FINE STRUCTURE OF AN UNUSUAL

PHOTOSYNTHETIC BACTERIUM

by

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INTRODUCTION

The elimination of increasing amounts of industrial, agricultural, and residential wastes presents a problem of national importance in which the pollution rate of natural resources dictates a need for efficient and economical methods in disposing of waste materials. One plausible solution to the problem has been proposed by Cooper, Oswald, and Bronson (1965) who suggested the disposal of organic wastes in oxidation ponds or lagoons. Of particular interest is the anaerobic pond which has the advantage of high organic loading rates on a small land area, but has the disadvantage of objectionable odor production. Such ponds occasionally assume a pink color produced by a bloom of sulfur purple bacteria belonging to the family Thiorhodaceae. These bacteria are anaerobic photoautotrophs capable of oxidizing reduced sulfur compounds. Thus, the sulfur purple bacteria have the facility of acting as biological deodorizers by partially eliminating odors produced from oxidation or organic wastes. According to Cooper (1963), this natural phenomenon of biological deodorization may be advantageously employed in anaerobic stabilization ponds.

Cooper, Oswald, and Bronson (1965) studied waste treatment at three California industrial sites: (1) an animal rendering plant located at Woodland; (2) a petroleum refinery near Richmond; and (3) chicken industries in the region of Morgan Hill. Microscopic examination of samples from the anaerobic lagoon at the rendering plant revealed two predominant microbial types: (1) a sulfur purple bacterium identified as Thiopedia rosea; and (2) the green alga, Chlorella vulgaris. The predominant

heterotrophic bacterium belonged to the genus Alcaligenes, and represent-atives of the following genera were also isolated: Pseudomonas; Bacillus; Flavobacterium; and Clostridium. In an earlier report by Cooper (1963), this pond was described as pink and odorless; the coloration was attrib-uted to the species Thiopedia rosea, which was presumed to render the pond odorless by oxidizing sulfides. Attempts to isolate the organism were un-successful; however a pure culture of another sulfur purple bacterium, belonging to the genus Chromatium, was obtained by the shake culture tech-nique. Samples from the waste pond at the petroleum refinery showed the presence of a sulfur purple bacterium characteristic of the species Chroma-tium vinosum accompanied by a large population of a green alga belonging to the genus Carteria. In the anaerobic ponds used in the waste treatment of the chicken manure, microscopic examination of samples revealed the presence of a sulfur purple bacterium, Thiocystis sp. A study of poultry manure lagoons by Hart and Turner (1964) associated a pink coloration with the organism Thiopedia rosea.

A local opportunity to study a "pink pond" and its microbial flora occurred in the vicinity of Ames, Iowa. The swine waste stabilization pond on the Iowa State University Agricultural Experimental Station Farm became a vivid pink in August, 1965, and this color persisted until the first ice cover in January, 1966. The pink coloration reappeared in August, 1966. This anaerobic lagoon, constructed in 1964, occupies an area of 0.64 acres and consists of two cells with the following dimensions: (1) 106' x 66' x 9'; and (2) 106' x 197' x 4'. Waste materials flow continuously from the swine finishing building into the pond where they are retained in the

combined cells for approximately 80 days, according to Mr. T. L. Willrich

(personal communication, Sanitary Engineering Department, Iowa State Uni-

versity, Ames, 1965). Each year prior to the occurrence of the pink bloom,

the pond showed evidence of gaseous evolution suggesting an appropriate en-

vironment for anaerobic, sulfur-oxidizing organisms. A preliminary study

of pond specimens indicated that the formation of the pink bloom and re-

duction of odor could be attributed to a sulfur purple bacterium character-

istic of the genus Rhodothece. Such information concerning the organism(s)

involved in such phenomena would eventually be of practical value in seek-

ing more efficient and odorless means of waste disposal.

From the point-of-view of fundamental biology, a careful study of the

fine structure of a sulfur purple bacterium was warranted. This study re-

vealed that the dominant organism in the bloom has some unusual features--

in particular a unique surface organization. The architecture of the photo-

synthetic apparatus and the organism's apparent association with a bacteri-

ophage are also of theoretical interest.

LITERATURE REVIEW

This review is composed of three sections, each bearing on a major aspect of this study. The first delves into the current concepts concerning the ultrastructure of the bacterial surface anatomy, consisting of cell wall and surface layers. Opinions regarding the morphology, origin and development of the bacterial photosynthetic apparatus are presented in the second section. Finally, the last section reviews several reports concerning the isolation and characterization of a virus infecting photosynthetic procaryotic cells.

Surface Anatomy

Cell wall

Much of the literature concerning the function, composition, and structure of the cell wall is correlated in the two monographs by Salton (1960; 1964). Salton (1964) defines the cell wall (envelope), "as the shape conferring structure which can be isolated as a single morphological entity".

Attention has been drawn to the bacterial cell wall partly because of its taxonomic significance, since the vast majority of bacteria can be subdivided into two groups--Gram-negative and Gram-positive organisms. The nature of the differences between cell walls of Gram-negative and Gram-positive organisms responsible for Gram-differentiation is not completely understood. Bartholomew and Cromwell (1965) have shown that the intact cell is the major factor contributing to the Gram-positive state,

and suggest that Gram-differentiation is based upon permeability dif-
ferences of the cell wall.

Gram-differentiation also reflects structural differences in the cell
wall. Micrographs of thin-section profiles of Gram-positive bacteria in-
dicate that the cell wall is homogeneous and ranges in thickness from 150
to 800 $\overset{o}{A}$. A multilayered cell wall has been reported in Streptomyces
coelicolor (Glauert and Hopwood, 1960; 1961). This multilayered appear-
ance in thin-section profiles could result from the layering of polymeric
constituents and not necessarily reflect chemically different layers
(Salton, 1964). Another explanation may be the formation of pseudo-unit
membranes produced by fresnel fringes (Saito, 1964). Ogura (1963) amply
demonstrated this phenomenon in his study of the surface structure of
Mycobacterium spp. A through-focus series of thin-section profiles
showed that various interpretations of the cell wall structure are pos-
sible depending on whether the micrographs were over or under focus. The
general model for the cell wall of Gram-positive bacteria consists of a
thick amorphous wall structure exterior and adjacent to the plasma membrane
(Salton, 1964). In contrast, the cell walls of Gram-negative bacteria
are composed of chemically discrete layers giving the appearance of a multi-
layered surface envelope. Claus and Roth (1964) have proposed a structural
model for the Gram-negative cell wall based on their interpretation of
micrographs from thin-section profiles of Acetobacter suboxydans; the
wall consists of an outer membrane and an inner homogenous layer located
between the plasmalemma and outer membrane. The authors postulate that
this inner homogenous layer is analogous to the entire wall structure of

Gram-positive bacteria.

Exceptions to these two major types of cell envelopes have been reported: structural simplicity in which a single "unit membrane" surrounds the cytoplasm as in the species of Mycoplasma and Halobacterium; and structural complexity as evidenced by the surface layers of Lampropedia hyalina and Micrococcus radiodurans.

Nelson and Lyons (1965), employing thin-section microscopy, reported that species of Mycoplasma possess a single limiting membrane 70 to 150 $\overset{o}{A}$ wide; however Morowitz and Maniloff (1966) indicate that the surface membrane of the species Mycoplasma gallisepticum is 110 $\overset{o}{A}$ wide and suggest that a very thin wall-like structure is attached to the "unit membrane".

Originally, Brown and Shorey (1962) reported that the cell envelope of species belonging to the genus Halobacterium consists of a single membrane as seen in micrographs of permanganate-fixed cells; however, further study (Brown and Shorey, 1963) indicated that the cell envelope of the species Halobacterium halobium can either appear as a "unit membrane" or a 5-layered "compound membrane". The authors also reported that the cell envelope of the species Halobacterim salinarum always resembles a 5-layered structure and conclude that the cell wall of the two halophiles are different from Gram-negative bacteria. They proposed that loss of a cell wall in halophilic bacteria is an adjustment of the organism to the high osmotic conditions of their natural environment.

Surface layers

In certain species such as Lampropedia hyalina and Micrococcus

radiodurans, the cell wall has a higher degree of complexity in relation to the typical envelope of Gram-negative and Gram-positive bacteria because of the additional surface layers. According to Salton (1964), "surface layers are independent structures that are separable from the underlying cell wall without impairing the functional and structural integrity of the cell".

The most obvious characteristic of Lampropedia hyalina is the orderly pattern of its spherical to cylindrical cells arranged in a monocellular, rectangular tablet; the tablets float because of the hydrophobic nature of the outer cellular layers (Kuhn, 1963). Electron micrographs of shadowed wall fragments reveal a component consisting of a hexagonal pattern which has a "honeycomb" appearance with holes at a repeat distance of 130 to 140 $\overset{o}{A}$ (Chapman and Salton, 1962). In further studies, Chapman, Murray, and Salton (1963) described the cell envelope as composed of two distinct structural layers: (1) a "honeycomb" or perforate layer which consists of a hexagonal pattern of holes with an average diameter of 75 $\overset{o}{A}$ and with a repeat distance between hole centers of 145 $\pm$ 10 $\overset{o}{A}$; and (2) a "punctate" layer consisting of a hexagonal lattice linked together by filaments with a repeat distance from unit to unit of 260 $\pm$ 20 $\overset{o}{A}$; the spines of the punctate layer are 40 to 60 $\overset{o}{A}$ wide, and surround the tablet rather than individual cells. The perforate and punctate layers are combined to form a single two component layer. Pangborn and Starr (1966) in their study of L. hyalina designated the punctate or outermost layer as the "echinulate" layer because of its spiny appearance. Each spine is an

extension of a hollow bulb interconnected to form a mat; the diameter of

the echinulate layer is 290 Å. Since the outer two layers, the echinulate

and perforate, are not seen in the division cleft; the authors propose

that these layers are synthesized at the site of separation within the

cleft from fibers in the intercalated zone. Murray(1963b) reported that

L. hyalina ATCC 11041 lost its peculiar characteristic of forming one-

celled tablets and exists as a non-sheeting form. Micrographs of thin-

section profiles showed that the intercalated layer responsible for "ce-

menting" cells together in tablet form and also the punctate layer were

absent in this particular strain. Such evidence clearly indicates that

the surface layers are superfluous to cell existence and survival.

A surface anatomy similar to L. hyalina has been reported in the

species Micrococcus radiodurans (Thornley, Horne, and Glauert, 1965). The

diameter of the cell wall is 80 to 200 Å as seen in thin-section profiles

of intact cells. In transverse sections, striations were observed in the

wall having a periodicity of 175 to 200 Å; whereas in sections tangential

to the periphery, a hexagonal array of 100 Å wide holes were seen having

a center-to-center spacing of about 180 Å. The sheath surrounding the

cells appeared either ragged or smooth and sometimes scalloped. The

sheath was occasionally bounded by a membrane 75 Å wide; an intermediate

layer was seen between the outer membrane of the sheath and cell wall.

Micrographs of negatively-stained preparations of surface fragments in-

dicated that the surface was composed of four distinct layers: (1) a

smooth membrane layer resembling a "grapeskin", the outermost layer;

(2) a "compartment" layer which is characterized by a pattern of curved lines forms the main part of the sheath; (3) a "holey" layer having a pattern of dark holes 100 $\overset{o}{A}$ in diameter forms either the inner layer of the sheath or the outer layer of the wall; and (4) a "hexagonal" layer consisting of a hexagonal array of hollow centered "pegs" with a diameter of 85 to 100 $\overset{o}{A}$ joined together by fine spokes. The authors concluded that the striations in the wall correspond to the hexagonal array of holes seen in the tangential sections and negatively-stained preparations. The outer membranous layer of the sheath is analogous to the grapeskin layer while the compartment layer forms the bulk of the sheath. They were not able to correlate the hexagonal layer seen in negatively-stained preparation with profiles of thin sections; however they postulated that the hexagonal layer is a sheath component located on one side of the compartment layer.

<div align="center">Photosynthetic Apparatus</div>

Chromatophores

The term chromatophore was originally designated by Pardee, Schachman, and Stanier (1952) to describe the pigment-bearing structures of Rhodospirillum rubrum; the distinguishing feature being the lack of structural complexity in comparison to chloroplasts. Electron micrographs of chromatophore fractions revealed flattened disks with a diameter of 1100 $\overset{o}{A}$ for which Schachman, Pardee, and Stanier (1952) postulated an equivalent spherical diameter of 600 $\overset{o}{A}$ with an

estimate of 5000 to 6000 chromatophores per cell. Vatter and Wolfe (1957;1958) employing thin-section electron microscopy measured the diameter of intact chromatophores of the following photosynthetic bacteria: R.rubrum, 500 to 1000 $\overset{o}{A}$; Rhodopseudomonas spheroides, 500 to 800 $\overset{o}{A}$; Chromatium sp. strain D, 200 to 400 $\overset{o}{A}$; and Chlorobium limicola, 100 to 300 $\overset{o}{A}$. The authors concluded that chromatophores are discrete vesicular structures and are characteristic of photosynthetic bacteria.

