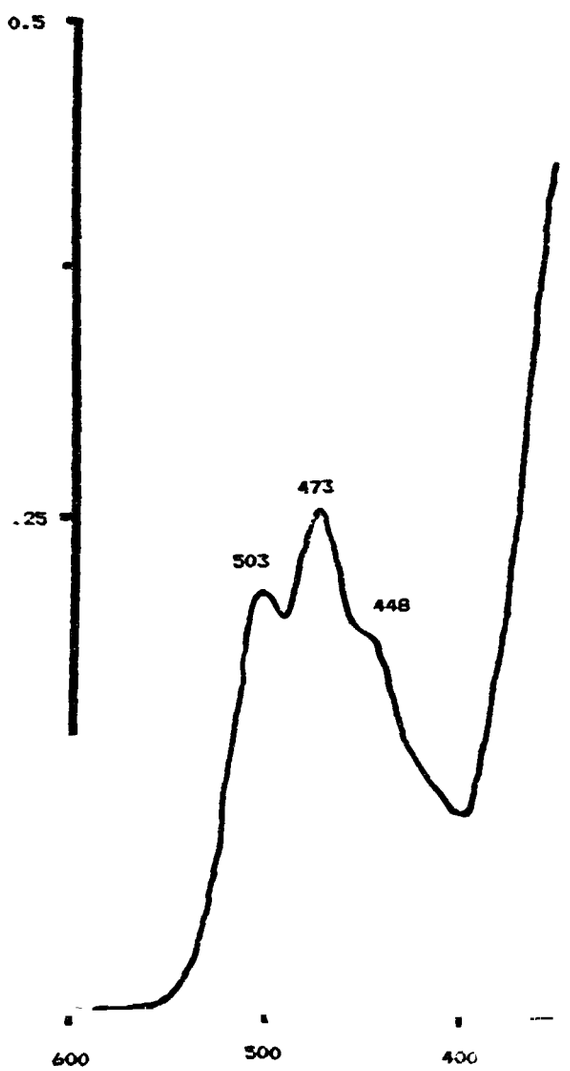

Figure II-16. Absorption spectrum of rhodopin (from *Chromatium vinosum*) in acetone.

Thiocapsa roseopersicina (Spirilloxanthin standard)		
Rf	Color	Identity
0	Blue	Bchl a?
0.62	Pink	Spirilloxanthin
0.73	Pale yellow	n.d.

Rhodopseudomonas capsulata (Spirilloxanthin should be present)		
Rf	Color	Identity
0.48	Pale yellow	n.d.
0.66	Pink	Spirilloxanthin?

Chromatium vinosum (Rhodopin standard)		
Rf	Color	Identity
0	Blue	Bchl a?
0.44	Peach	Rhodopin
0.60	Pink	Spirilloxanthin

Thiocystis gelatinosa (Okenone standard)		
Rf	Color	Identity
0.48	Pink	Okenone

Big Soda Lake Sample		
Rf	Color	Identity
0.56	Pink	Okenone

Gypsum Crust Purple Bacteria		
Rf	Color	Identity
0	Orange	n.d.
0.62	Pink	Spirilloxanthin
0.73	Yellow	n.d.
(Several other faint bands were also observed.)		

n.d. Not determined

? The spot was not examined spectroscopically, but a likely identity is suggested.

Table II-1. Separation of bacterial carotenoids on silica gel with Rf values, colors of observed spots and the identities of the spots listed under each bacterial species.

Thiocapsa roseopersicina

Rf	Color	Identity
0	Blue	Bchl a?
0.[illegible]	[illegible]	[illegible]

Rhodopseudomonas capsulata

Rf	Color	Identity
0.42	Peach	n.d.
0.78	Pink	Spirilloxanthin?

Chromatium vinosum

Rf	Color	Identity
0	Blue	Bchl a?
0.65	Peach	Rhodopin
0.78	Pink	Spirilloxanthin

Thiocystis gelatinosa

Rf	Color	Identity
0.49-0.67+	Pink	Okenone

Big Soda Lake Sample

Rf	Color	Identity
0.77	Pink	Okenone

Gypsum Crust Purple Bacteria

Rf	Color	Identity
0	Orange	n.d.
0.75	Pink	Spirilloxanthin
0.84	Yellow	n.d.

+ The spot was a smear, probably because an excess of pigment was applied.

n.d. Not determined

? The spot was not examined spectroscopically, but a likely identity is suggested.

Table II-2. Separation of bacterial carotenoids on alumina with Rf values, colors of observed spots and the identities of the spots listed under each bacterial species.

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