Gibson (1965) substantiated the spherical shape in his electron microscopic studies of thin-sectioned osmium-fixed chromatophore fractions of Rhodopseudomonas spheroides. He found them to have a mean diameter of 570 $\overset{o}{A}$ with a standard deviation of 40 $\overset{o}{A}$ which correlates well with the 600 $\overset{o}{A}$ measurement obtained by Worden and Sistrom (1964) from X-ray diffraction studies.

Bergeron (1958) reported that lyophilized chromatophores of Chromatium sp. have the following composition calculated on a dry weight basis: 300 phospholipid molecules; and a protein equivalent of 67,000 amino acids. His model of the chromatophore of Chromatium sp. is a hollow sphere 320 $\overset{o}{A}$ in diameter with a cortex 90 $\overset{o}{A}$ wide; the core or central region being surrounded by two monolayers: (1) an inner 30 $\overset{o}{A}$ phospholipid layer; and (2) an outer 60 $\overset{o}{A}$ thick protein layer. Bacteriochlorophyll is presumably dispersed between the monolayers, but no suggestion was made concerning the composition of the core. Frenkel (1959) postulated that the core is not a hollow region. According to Bergeron and Fuller (1961) the chromatophore con-

cept is not applicable to the green sulfur bacterium, <u>Chlorobium</u> <u>thio-</u><u>sulfatophilum</u>, for there is apparently no indication of chromatophores typical of those found in <u>Chromatium</u> sp. They observed 150 $\overset{o}{A}$ spherical particles which were less highly organized than the chromatophores of <u>Chromatium</u> spp.

Fractionated extracts from cells of <u>Rhodospirillum</u> <u>rubrum</u> yield 250 $\overset{o}{A}$ particles which exist as part of a repeating unit within the chromatophore. These particles designated as "subchromatophores" are capable of photophosphorylation and photoreduction of DPN (Hickman and Frenkel, 1959). Other investigators such as Oda and Horio (1964) maintain that the chromatophore is the basic structural unit involved in photophosphorylation. Elementary particles similar in appearance to those found associated with mitochondrial membranes were observed on the chromatophores of <u>R</u>. <u>rubrum</u> by using the same techniques employed by Fernandez-Moran (1962). Since cytochrome oxidase is absent from this particular system, the 120 $\overset{o}{A}$ subunit structures are presumably characteristic of membranous structures in general and do not relate to any electron transport function (Low and Afzelius 1964; Afzelius and Low, 1965).

There is little or no support in the literature for the concept of <u>de</u> <u>novo</u> synthesis of chromatophores. The several mechanisms that have been proposed to account for the origin of chromatophores can be summarized as follows: (1) formation of a reticulum by infoldings of the cytoplasmic membrane into the cytoplasm as observed in <u>Rhodo-</u><u>spirillum</u> <u>rubrum</u> (Cohen-Bazire and Kunisawa, 1963); (2) direct forma-

tion by invagination of the cytoplasmic membrane from which discrete, uniform vesicles are formed in: R. rubrum (Cohen-Bazire and Kunisawa, 1963; Boatman, 1964; Oda and Horio, 1964); and in Rhodopseudomonas spheroides (Gibson, 1965); (3) by binary fission as indicated by the "dumb-bell" shaped chromatophores of R. spheroides (Gibson, 1965); and (4) fragmentation of the intracytoplasmic membranes as in Rhodospirillum rubrum (Hickman and Frenkel, 1965b; Holt and Marr, 1965b).

The photosynthetic apparatus is described as a lamellar array rather than a vesicular system in Rhodospirillum molischianum (Drews, 1960; Giesbrecht and Drews, 1962; Hickman and Frenkel, 1965a; Gibbs, Sistrom, and Worden, 1965); Rhodomicrobium vannielli (Boatman and Douglas, 1961; Vatter, Douglas, and Wolfe, 1959); and Rhodopseudomonas viridis (Drews and Giesbrecht, 1965; 1966).

According to Holt and Marr (1965a) the internal membranes of Rhodospirillum rubrum originate as spherical vesicles from the cytoplasmic membrane and can further develop into a more or less flattened tubular system appearing as lamellar profiles. The lamellae of Rhodospirillum molischianum also originate as infoldings of the cytoplasmic membrane which elongate to form a paired lamellar structure approximately 180 Å wide consisting of two nearly appressed 80 Å membranes (Hickman and Frenkel, 1965a).

Environmental effects

Cultural conditions, especially age, light intensity, and oxygen tension have a pronounced effect upon the morphology of the photosynthetic apparatus.

According to Hickman and Frenkel (1959), young cultures of Rhodo-
spirillum rubrum show typical chromatophores; whereas, both lamellae and
chromatophores appear in cultures 8 days and older.

Pigment synthesis is inversely related to light intensity in the
species Rhodopseudomonas spheroides and Rhodospirillum rubrum (Cohen-
Bazire, Sistrom, and Stanier, 1957). Dark grown cells of R. rubrum con-
tain neither pigment nor chromatophores (Vatter and Wolfe, 1958); where-
as, cells grown at a light intensity below 100 ft-c contain more internal
membranes than cells grown above 400 ft-c (Holt and Marr, 1965b). Such
observations have led Holt, Conti, and Fuller (1966b) to conclude that
the formation of the photosynthetic apparatus is an inverse function
of light intensity which correlates with the proposal by Cohen-Bazire
and Kunisawa (1963), that the amount of pigment in cells of R. rubrum
is inversely related to light intensity and oxygen tension. Gibbs,
Sistrom, and Worden (1965) showed that a paradox existed between elec-
tron microscopic observations and analytical results regarding the
amount of bacteriochlorophyll and chromatophore material in the cell.
The amount of internal membrane in cells of Rhodospirillum molischianum
apparently has no absolute relationship to the amount of bacteriochloro-
phyll, but it is rather the total membrane content of the cell including
the cytoplasmic membrane which is proportional to the bacteriochlorophyll
content. The authors explain the paradox in proposing that bacteriochloro-
phyll is localized on both the cytoplasmic and internal membranes, thus
supporting an earlier viewpoint of Cohen-Bazire and Kunisawa (1963).

Virus Particles

The author has found no report in the literature of a bacteriophage infecting a photosynthetic bacterium. The only known virus infecting photosynthetic procaryotic cells has been isolated by Safferman and Morris (1963); a blue-green algal virus (BGA) designated as strain LPP-1, which is capable of lysing cells of the following three genera: Lyngbya, Phormidium, and Plectonema. Schneider, Diener, and Safferman (1964) proposed the general term "Phycovirus" for viruses infecting algae. Electron micrographs of shadowed preparations of the BGA virus indicated that the particles were either hexagonal or pentagonal with an average diameter of 660 $\overset{o}{A}$. These workers found no evidence of a tail structure; however, Dr. P. Walne (personal communication, Department of Botany, U. of Tennessee, Knoxville, 1965) revealed the existence of a tail structure typical of a bacteriophage. The BGA virus was further characterized by Schneider, Diener, and Safferman (1964) as having a sedimentation coefficient of 548S and containing deoxyribonucleic acid. Safferman and Morris (1964a) showed that the established plaque assay techniques are applicable in the study of the BGA virus which forms two plaque variants. The BGA virus may be of practical value in its potential use as a "biologic algicide" employed in selective algae control (Safferman and Morris, 1964b).

MATERIALS AND METHODS

Isolation and Cultivation

Organisms

Microscopic examination of samples from the swine waste stabilization pond indicated that the predominant organism was a sulfur purple bacterium which closely adhered to the description in Bergey's Manual of Determinative Bacteriology (Breed et al., 1957) as belonging to the genus Rhodothece; the identification was confirmed by Dr. J. Holt, Department of Bacteriology, Iowa State University, Ames. Attempts to isolate Rhodothece sp. were unsuccessful; however another sulfur purple bacterium was repeatedly isolated in pure culture and designated as Chromatium sp. strain Ps 668; the identification was confirmed by Professor Dr. N. Pfennig, Institut fur Mikrobiologie, Gottingen, Germany.

A green alga, Euglena sp., was also common to the microbial flora and occurred mainly in its encysted form. The species Nitzchia thermalis appeared to be the only representative of the family Diatomaceae. The algal representatives are mentioned only for their ecological interest and are not incorporated in this study.

Enrichment technique

The merits of the enrichment culture technique are amply discussed by Van Niel (1949). The basic principle is to selectively enhance growth to favor one or a specific group of organisms from a mixture by adjusting the nutritional and environmental conditions. An enrichment medium for

sulfur purple bacteria as described by Stanier, Doudoroff, and Adelberg

(1963, p. 458: see Appendix B) was inoculated with aliquots of liquid

sewage. Since members of the family Thiorhodaceae are obligate anaerobes

requiring a low oxidation-reduction (O/R) potential, the organisms were

cultivated in completely filled glass-stoppered bottles. Cultures were

incubated at 25 C under constant illumination of 50 ft-c from an incan-

descent source.

In principle, the isolation of anaerobic photoautotrophic bacteria

from enrichment cultures should be readily accomplished on the assumption

that an anaerobic environment would eliminate aerobic bacteria; however

the assumption is not valid and further procedures for isolation were

necessary.

Dilution method

The streak and pour plate methods accompanied by conditions for ana-

erobiosis as described by Society of American Bacteriologists, (1957) were

also unsuccessful in obtaining pure cultures. Chemical methods using alka-

line pyrogallol for the absorption of oxygen and a commercially available

anaerobic incubator based on the principle of gas exchange failed to lower

the O/R potential enough to permit anaerobic growth. Thus, it was neces-

sary to adopt the classic method of dilution. A completely filled 4 oz.

screw-capped bottle (approximately 127 ml) containing the defined synthetic

medium described by Pfennig (1961; 1962: see Appendix B) was inoculated

with a 1.0 ml aliquot from the enrichment culture. Serial dilutions were

made to extinction and the cultures incubated at 30 C under an illumina-

tion of 50 to 100 ft-c from an incandescent source with alternating light and dark periods of 16 and 8 hr respectively. This procedure was repeated until a culture showed evidence of purity by light microscopic observations.

Dilution shake culture

A pure culture obtained by the dilution method is questionable because the sole criterion is the direct microscopic examination of small specimen samples from a relatively large volume of material. The dilution shake culture has the advantages of visualizing and isolating individual colonies embedded in agar. A series of ten sterile tubes 16 x 150 mm were one-half to two-thirds filled with a defined synthetic medium containing 1.5% Difco Purified Agar (Pfennig 1961; 1962: see Appendix B). An inoculum was added to the first tube containing cooled but unsolidified agar and shaken well using a super-mixer (Matheson Scientific). Approximately one-tenth of the seeded agar from the first tube was added to the second tube and the procedure repeated until all ten tubes had been serially diluted. After solidification the tubes were sealed with a mixture of petrolatum and paraffin (1:1 W/W). The culture tubes were incubated at 30 C under constant illumination of 50 to 100 ft-c from an incandescent source. If the tube is slightly warmed in a water bath, the plug sealing the tubes can be easily removed with a sterile wire. Filtered compressed air passing through a sterile Pasteur pipette placed between the column and glass wall forced the agar column out of the tube into a sterile petri dish. The column was sectioned with a sterile scalpel into small blocks containing individual colonies, which were then placed in a liquid growth

medium.

Growth medium

A defined synthetic medium for the growth of sulfur purple bacteria

was suggested by Dr. R. Y. Stanier, University of California, Berkeley;

the medium was originally formulated by Pfennig (1961; 1962: see Appendix

B). Modifications of the heavy metal solution and calcium concentration

necessary to specifically enhance the growth of Chromatium sp. Ps 668

were suggested by Professor Dr. N. Pfennig (personal communication, 1966:

see Appendix B). Organisms were subsequently grown in the modified medium

as described in Appendix B at 30 C under an illumination of 50 to 100 ft-c

from an incandescent source with alternating light and dark periods of 16

and 8 hr respectively.

Natural environmental conditions

Two methods were used to mimic the natural environment under labor-

atory conditions: (1) growth of organisms in sterilized pond water; and

(2) substitution of pond water for distilled water in the preparation of

Solution 1 as described in Appendix B. Liquid sewage was first filtered

through glass wool to remove large debris and then centrifuged (Sorvall

Model SS-3; GSA rotor) at 5.500 x g for 30 min. to remove most of the

bacterial cells. The supernatant was an amber colored colloidal suspen-

sion which either served directly as a growth medium after sterilization

by autoclaving, or was added in appropriate amounts to prepare Solution 1.

In order to ascertain environmental influences, cells of Chromatium

sp. Ps 668 grown under described laboratory conditions were replaced in
the environment from which the strain was isolated. Approximately 50 ml
of a 7 day culture were dispensed into a sterile dialysis bag, sealed with
twine and floated on the swine waste stabilization pond for a 7 day period.

Microscopic Techniques

Light microscopy

Viable cells were placed on a slide with a non-hardening medium,
Zeiss Enschlussmittell W15, the refractive index of N_D-1.515 enhanced
phase contrast and its viscosity decreased bacterial motility. Slides
were rendered semi-permanent by ringing the appressed cover glass with
Zeiss edging lacquer. Micrographs were taken with either a Zeiss photo-
microscope or a Leitz Ortholux using an apochromat 100/1.30 phase oil ob-
jective; magnifications were calibrated with a Lafayette stage micrometer.
All light micrographs were taken on Adox KB-14 film which was processed
according to standard photographic procedures.

Electron microscopy

The majority of the specimens studied were fixed at room temperature
for 3 hours in 1% osmium tetroxide with added calcium and sodium chloride
as described in Appendix A. This modification of the standard fixation of
Kellenberger, Ryter, and Séchaud (1958) consists of: (1) elimination of
post-fixation treatment with uranyl acetate; and (2) elimination of amino
acid additives. Partial justification for this modification is based on
the findings of Schreil (1964). The author suggests that an artificial

ordering of deoxyribonucleic acid (DNA) results from exposure of specimens to uranyl salts.

Two experimental fixation techniques were employed: (1) addition of osmium tetroxide directly to the natural substrate to a final concentration of 1% osmium and (2) potassium permanganate added to the natural substrate to yield a final concentration of 2% permanganate. The addition of these oxidizing agents directly to the growth medium was an attempt to minimize mechanical damage to cell surface layers during harvesting procedures prior to fixation. Three percent glutaraldehyde (Sabatini, Bensch, and Barnett, 1963) with post-osmification as described above was also used.

The procedure for dehydration with increasing concentrations of ethanol and the detailed process of embedding in Epon 812 (Luft, 1961) are given in Appendix A. Ultrathin sections of Epon embedded material were cut with a DuPont diamond knife using an LKB ultramicrotome; sections ranged in thickness from 50 to 100 mμ as determined by diffraction colors. Naked 400 mesh copper grids and formvar-coated 150 mesh copper grids were used. Increased contrast of sectioned material was obtained with either methanol uranyl acetate (Stempak and Ward, 1964) or lead citrate (Venable and Coggeshall, 1965).

Whole bacteria and brei preparations were negatively-stained with phosphotungstic acid (PTA) according to the loop film technique described by Murray (1963a). This was done by dispensing 100 ml aliquots of liquid sewage into 250 ml centrifuge bottles. Cells were harvested by centrifugation at 3000 rpm for 30 min at 4 C. The pellet was resuspended in several ml of distilled water and the cells were then transferred either to a

Mickle tube containing glass beads (0.2mm) or stained directly with PTA. The Mickle disintegrator was operated in a commercial deep freezer for 5 min. at 4 C. Three drops of the resulting brei were immediately transferred to a spot plate containing a mixture of 3 drops of 4% PTA, 3 drops of distilled water, and a trace of bovine serum albumin (Calbiochem). Carbon-coated 150 mesh copper grids were placed on an absorbent blotter; a 5 mm diameter platinum loop containing a portion of the specimen mixture was lowered over the grid and appressed to the blotter until the excess mixture was absorbed.

Specimens were viewed with an RCA EMU-3F electron microscope using a 25 to 40 μ objective aperture and operated at either 50 or 100 KV. Micrographs were taken on either Kodak Projector Slide Plates or "Estar" (Kodak), and "Cronar" (DuPont) films. Routine photographic procedures were followed in development of negatives and printing on Kodabromide F single weight paper.

Laser-light Diffraction

The technique of laser-light diffraction as described by DiBona (1966) was used in this study to ascertain the geometric configuration of the subunit structure observed in some negatively-stained fragments from cells of Rhdothece sp. The laser emission source was supplied by a helium-neon (HeNe) gas laser (Model TL-1, Bendix Corporation); the optical power output is 0.5 mW and the spatially coherent plane polarized light has a precise wave length of 6238 Å. The laser chasis was positioned 2.86 meters from a mirrored surface which directed the laser emission down an optical path through the sample, focusing lens, and reflection shield to

the film holder. All elements were mounted on a three meter optical

bench and supported by three-pronged lens holders on centerable mounts.

The samples were positive transparencies of maximum contrast which were

printed by contact on Kodak Projector Slide Plates from the negative micro-

graph with the appropriate electron microscopic images. Opaque adhesive

tape was used to frame the desired specimen area on the positive plate to

prevent diffraction from the surrounding areas. According to the termin-

ology of DiBona (1966), the fully-framed specimen will be referred to as

a "mask", and the optical diffraction pattern on the film negative as a

transform. A Tri-X Film Pack (Kodak) was a convenient means of recording

the resulting optical diffraction patterns. Exposed film was processed

in Microdol-X (Kodak) developer.

OBSERVATIONS

Rhodothece sp. (liquid sewage)

General morphology

Spherical, non-motile cells usually arranged in pairs were observed
in pond specimens with phase-contrast microscopy (Figure 1). The cellu-
lar diameter of 1.2 µ correlates with measurements obtained from both
negatively-stained (Figure 2) and thin-sectioned material (Figure 3).
The absence of flagella was substantiated by negatively-stained prepara-
tions of intact cells, but the capsular material surrounding the cell
increases the electron density of such preparations and obscures further
detail (Figure 2). In thin-section profiles, a diplococcoid arrangement
of cells is frequently indicated (Figure 3).

Highly refractile areas as seen in Figure 1 represent granular in-
clusions; usually one per cell, but three in a diplococcus. In electron
micrographs, two classes of inclusions are observed: (1) large amorphous
granules surrounded by a membrane 90 $\overset{o}{A}$ wide; and (2) smaller electron-
transparent areas which are not delimited by a membrane structure (Figure
4). Further evidence for a membrane closely adhering to the undulating
surface of the granules is demonstrated in Figure 5; the actual movement
and displacement of these membranes by the electron beam was observed
during study of negatively-stained preparations.

Photosynthetic apparatus

Intracytoplasmic membranes characterize the photosynthetic apparatus

in which anastomosing tubes function as the pigment-bearing structures.
The width of these membranes as determined from micrographs of osmium-
fixed, thin-sectioned material is 60 $\overset{c}{A}$. In profile, the tubes usually
appear randomly distributed throughout the cytoplasm (Figure 6); however
they were occasionally seen in concentric arrangements (Figure 7) and in
flattened arrays near the cell periphery (Figure 8). Continuity between
the tubular elements and the plasmalemma was frequently observed forming
a tubular intracytoplasmic reticulum (Figure 9). Micrographs of negatively-
stained disrupted cells support this observation (Figures 10 and 11). Thus
the cytoplasm of the cell is divided almost equally into two phases: (1)
the relatively electron-dense extratubular phase containing ribosomes, in-
clusions and nucleoplasm; and (2) the less dense intratubular phase (Fig-
ures 12 and 15). The plasmalemma is not appressed to the innermost layer
of the cell wall and the resulting space has the same appearance as the
intratubular phase of the cell to which it connects.

Surface anatomy

Thin-section profiles   Cells of Rhodothece sp. have an unusual sur-
face anatomy due to several complex extracellular layers. Chapman, Murray,
and Salton (1963) observed somewhat similar structures in cells of Lam-
propedia hyalina and their terminology will be followed. The surface
layers are fragile and easily lost during the preparative techniques for
electron microscopy. Even with utmost care taken to prevent mechanical
damage, relatively few cells were observed with the outer layers intact.
The cell envelope of ·osmium-fixed cells (Figure 12) can be subdivided into
four distinct components: (1) capsule (outermost layer); (2) punctate

layer composed of complex spines projecting outward from the cell; (3)
a perforate layer consisting of overlapping platelike structures; and
(4) a complex cell wall structure similar to other Gram-negative bacteria.

The capsule completely surrounds the cell as indicated in Figures 12
and 14. Fibrous elements extend between the capsule and the spines of
the punctate layer (Figure 14). These fibers may serve to connect the two
layers, represent a diffuse inner component of the capsule, or may be en-
tirely a fixation artifact.

The punctate layer, immediately adjacent to the capsule, (Figures 12
and 14), is characterized by a geometric arrangement of spines each con-
sisting of a tapered bulb on a stalk (Figure 15, insert). The thickness
of the punctate layer is approximately (ca.) 500 $\overset{o}{A}$; the bulbs and stalks
are 270 $\overset{o}{A}$ and 230 $\overset{o}{A}$ long respectively. A bulb is 180 $\overset{o}{A}$ in diameter at its
widest point and the inner electron-transparent region is 60 $\overset{o}{A}$ wide; the
tapered portion or tip is 75 $\overset{o}{A}$ wide. In some profiles of the cell envelope
in median sections of cells, the spines are ca. 200 $\overset{o}{A}$ apart (Figure 12);
whereas a spacing of 180 $\overset{o}{A}$ is observed in other profiles (Figure 15).
These spines, readily seen in oblique sections, appear in rows that are
180 $\overset{o}{A}$ apart measured from center-to-center (Figures 13 and 14).

Most thin-section profiles of osmium-fixed cells lack distal capsules
and punctate layers so that the perforate layer appears outermost (Figure
16). The thickness of this layer is 80 $\overset{o}{A}$ and appears discontinuous in
profile with a periodicity of 80 to 85 $\overset{o}{A}$. In a "plan-view" as seen in
sections tangential to the cell periphery (Figure 19), the layer consists
of 30 $\overset{o}{A}$ densities arranged in a 90 degree lattice with a center-to-center

spacing of 80 Å. Thus, the discontinuities seen in transverse sections

appear to be views of the linear arrays of these densities. Where rows

of these densities are not normal to the plane of section, this layer

appears as a continuous line. The perforate layer is organized as sep-

arate overlapping platelike structures (Figure 16). Fixation employing

osmium in the natural substrate tended to distort these plates, thus

exaggerating the overlapping arrangement (Figure 17).

Structural elements perpendicular to the cell surface are observed

in the 100-200 Å thick "sub-perforate" region located between the perforate

and cell wall layers of osmium-fixed cells (Figure 16); however these ele-

ments are not preserved with either permanganate-fixation (Figure 18), or

with osmium in the natural substrate (Figure 17). Frequently, it appears

as if the spines penetrate this region (Figure 15); the stalks of these

spines probably forming some of these structural elements.

It is difficult to reconstruct the geometry of sectioned cell frag-

ments and to relate the configuration to cell structure. The variable of

an impure culture provides an additional problem; however it is believed

that Figures 20 and 21 represent tangential sections through detached

fragments of the cell envelope probably portions of either the cell wall,

sub-perforate, or perforate layers. Both exhibit hexagonally arranged

subunits, but with different repeat distances. In Figure 21, the pattern

appears skewed such that the center-to-center spacing of the subunits

ranges between 195 Å and 250 Å. A set of parallel densities, each 60 Å

wide, is also observed in this profile with a single row of subunits be-

tween each density. The fragment in Figure 20 has a subunit arrangement

with a center-to-center distance of 120 Å.

The cell wall proper of Rhodothece sp. is located between the rel-
atively diffuse sub-perforate layer and the plasmalemma. In micrographs
of osmium-fixed, sectioned cells, profiles of the cell wall appear multi-
layered (Figure 16); (1) An outer membrane which is an undulating structure
75 Å thick has the appearance of a unit membrane. (2) An inner zone con-
sists primarily of a relatively uniform 35 Å thick electron-dense inner
layer, and scattered less dense local regions of varying thickness beneath
the outfoldings of the undulating outer membrane. In permanganate-fixed
material (Figure 18), the outer membrane does not undulate and is generally
appressed to the dense inner layer so that these less dense regions between
the outer membrane and inner layer are rarely observed. (3) A relatively
electron-transparent space of varying width appears between the inner layer
and the 70 Å thick plasmalemma (Figure 16). This space, the vestibule, as
previously pointed out, is continuous with the intratubular cytoplasm.
After permanganate-fixation this space is greatly expanded suggesting a
plasmolytic effect (Figure 18).

Negative-staining   Fragments of disrupted cells negatively-stained
with PTA often showed a structural pattern consisting of hexagonally
arranged subunits. In most of the observed fragments, the diameter of
the individual subunits was 125 Å with a center-to-center spacing of 230 Å
(Figure 22). The laser-light diffraction pattern from the mask of Figure
22 substantiated the arrangement of subunits as a "perfect" hexagon (Fig-
ure 23). The center-to-center spacing of these subunits calculated from

the optical transform was 232 $\overset{o}{A}$ (see Appendix C). These fragments ap-
peared to have been elements of the cell envelope from their position in
relation to other components of the disrupted cells. Other negatively-
stained fragments revealed a similar hexagonal subunit arrangement, but
the diameter of these individual subunits was ca. 250 $\overset{o}{A}$ with a center-
to-center spacing of 395 $\overset{o}{A}$ (Figure 24). Again a nearly "perfect" hex-
agonal configuration of subunits was confirmed by the diffraction pattern
from the optical transform (Figure 25). The calculated point-to-point
separation was 382 $\overset{o}{A}$ (see Appendix C). None of these fragments were ever
observed in association with other cellular elements so that their rel-
ative position in the cell was never established with certainty.

Phage-like particles

In micrographs of thin-sectioned specimens of Rhodothece sp., intra-
cellular structures resembling phage-heads were occasionally seen in the
nucleoid region of the cell (Figure 26). Phage-like particles also appeared
to be adsorbed to the cell walls of both intact cells (Figure 27) and cell
fragments (Figure 28).

Micrographs of negatively-stained preparations of disrupted cells of
Rhodothece sp. revealed numerous contracted phage-like tails attached to
cell fragments (Figure 29). Particles still intact after cell disruption
appear intermingled with the tubular elements of the photosynthetic appa-
ratus (Figure 30) and occasionally appear to be adsorbed to the membranes

of these elements (Figure 31). The contractile mechanism is peculiar in that the tail core often appears elongate rather than shortened as would be expected due to sheath contraction (Figure 32).

The phage-like particles observed in this study can be morphologically characterized as follows: a head structure 450 $\overset{o}{A}$ wide; tail core 60 $\overset{o}{A}$ wide and 450 $\overset{o}{A}$ long (sheath contracted); and a contractile sheath 200 X 325 $\overset{o}{A}$. Base plate and tail fibers were not observed; however structures resembling typical spikes are present (Figure 33).

<div align="center">Chromatium sp. (pure culture)</div>

General morphology

Despite considerable effort and the employment of various isolation procedures, an organism characteristic of the species Rhodothece was never obtained in pure culture. Instead, pure cultures of a motile, sulfur purple bacterium were repeatedly isolated from "pink pond" specimens. One such culture of this organism was identified as Chromatium sp. strain Ps 668. Examination of specimens with phase-contrast microscopy revealed short rod-shaped cells occurring singly (Figure 34). Size varied in width from 1.5 to 2.0 μ and in length from 3.0 to 4.5 μ. Highly refractile granular inclusions were characteristic of actively growing cells (Figure 34). Profiles of these inclusions seen in thin-sections indicate that they are amorphous, and that at least some are membrane limited (Figure 38). Cells are motile by means of a polar flagellum 120 $\overset{o}{A}$ wide (Figure 35).

In profile, the cell envelope of osmium-fixed cells appears as a

multilayered structure differing from the cell wall of <u>Rhodothece</u> sp. in several respects. In cells from a four day old culture, a dense "encrustation" 150 $\overset{o}{A}$ thick is appressed to the exterior portion of the outer 80 $\overset{o}{A}$ membrane (Figure 36). In turn, this outer membrane is occasionally appressed to the electron-dense 40 $\overset{o}{A}$ thick inner layer; however a less dense region of varying thickness is generally visible between the slightly undulating outer membrane and the inner layer. In places, the 75 $\overset{o}{A}$ plasmalemma is appressed to the inner layer of the wall; whereas, in other areas, an electron-transparent space up to 200 $\overset{o}{A}$ in width appears between the inner layer and the plasmalemma. This space corresponds to the vestibule as observed in cells of <u>Rhodothece</u> sp., but appears less extensive than the latter. It may, at least in part, be the result of plasmolysis.

Spherical vesicles, about 350 $\overset{o}{A}$ in diameter, each bounded by a unit membrane 70 $\overset{o}{A}$ wide constitute the chromatophores which are characteristic of the photosynthetic apparatus of <u>Chromatium</u> spp. (Figure 37).

Cylindrical elements were occasionally observed near the cell periphery. These structures are ca. 250 $\overset{o}{A}$ in diameter and consist of a 60 $\overset{o}{A}$ wide dense central zone or core surrounded by an electron-transparent zone 65 $\overset{o}{A}$ wide, which is bounded by an electron-dense layer 30 $\overset{o}{A}$ wide (Figure 38).

Aging and environmental effects

Osmium-fixed, thin-sectioned cells from a 4 month culture of <u>Chromatium</u> sp. were studied to observe effects of aging (Figure 39). Micrographs of thin-sectioned cells revealed a papillose encrustation covering

the outer membrane of the cell wall. The individual electron-dense papillae measure ca. 300 $\overset{o}{A}$ at their base and 150 $\overset{o}{A}$ in total height. Between papillae, the encrustation appears to be ca. 60 $\overset{o}{A}$ thick and is appressed to the outer membrane as observed in younger cells.

Cells of Chromatium sp. Ps 668 that were placed in dialysis bags and kept in the stabilization pond for 7 days were still identifiable as Chromatium sp. and bore no resemblance to organisms characteristic of the genus Rhodothece. Apparently this environment did not favor growth, and in general the cells resembled those of aged cultures exhibiting many of the usually accepted signs of necrosis. The most prominent cytological features were: the development of myelin-like material and lamellar structures (Figure 40); formation of complex lamellar inclusions or membranous structures (Figures 42 and 43); gross local evaginations of the cell wall (Figure 41); and the proliferation of the 250 $\overset{o}{A}$ wide cylindrical bodies seen occasionally in young cultures (Figure 41).

DISCUSSION

Taxonomic Considerations

The primary criterion for generic classification in the family Thiorhodaceae is based on the development of cell aggregations--an unstable feature under the influence of changing environmental conditions. Thus, three generic possibilities were examined in the final identification of the sulfur purple bacterium in the "pink pond": (1) Thiocapsa; (2) Thiopedia; and (3) Rhodothece. Of the three, the genus Rhodothece was chosen as being most descriptive of the organism, since microscopic examination of pond specimens failed to reveal any colonial aggregations characteristic of the other genera. Cells were always observed as individual diplococci both with light (Figure 1) and electron microscopy (Figure 3); however "it is quite conceivable that the genus Rhodothece is identical with some other genus, e.g., Thiopedia or Lamprocystis, and that these genera represent different growth forms induced by environmental conditions" (Breed et al., 1957). The genus Lamprocystis was not considered since its representatives are characterized as being motile; motility was never observed in the organism under consideration.

Repeated attempts to obtain isolates of Rhodothece sp. in pure culture using the described techniques yielded a characteristically different sulfur purple bacterium designated as Chromatium sp. Ps 668. Cooper (1963) reported a similar situation in which he identified an organism characteristic of the species Thiopedia rosea in "pink pond" specimens, but managed only to obtain isolates of Chromatium vinosum. Such evidence suggested a

possible relationship between organisms of the genera designated Rhodo-
thece and Chromatium. The two alternatives examined were: (1) each
organism represented some stage in a life cycle of a single species; and
(2) each organism was an ecotype of one and the same organism. If the
first assumption was valid, then transitional or intermediate stages of
the two forms ought to be observed at various points in time. There
were no significant morphological or cytological changes in aging cells
of Chromatium sp. Ps 668 that would lend credence to such cyclical events
(Figure 39). In the other case, if the distinctive features of the genus
designated Chromatium were the result of laboratory induced conditions,
then replacing the cells in their original environment would elicit some
features resembling the genus Rhodothece. An experiment designed to test
this hypothesis involved placing cells of Chromatium sp. Ps 668 in dialysis
bags, which were then incubated in the sewage stabilization pond. Although
there was some necrosis, cells cultured in their original environment were
still identifiable as belonging to the genus Chromatium (Figures 40, 41,
42 and 43) and showed none of the striking features of cells of Rhodothece
sp. It was not determined whether the necrotic state of the cells was
directly a result of unfavorable growth conditions within the dialysis
bag or of the pond substrate; however large cell numbers of the genus
Chromatium were not observed in microscopic examination of pond specimens.
Therefore, it seems that the pond substrate does not favor a population
of organisms belonging to this genus, though they probably occur in small
numbers. If the media and environmental conditions used in the isolation
procedures selectively enhanced their growth, then this would account for

their repeated isolation. It seems evident that the representatives

of the two genera are separate and distinct organisms unrelated either

through a cyclical event or as ecotypes.

Cytoplasm

Photosynthetic apparatus

Worden and Sistrom (1964) proposed that the structure of the

photosynthetic apparatus of all sulfur purple bacteria resemble the

lamellae of Rhodospirillum molischianum; however this assumption is not

substantiated in this study. In cells of Rhodothece sp., the photo-

synthetic apparatus appears as an anastomosing network of tubular

structures which topologically consist of the plasmalemma forming a

continuous intracytoplasmic reticulum (Figures 6, 9, and 11). A photo-

synthetic apparatus consisting of a single continuous system of "spaces"

throughout the cytoplasm which are in continuity with the space exterior

to the protoplast proper has not been previously reported. It is true,

that enclosed lamellar elements (chromatophores and thylakoids) of

photosynthetic organisms not only in the bacteria, but also in cells of

blue-green algae and the chloroplasts of higher plants, have been shown

to be derived ontogenetically from invaginations of the surrounding

membranes. However in most cases, these elements lose their continuity

as the cell matures. Such a relationship has a physiological basis in

that bacteriochlorophyll is localized on both the plasmalemma as well as

the intracyoplasmic membranes (Cohen-Bazire and Kunisawa, 1963; Gibbs,

Sistrom, and Worden, 1965). The photosynthetic system in Rhodospirillum

rubrum as described by Holt and Marr (1965c) is somewhat like

the system in cells of <u>Rhodothece</u> sp. The authors believe that this organism possesses many tubular elements in the cytoplasm, each continuous with the "peripheral membrane". These "tubes" are thought to be constricted at intervals, thus giving rise to the many circular profiles characteristic of chromatophores.

Descriptions of the architecture of the photosynthetic apparatus in photosynthetic bacteria differ considerably. These include accounts of: chromatophores (Pardee, Schachman, and Stanier, 1952); chlorobium vesicles (Cohen-Bazire, Pfennig, and Kunisawa, 1964); thylakoids (Menke, 1961); alternately constricted and expanded tubes (Holt and Marr, 1965c); and the ramiform tubular structures described here. Some of the architecture is strikingly different from others, but in some cases the differences may be merely semantic. If there is any unifying concept for this apparent structural diversity of the photosynthetic apparatus, it is that they are all membranous systems. Echlin and Morris (1964) proposed a basic pattern for the photosynthetic apparatus, "basic structure of the membrane system of the photochemical apparatus is a pair of unit membranes enclosing a space...". Observations of the photochemical apparatus in cells of <u>Rhodo-thece</u> sp. point out that this space need not be completely enclosed by unit membranes, and that it may be continuous with the space usually considered as exterior to the protoplast (Figures 6 and 9).

It should be noted that the appearance of the intratubular space is similar to that of the narrow zone between the plasmalemma and the innermost elements of the cell wall. This latter region appears to be a con-

sistent component of these cells, and since it is exterior to but continuous with the intratubular phase of the cytoplasm, it is termed the "vestibule" (Figures 6 and 46).

Inclusions   The intratubular phase of the cytoplasm in cells of Rhodothece sp., in addition to the nucleoplasm and ribosomes was seen to contain three kinds of inclusions. Occasionally, structures resembling phage-heads were observed in the nucleoplasm (Figure 26). These will be discussed later as a separate topic. Two kinds of granules were found in most, if not all, cells.

The sulfur granules are characteristic of the sulfur purple bacteria, which utilize hydrogen sulfide as the hydrogen donor in photosynthesis rather than water as is characteristic of most other photosynthetic organisms. This results in some form of oxidized sulfur stored within the cell, usually as granules of elemental sulfur in young cells or as sulfate in aged cells. These granules are membrane limited and exhibit a slightly undulating surface structure in negatively-stained preparations which aids in their identification (Figures 4 and 6).

The last class of inclusions probably represents deposits of poly-hydroxybutyric acid (PHBA) which appear as non-membrane limited, electron-transparent areas in sectioned material (Figure 4). Stanier, Doudoroff, Kunisawa, and Contoupoulou (1959) gravimetrically determined the presence of PHBA in cells of Rhodospirillum rubrum and proposed that its accumulation represented storage of organic carbon and also reserve reducing power

for CO_2 fixation. Formation of PHBA is strongly favored in a medium containing acetate and H_2S with CO_2 absent; the latter being essential to PHBA utilization. Since cells of Rhodothece sp. appeared to have an abundance of PHBA, this may indicate that the pond contained acetate, with a limited amount of CO_2.

Intracellular deposits of polysaccharide were reported in cells of Chromatium okenii (Kran, Schlote, and Schlegel, 1963). Apparently the presence of CO_2, H_2S and succinate in the growth medium promotes polysaccharide storage. Such deposits were not identified in cells Rhodothece sp.

Membrane limited granules indicative of sulfur deposits were observed in cells of Chromatium sp. Ps 668, but neither PHBA nor polysaccharide granules were present (Figures 38 and 39). Their absence may have been due in part to the omission of acetate and succinate from the growth medium which otherwise would have stimulated the formation of these compounds.

Nothing resembling the "cylindrical structures" seen in Chromatium sp. Ps 668 was observed in cells of Rhodothece sp. These structures are similar to the inclusions described by Kran, Schlote, and Schlegel (1963) in the cytoplasm of Chromatium okenii (Figures 38 and 41). The origin, composition, and function of these structures remain unknown.

Cell Envelope

The complex surface anatomy of organisms belonging to the genus Rhodothece has not been previously reported. The only observation of structures resembling the arrays of spines in the punctate layer was made

on wall components of the apparently unrelated non-photosynthetic species, Lampropedia hyalina (Chapman and Salton, 1962; Chapman, Murray, and Salton, 1963; Pangborn and Starr, 1966). The organisms of both species have some morphological similarities, but are easily distinguishable at the ultra-structural level. Murray (1936b) suggested an evolutionary interrela-tionship among representatives of the following genera: Lampropedia, (heterotrophic); Thiopedia, (thiotrophic); and Merismopedia, (photoauto-trophic). This study partially supports this concept in that unique structural relationships were demonstrated between organisms of the genus Lampropedia and its possible thiotrophic counterpart, the genus Rhodothece.

It would be desirable to relate each layer of the cell envelope as seen in median sections with their corresponding "plan-views" as observed in tangential sections and negatively-stained material. A variety of such. views have been seen. In most cases, it is difficult enough to ascertain whether or not the observed structures are actually from cells of Rhodo thece sp. without further identifying their specific location in the cell envelope. Plan-views of the punctate and perforate layers were seen occa-sionally in sectioned material (Figures 13, 14 and 19); however, it was impossible to accurately relate the position of some ordered structures to the cell envelope (Figures 20, 21, 22, and 24). The problem was complicated in that the spacing of units in plan view did not correlate with any of the periodicities seen in profile. This is not entirely surprising since there could be considerable dimensional differences resulting from the type of fixation and the microscopic preparation. In fact, "fixation" for negative-staining consists of drying-down cells and cell components in PTA, which un-

doubtedly has an effect upon unit dimensions.

Two dimensional arrays of hexagonally arranged densities have been shown by other workers in negatively-stained preparations of cell fragments from several species of bacteria (Chapman and Salton, 1962; Chapman, Murray and Salton, 1963; Thornley, Horne, and Glauert, 1965). Chapman and Salton, (1962) identified one such pattern as the "punctate" layer of Lampropedia hyalina and indeed, the spacing seems in accord with their concept of the relationship of the spines to the underlying "perforate" layer. In negatively-stained preparations of disrupted cells of Rhodothece sp., structures were seen with a hexagonal pattern (Figures 22 and 24); however none of the dimensions corresponded with any conceivable configurations of either the punctate or perforate layers observed in our study. Similar subunit arrangements were reported in negatively-stained cell wall fragments of Micrococcus radiodurans (Thornley, Horne and Glauert, 1965). Since this species does not have structures corresponding to the punctate and perforate layers observed in cells of Rhodothece sp. and Lampropedia hyalina; it is entirely possible that the structures seen in Figures 22 and 24 are components of the cell wall proper. Chapman, Murray and Salton (1963) showed that both negatively-stained and shadowed fragments from cells of L. hyalina exhibit a hexagonal arrangement of "holes". They termed this the "perforate" layer and suggested that it probably lies inside the punctate layer. The authors visualize the spines as being geometrically located in relation to the holes of the perforate layer in a ratio of one spine for every three holes. Such an arrangement would have center-to-center spacings of holes and spines in a ratio of 1:1.73.

Thus, with the spines in cells of <u>Rhodothece</u> sp. spaced 205 Å measured

from center-to-center, the perforate layer should have a hole spacing of

118 Å. Sectioned fragments were observed with such a subunit spacing

(Figure 20); however, there is no positive evidence that these were frag-

ments of cells of <u>Rhodothece</u> sp. It is interesting to note that similar

fragments were observed with double this spacing, 236 Å center-to-center.

The relationship of these fragments to the cellular envelope of <u>Rhodothece</u>

sp. is even more obscure. The only pattern associated with an envelope

fragment that was in a position appropriate for identification as the per-

forate layer is shown in Figure 19. This pattern consists of discrete

elements ca. 80 Å center-to-center in a 90 Å lattice, which is not in-

compatible with the periodicity of the perforate layer as seen in Figure

16. In profile, it appears as if the spines radiate from every other

density of this layer (Figure 12, 15 and 44).

The cell wall proper of cells of <u>Rhodothece</u> sp. consists of an un-

dulating unit membrane in contact with the sub-perforate region and a

discrete inner layer of uniform thickness (Figures 16 and 18). Unlike

the generally accepted model of the wall of a gram-negative bacterium,

the inner layer is discrete and not completely appressed throughout to the

inner portion of the outer membrane (Figures 16 and 17). Also the elec-

tron-transparent vestibule intervenes in certain areas between the inner

layer and the plasmalemma (Figure 16). It is interesting, that the inner

layer of the wall of <u>Chromatium</u> sp. Ps 668 also differs from the general

model, and has a similar relationship to the outer membrane and the plas-

malemma as observed in cells of <u>Rhodothece</u> sp. (Figure 36). Another

difference is a 150 $\overset{o}{A}$ thick "encrustation" that is distal to the outer

membrane. This electron-dense structure is so closely appressed to the

outer membrane that the latter is barely distinguishable from the adjacent

encrustation. In older cultures, this encrustation appears papillose;

never being less than 60 $\overset{o}{A}$ and only at each papilla is the original 150 $\overset{o}{A}$

thickness observed. It is possible that this variation in thickness may

be due to local differences in "plasticity" as the cell enlarges during

growth; the encrustation stretching at some points and not others. On

the other hand, they may result from local losses or erosion of the en-

crustation leaving the papillae as vestiges of the original intact en-

crustation.

The cell envelopes of cells of <u>Rhodothece</u> sp. and of young and old

cells of <u>Chromatium</u> sp. Ps 668 are represented schematically in Figures

45, 46 and 47 respectively.

Virus-like Particles

The observations in this study suggest that structures morphologically

resembling in some respects the T strains of bacteriophage occur in asso-

ciation with cells of <u>Rhodothece</u> sp. The existing evidence for a phage

infecting photosynthetic bacteria is based on "guilt by association",

since such a relationship was observed solely through electron microscopic

studies. There are two alternatives for the association of these phage-

like particles with cells of <u>Rhodothece</u> sp.: (1) non-host specific in

adsorption to receptor sites; and (2) true host-parasite relationship.

Evidence for the latter assumption is seen in Figure 26 in which phage
head-like structures appear in the nucleoid region of the cell suggesting
a phase in the normal vegatative cycle. However, the adsorption of num-
erous tail structures and intact particles to both wall fragments and
tubular structures of the photosynthetic apparatus, suggests a non-spe-
cific adsorption (Figures 29, 31 and 32). These observations though in-
conclusive could provide the stimulus for the exploration and subsequent
isolation of a phage and its photosynthetic host. Sistrom and Worden
(1964) have reported the isolation of both carotenoid and photosynthetic
mutants of the photosynthetic bacterium, Rhodopseudomonas spheroides.
A phage vector used with such a series of mutants could provide a system
of studies for the elucidation of loci involved in light reactions of
photosynthesis.

SUMMARY

The pink coloration of the swine waste stabilization pond located on the Iowa State University Agricultural Experimental Farm is attributed to the sulfur purple bacterium identified as Rhodothece sp. The organism has an unusual surface anatomy characterized by extracellular layers which completely surround individual cells. These surface layers consist of: a capsule; a 500 $\overset{o}{A}$ wide punctate layer composed of a geometric arrangement of spines; and a 80 $\overset{o}{A}$ perforate layer appearing as overlapping platelike structures. The space between the perforate layer and the outer membrane of the wall is termed the "sub-perforate" region. The cell wall proper consists of an outer undulating 75 $\overset{o}{A}$ wide membrane and a discrete 35 $\overset{o}{A}$ thick electron-dense inner layer which surrounds the 70 $\overset{o}{A}$ wide plasmalemma. A "vestibule" is designated as that space between the inner layer and plasmalemma which appears continuous with the intratubular cytoplasm. The architecture of the photosynthetic apparatus is described as an anostomosing network of large tubular structures. Phage-like structures were observed in association with cells of Rhodothece sp., but a true host-paracite relationship was not established.

A sulfur purple bacterium designated as Chromatium sp. Ps 668 was isolated from the "pink pond". Aging and environmental studies indicated that the organism was distinct from cells of Rhodothece sp. The outer membrane of the cell wall of Chromatium sp. Ps 668 appeared encrusted becoming papillose with age. Typical chromatophores represent the photochemical apparatus. Cylindrical structures of undetermined function and composition were observed in both young and aged cells of Chromatium sp.

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APPENDIX A: FIXATION, DEHYDRATION AND EMBEDDING

Fixation

Osmium tetroxide

1. 1.0% osmium tetroxide in Michaelis buffer at pH 6.1 for 3 hr

   at 25 C (Kellenberger, Ryter, and Séchaud, 1958)

   a. 5 ml Michaelis buffer

$NaC_2H_3O_2$ $3H_2O$	1.94 g
Sodium diethylbarbiturate	2.94 g
NaCl	3.4 g
Distilled water	q.s. 100 ml

   7 ml 0.1N HCl

   13 ml distilled water

   0.25 ml 1.0M $CaCl_2$

   b. Dilute mixture (a) with 2% osmium tetroxide 1:1

2. 1.0% osmium tetroxide in liquid sewage at pH 7.4 for 3 hr

   at 25 C

   a. 10 ml of liquid sewage from stabilization pond

   b. Dilute mixture (2) with 2% osmium tetroxide 1:1

Glutaraldehyde

1. 3% glutaraldehyde in phosphate buffer at pH 7.2 and 4 hr

   at 25 C (Sabatini, Bensch, and Barrnett, 1963); post-

   fixation with 1% osmium tetroxide

   a. Phosphate buffer

0.1M KH_2PO_4	13 ml

$$0.1M \ Na_2HPO_4 \qquad\qquad 37 \ ml$$

    b. Dilute phosphate buffer (a) with 50% glutaraldehyde to

        obtain a final concentration of 3%

    c. Rinse specimens 3 x 5 minutes in phosphate buffer prior

        to osmium fixation as described above.

Potassium permanganate

1.   2% $KMnO_4$ in liquid sewage at pH 7.4 for 15 min at 25 C

    a. 10 ml of liquid sewage from stabilization pond

    b. Add 10 ml of filtered (Millipore) 4% $KmnO_4$ to mixture (a)

    c. Rinse specimen after fixation with several changes of 30%

        ethanol until solution is no longer colored.

Dehydration and Embedding

All specimens were dehydrated and embedded in Epon 812 according to a procedure modified from Luft (1961) as follows:

Schedule

1.   5 minutes each in 50, 70, and 95 percent ethanol at 25 C

2.   3 rinses of 5 minutes each in 100 percent ethanol at 25 C

3.   3 rinses of 5 minutes each in propylene oxide at 25 C

4.   15, 30, and 45 minutes in the respective Epon-propylene oxide

       mixtures of 1:3, 1:1, and 3:1 at 25 C

5.   12 hours at 25 C in 100 percent Epon swirled in specimen vials

       on a slow rotating mixer

6.   Embeddment in shallow aluminum boats or Beem (LKB) capsules

7. Stepwise polymerization at 35 C for 12 hours, 45 C for 12 hours and 60 C for 3 to 5 days

Epon 812

1. Mixture A

   a. 62 ml of Epon 812

      100 ml of Dodecenyl succinic anhydride (DDSA)

2. Mixture B

   a. 100 ml of Epon 812

      89 ml of Nadic Methyl Anhydride (NMA)

3. Add mixture A to B in 3:2 ratio

4. Add 0.2 ml of DMP-30 per 10 ml of Epon mixture

APPENDIX B: MEDIA

Enrichment Medium

Enrichment medium for sulfur purple bacteria as described by Stanier, Doudoroff, and Adelberg (1963, p. 458) as follows:

Compound	Grams per liter
$MgSO_4 \cdot 7H_2O$	0.2
K_2HPO_4	1.0
$FeSO_4 \cdot 7H_2O$	0.01
$CACl_2$	0.02
$MnCl_2 \cdot 4H_2O$	0.002
$NaMoO_4 \cdot 2H_2O$	0.001
$NaCl$	0.5
NH_4Cl	1.0
$Na_2S \cdot 9H_2O$	1.0
$NaHCO_3$	5.0

Growth conditions

The special environmental features for growth of organisms in the enrichment medium described are as follows:

1. Adjustment of pH to 8.0-8.5

2. Culture in completely filled glass-stoppered bottles

3. Constant illumination

4. Incubate at 25 C

Culture Medium

Preparation of a defined synthetic medium for red and green sulfur bacteria as described by Pfennig (1961; 1962)

Solution 1

1.	$CaCl_2$ (anhydrous)	2 g
2.	Distilled H_2O	2500 ml

a. Approximately 500 ml are autoclaved in an Erlenmeyer flask and the remaining 2000 ml are distributed in amounts of 75 to 80 ml into 4 oz screw capped bottles and autoclaved. The bottles are refrigerated and kept at approximately 4 C before adding solutions 2 and 3 (below).

Solution 2

1.	Distilled water	32 ml
2.	Heavy metal solution	50 ml
3.	Vitamin B_{12} solution	3.0 ml
4.	KH_2PO_4	1.0 g
5.	KCl	1.0 g
6.	$MgCl_2 \cdot 6H_2O$	0.8 g
7.	NH_4Cl	0.8 g
8.	Na-ascorbate	2.4 g
9.	Vitamin solution	15 ml

a. If a small inoculum is used, then the Na-ascorbate can be eliminated.

b. 1.0 ml Betalin Complex (Eli Lilly) can be substituted for
the vitamin solutions

Solution 3

1.	$NaHCO_3$	4.5 g
2.	Distilled water	100 ml

a. Bubble gaseous CO_2 through the solution for at least 30
minutes or until the pH is 6.1

Solution 4

1.	$Na_2S. 9H_2O$	3.0 g
2.	Distilled water	200 ml

a. Add a magnetic stirring rod to the flask before autoclaving
the solution

Heavy metal solution

1.	Ethylendiaminetetraacetic acid (EDTA) (Tetra-sodium salt)	1.5 g
2.	Modified Hoaglund-trace-element-solution	6.0 ml
3.	$FeSO_4. 7H_2O$	200 mg
4.	$ZnSO_4. 7H_2O$	100 mg
5.	$MnCl_2. 4H_2O$	20 mg
6.	Distilled water	1000 ml

Vitamin B_{12} solution

1.	Vitamin B_{12}	200 mg/100 ml

Vitamin solution

1.	Biotin	0.2 mg
2.	Nicotinic acid	2.0 mg

3.	Thiamine	1.0 mg
4.	p-Aminobenzoic acid	1.0 mg
5.	Pantothenic acid	0.5 mg
6.	Pyridoxamin-HCl	5.0 mg
7.	Distilled H_2O	100 ml

Modified "Hoaglund-trace-element-solution"

1.	$AlCl_3$	1.0 g
2.	KI	0.5 g
3.	KBr	0.5 g
4.	LiCl	0.5 g
5.	$MnCl_2 \cdot 4H_2O$	7.0 g
6.	H_3BO_3	11.0 g
7.	$ZnCl_2$	1.0 g
8.	$CuCl_2$	1.0 g
9.	$NiCl_2$	1.0 g
10.	$CoCl_2$	1.0 g
11.	$SnCl_2 \cdot 2H_2O$	0.5 g
12.	$BaCl_2$	0.5 g
13.	Na_2MoO_4	0.5 g
14.	$NaVO_3 \cdot H_2O$	0.1 g
15.	Selenium salt	0.5 g

a. Each salt is dissolved separately in distilled water with a total final volume of 3.6 liters. Each salt solution is adjusted below pH 7.0 prior to mixing and the pH of the final salt solution is adjusted

to pH 3-4. The immediate formation of a flaky yellow precipitate transforms to a fine white precipitate within a few days.

b. 5 ml of commercially prepared trace element solution (Aquarium Systems) can be substituted for "Hoaglund-trace-element-solution"

Solution (2) is added to Solution (3) after CO_2 saturation and the mixture is immediately filter sterilized using CO_2 pressure with a Millipore filter. The sterile mixture is added to the 4 oz bottles containing the cold 75 to 80 ml $CaCl_2$ (Solution 1).

The sterilized Solution (4) is partially neutralized by adding dropwise 2 ml of sterilized 2.0M H_2SO_4 while stirring on a magnetic stirrer. The partially neutralized solution is added to the 4 oz bottles in 6 ml amounts. The bottles are nearly filled with Solution (1) from the 500 ml flask which was autoclaved separately. The bottles are immediately sealed leaving only a small air bubble. If the procedure is followed exactly the final pH will be 6.6-6.8.

The freshly prepared medium appears slightly turbid because of oxidation of some H_2S to elemental sulfur by dissolved oxygen. The turbidity disappears after storage of the medium for 1 to 2 days and a slight black sediment is formed. Tightly closed bottles should be stored in the dark and the medium will remain stable for several months.

Disappearance of the chalky characteristic of the culture indicates that the organisms have depleted both the H_2S and the stored sulfur, thereby requiring the addition of neutralized Na_2S solution. A suitable volume of Solution (4) is neutralized by adding dropwise sterile 2.0 M H_2SO_4 until a slight sulfur turbidity appears. The tubidity will dissipate if an ex-

cess of H_2SO_4 is not added. The slightly yellow neutralized solution is added in the amount of 5 to 6 ml to the sulfur-depleted cultures. The cultures should be kept in the dark for a few hours after the addition of the sulfide solution.

Modified heavy metal solution (personal communication, Pfennig, 1966)

1. Ethylendiaminetetraacetic acid (EDTA)   500 mg

2. $FeSO_4$. 7 H_2O 200 mg

3. $ZnSO_4$. 7 H_2O 10 mg

4. $MnCl_2$. 4 H_2O 3 mg

5. H_3BO_3 30 mg

6. $CoCl_2$. 6 H_2O 20 mg

7. $CuCl_2$. 2 H_2O ·1 mg

8. $NiCl_2$. 6 H_2O 2 mg

9. Na_2MoO_4 . 2H_2O 3 mg

10. Distilled water   1000 ml

a. The solution should have a pH of 3-4

b. Add 10 ml per liter of culture medium

c. Reduce $CaCl_2$. 2 H_2O concentration to 1.0 g per 3 liters

of final culture medium.

APPENDIX C: CALCULATIONS

Calculations for Laser-Light Diffraction Patterns

Calibration

1. 200 mesh copper electron microscopic grid

2. 0.985 mm on mask (positive plate) corresponds to 1.000 mm on transform (negative)

Lattice dimensions (Fig. 23)

1. Spot-to-center = 3.24 mm on transform

2. 0.985/3.24 = 0.321 mm point separation on mask

3. 0.321 mm @ 15,500 = 0.321/17,100 = 201 $\overset{o}{A}$

4. 201 $\overset{o}{A}$ = separation of lattice lines

5. Uniform spacing in 6 directions (60° separation); therefore perfect hexagon

    a. $x = 60^o$

    b. $\sin x = 0.8660$

    c. $\sin x = 201/x$

    d. $x \sin x = 201$

    e. $x = 188/\sin x = 188/0.8660 = 232$ $\overset{o}{A}$

6. 232 $\overset{o}{A}$ = center-to-center distance

Lattice dimensions (Fig. 25)

1. Spot-to-center = 1.93 mm on transform

2. 0.985/1.93 = 0.513 mm on mask

3. 0.513 mm @ 15,500 = 0.513/17,100 = 302 $\overset{o}{A}$

4. 331 $\overset{o}{A}$ = separation of lattice lines

5. x = 331 $\overset{o}{A}$ (same as Step 5 above)

6. 382 $\overset{o}{A}$ = center-to-center distance

APPENDIX D: ABBREVIATIONS

b	-	bulb	ɳp -	nucleoplasm
ch	-	chromatophore	om -	outer membrane
cl	-	cylindrical structures	p -	particle
cp	-	capsule	pc -	punctate layer
cs	-	contractile sheath	pf -	perforate layer
cw	-	cell wall	pl -	plasmalemma
ec	-	encrustation	pt -	plate
et	-	electron-transparent	sf -	sub-perforate
f	-	fiber	sk -	spike
g	-	granule	sp -	spine
h	-	head	t -	tubular structure
il	-	inner layer	tc -	tail core
la	-	lamellae	v -	vestibule
ms	-	membranous structure		

APPENDIX E: FIGURES
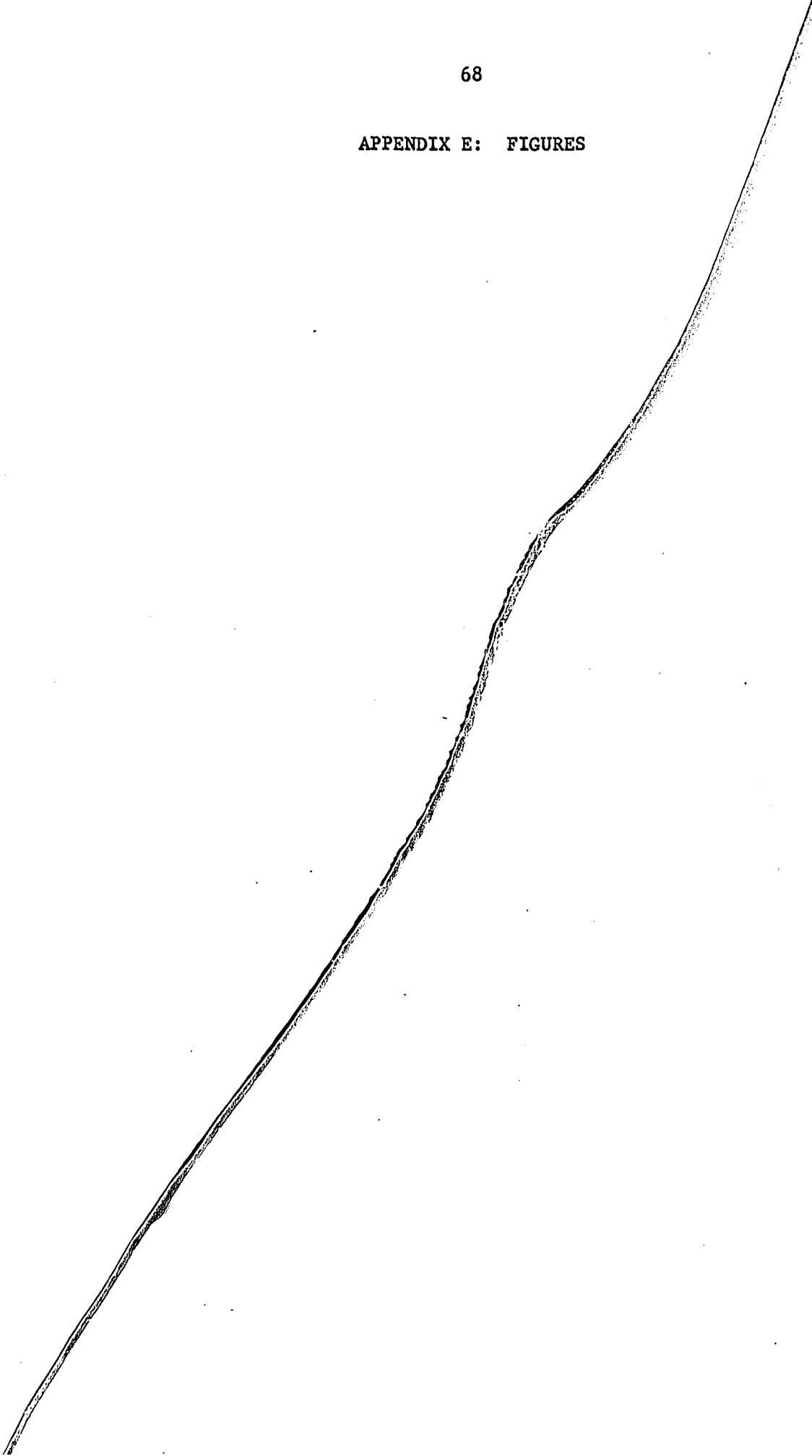
Figure 1. Phase-contrast photomicrograph of living cells from pond
           specimen. Spherical cells arranged in pairs with intra-
           cellular granules appearing as highly refractile areas (g).

Figure 2. Capsule (cp) surrounding cell of Rhodothece sp. negatively-
           stained with PTA.

Figure 3. Diplococcoid arrangement of cells of Rhodothece sp. Osmium-
           fixation with methanol uranyl acetate staining.

Figure 4. Portion of cell of Rhodothece sp. revealing an amorphous
           granule (g) surrounded by a membrane (arrow) and electron-
           transparent areas not surrounded by a membrane (et).

Figure 5. Granule (g) negatively-stained with PTA showing undulating
           surface. Note partial displacement of the surrounding mem-
           brane (arrows).
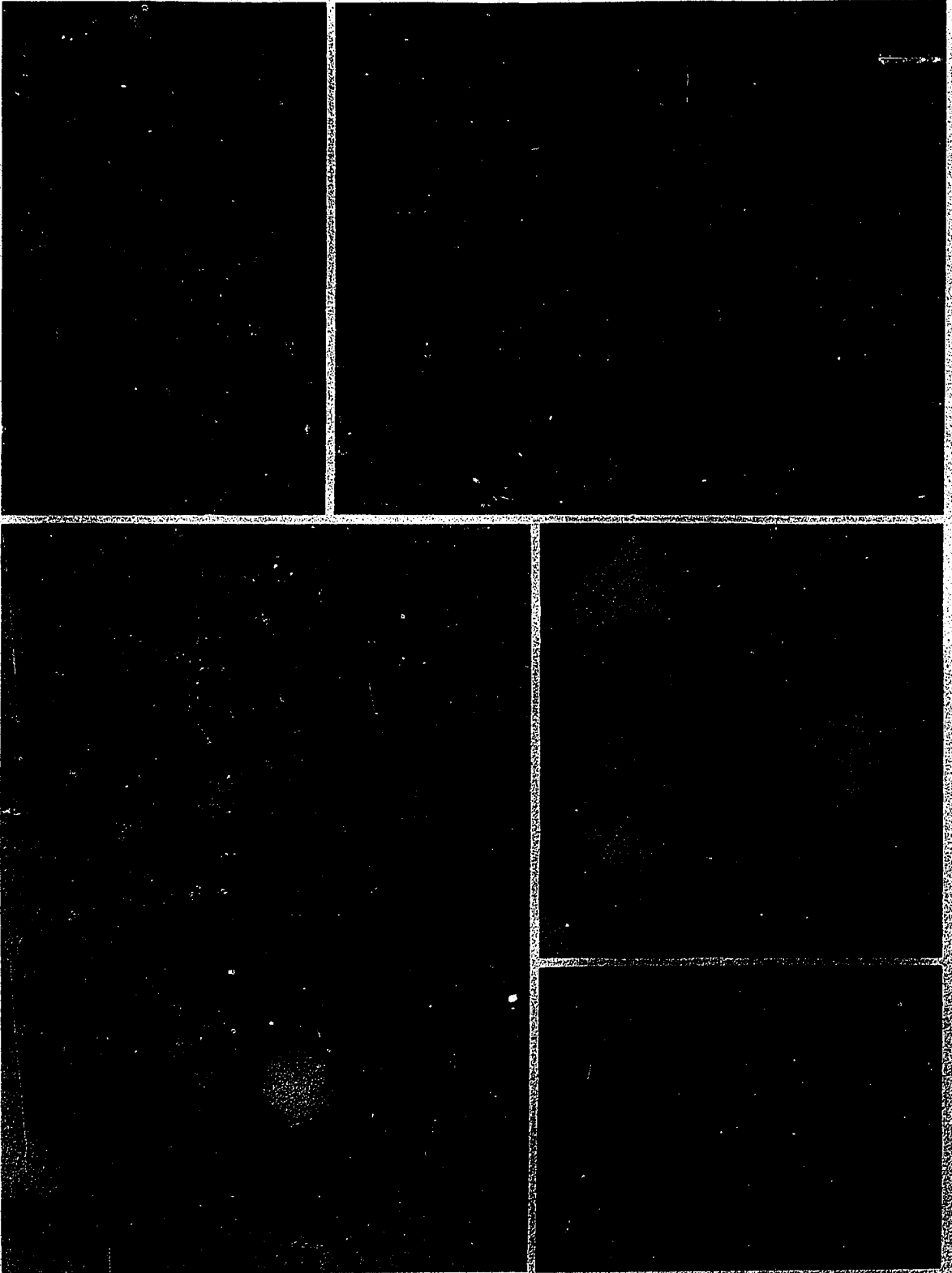
Figure 6.   Portion of cell of <u>Rhodothece</u> sp. Ramifying tubular struc-
            tures (t) randomly dispersed throughout the cytoplasm. Note
            vestibule (v) located between the plasmalemma and the inner-
            most elements of the cell wall. Osmium-fixation with methanol
            uranyl acetate staining.

Figure 7.   Portion of a cell of <u>Rhodothece</u> sp. Tubular structures (t)
            are arranged concentrically near the cell periphery. Per-
            manganate-fixation.

Figure 8.   Portion of a cell of <u>Rhodothece</u> sp. Parallel tubular struc-
            tures (t) appear in a flattened array at the cell periphery.
            Permanganate-fixation.

Figure 9.   Portion of a cell of <u>Rhodothece</u> sp. Indication of continuity
            between the plasmalemma and membranes of the tubular struc-
            tures (arrows). Permanganate-fixation.

Figure 10. Fragment of tubular system from distrupted cells of <u>Rhodothece</u>
            sp. negatively-stained with PTA.

Figure 11. Partially disrupted cell of <u>Rhotothece</u> sp. negatively-stained
            with PTA revealing an anastomosing three-dimensional network
            of tubular structures (arrow).
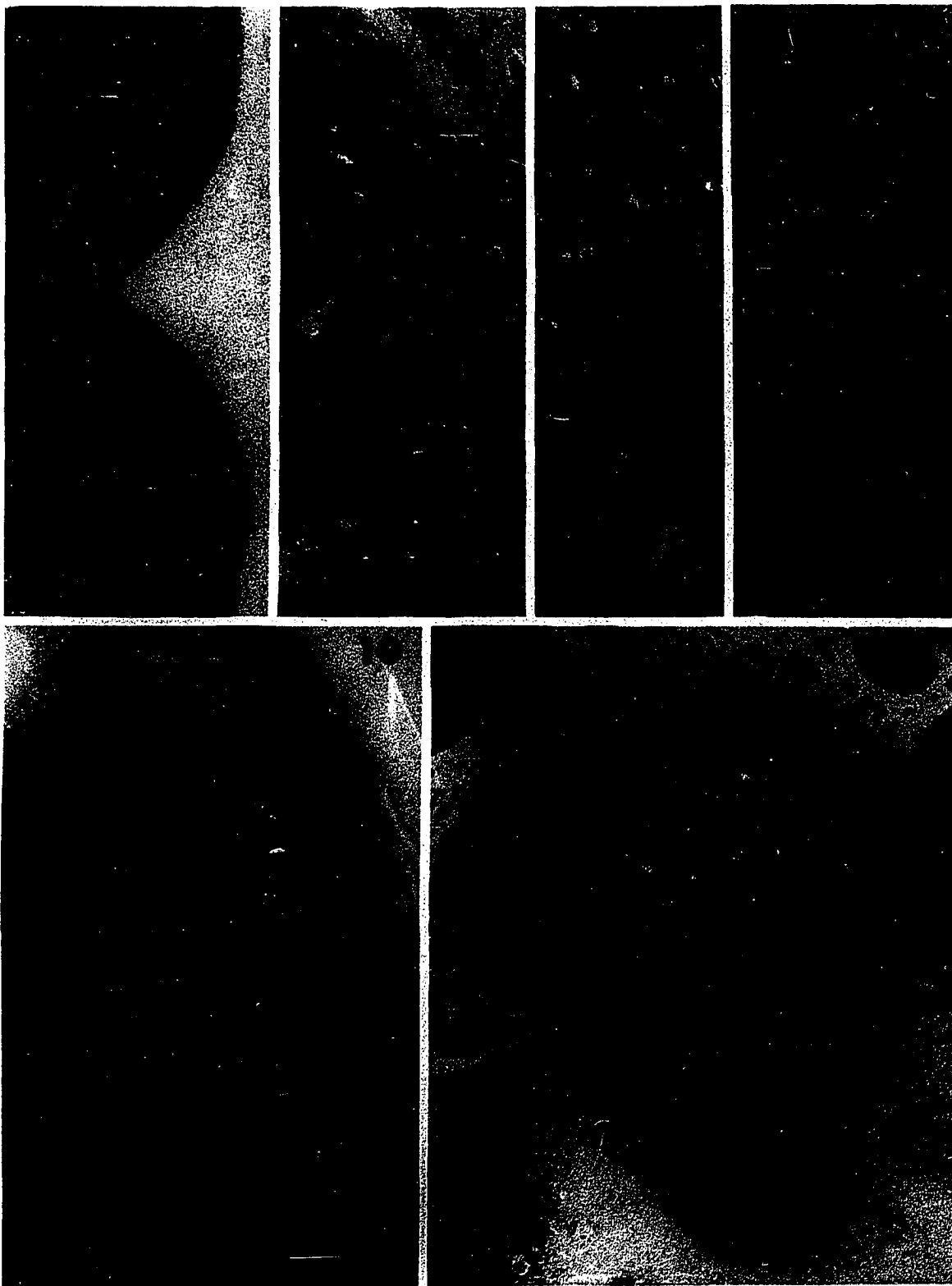
Figure 12.   Median section of a cell of <u>Rhodothece</u> sp. The surface
             layers (sl) consist of an outer capsule (cp) surrounding
             a punctate layer (pc) composed of spines which appear to
             penetrate the adjacent perforate layer (pf). The sub-per-
             forate (sf) region is located between the perforate layer
             and cell wall proper (cw) and adjacent plasmalemma (pl).
             Osmium-fixation with methanol uranyl acetate staining.

Figure 13.   Oblique section through paired cells of <u>Rhodothece</u> sp.
             revealing ordered configuration of the punctate layer
             (arrows). Osmium-fixation with methanol uranyl acetate
             staining.

Figure 14.   Tangential section of a cell of <u>Rhodothece</u> sp. showing
             spines of punctate layer (arrow) and fibers (f) extending
             between the capsule and punctate layer. Osmium-fixation
             with methanol uranyl acetate staining.
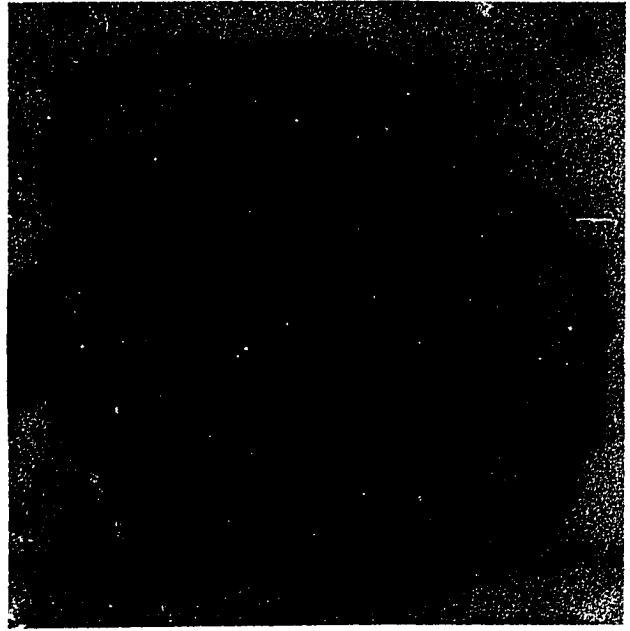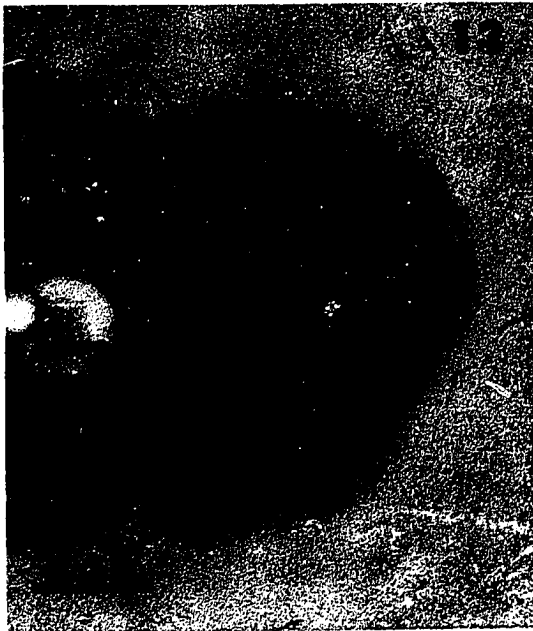
Figure 15. Median section of a cell of _Rhodothece_ sp. showing punctate layer of spines (sp) completely surrounding cell. The spines (insert) consist of bulbs (b) supported by stalks (st). Osmium-fixation with methanol uranyl acetate staining.
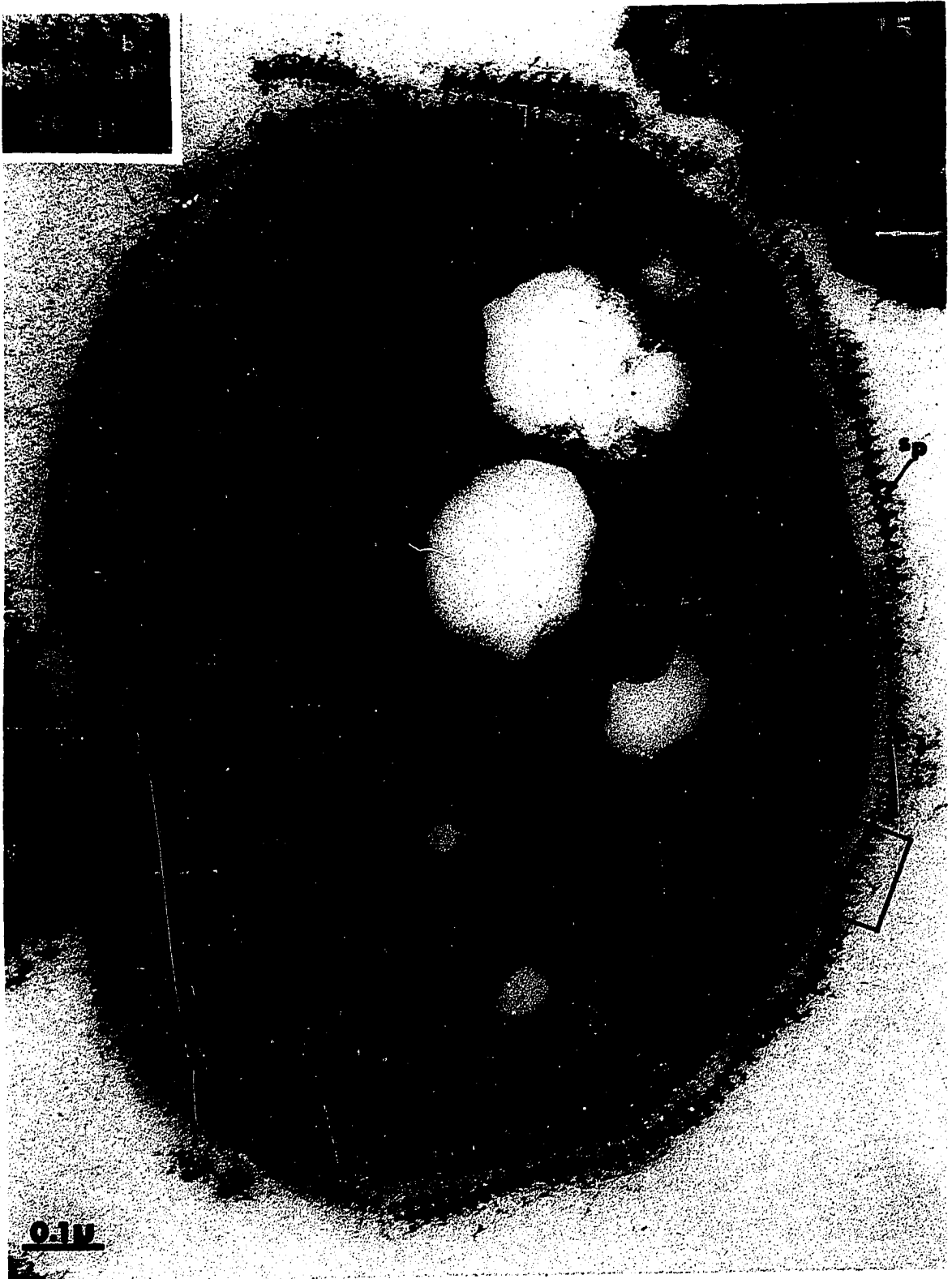
Figure 16.   Portion of a cell of _Rhodothece_ sp. lacking punctate layer.
             The outermost layer of the cell envelope is the perforate
             layer consisting of platelike structures (pt).   Subunit
             structure of the plate appears as an alternating series of
             electron dense and transparent areas.   The sub-perforate
             region also shows an alternating pattern though less defined
             than the perforate layer (arrow).   The cell wall is composed
             of an undulating outer membrane (om) and an innter electron
             dense layer (il).   The vestibule (v) separates the plas-
             malemma from the cell wall.   Osmium-fixation with methanol--
             uranyl acetate staining.

Figure 17.   Portion of a cell of _Rhodothece_ sp. demonstrating the plate-
             like structure (pt) of the perforate layer.   Note apparent
             lack of sub-perforate region.   Osmium-fixation employed in
             natural substrate with methanol uranyl acetate staining.

Figure 18.   Portion of a cell of _Rhodothece_ sp. revealing a three-
             layered cell envelope: an outer membrane (om); an inner
             layer (il); and the plasmalemma (pl).   Note greatly ex-
             panded vestibule (v) which may be a fixation artifact.
             Permanganate-fixation.

Figure 19.   Tangential section of the perforate layer of _Rhodothece_ sp.
             revealing structural periodicity (arrows).   Permanganate-
             fixation with methanol uranyl acetate staining.

Figure 20.   Oblique section of detached cell fragments from cells of
             Rhodothece sp. demonstrating a hexagonal pattern (arrows).
             Glutaraldehyde-fixation followed by osmium post-fixation
             with methanol uranyl acetate staining.

Figure 21.   Tangential section of detached cell fragment from cells of
             Rhodothece sp. revealing a hexagonal arrangement of subunits
             (arrows).   Note parallel electron dense lines running from
             upper-left to lower-right.   Osmium-fixation with lead citrate
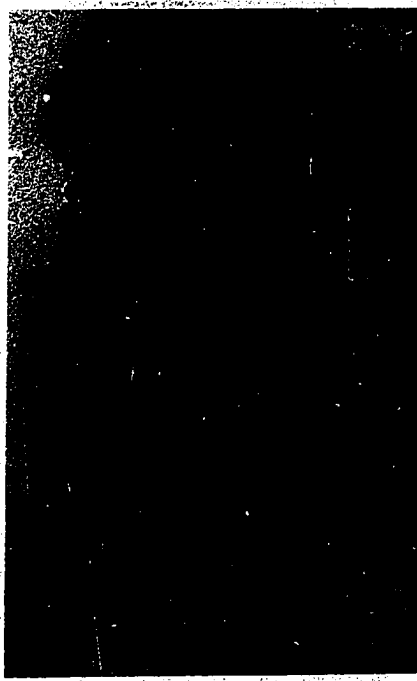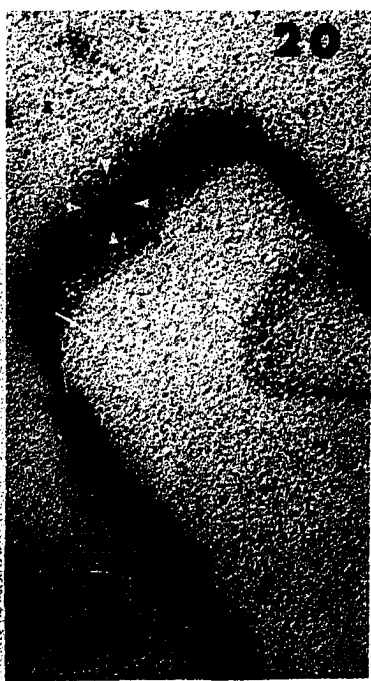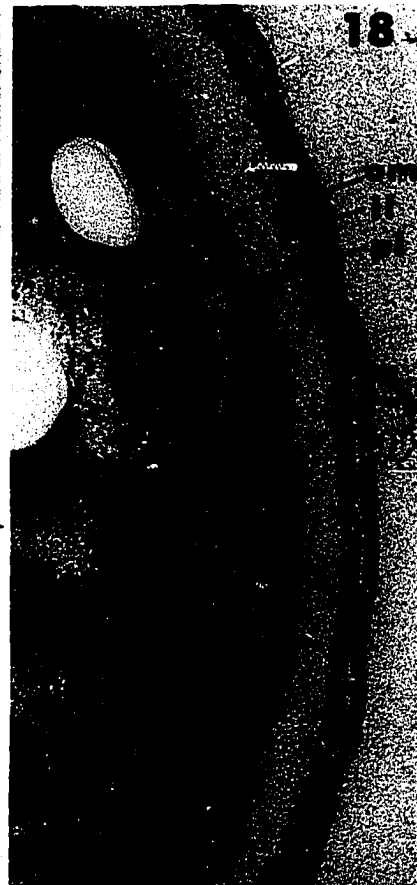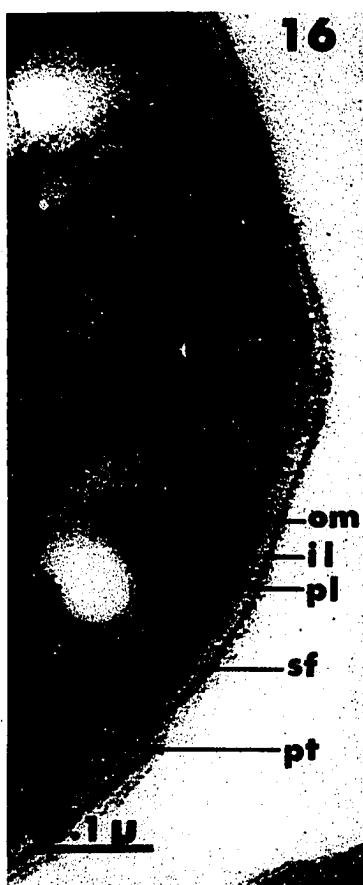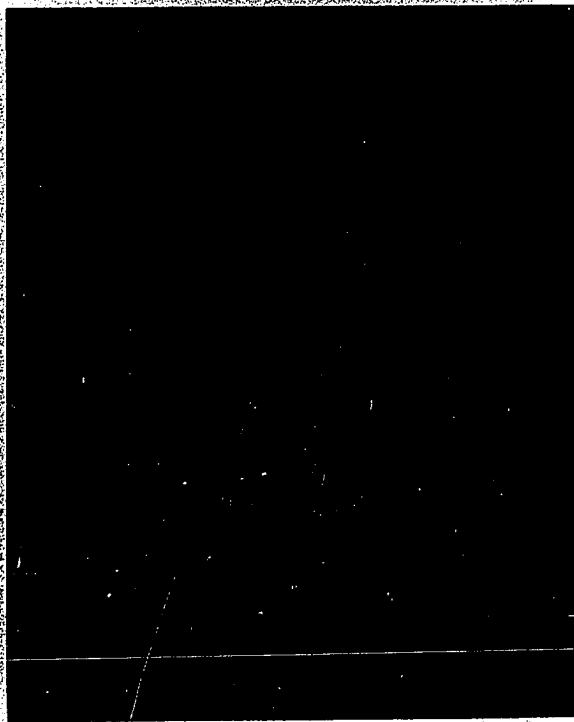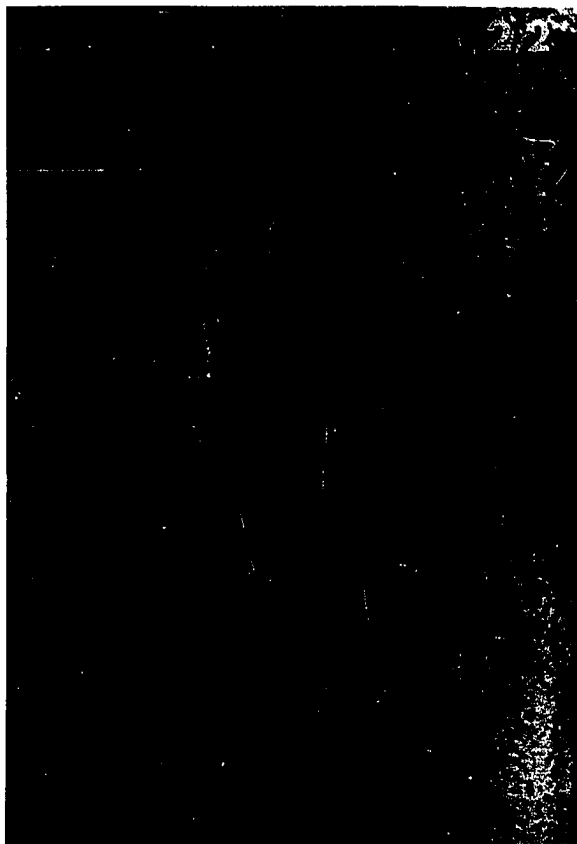   staining.

om
il
pl
sf
pt

Figure 22. Cell fragment of Rhodothece sp. negatively-stained with PTA.
Individual subunits arranged in a hexagonal configuration
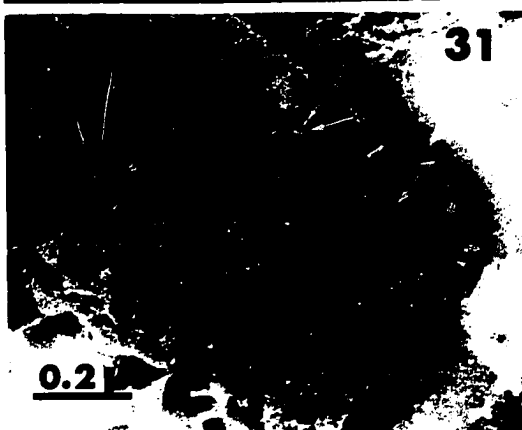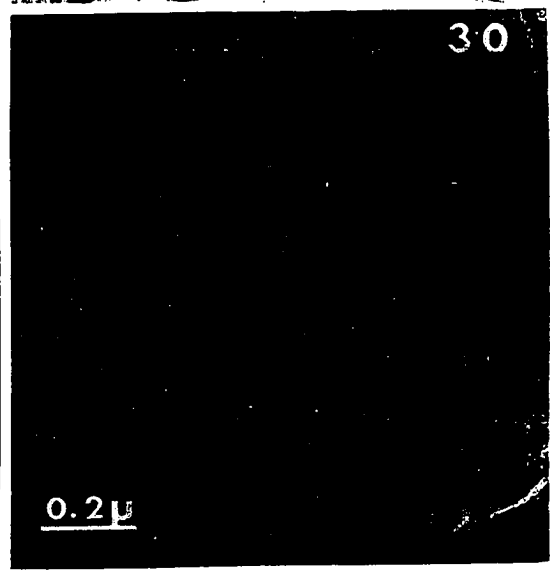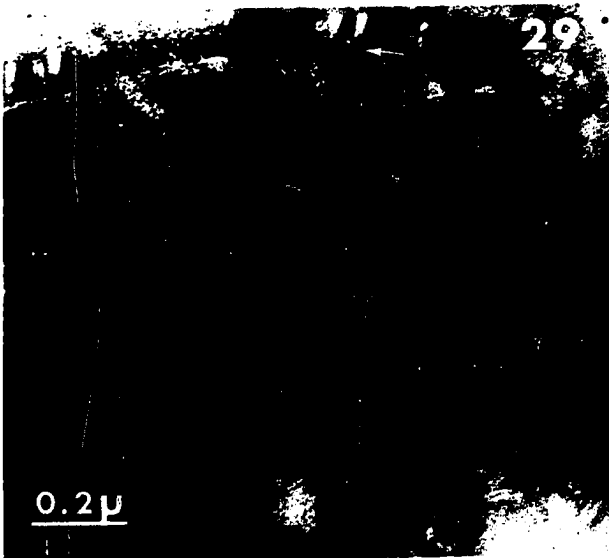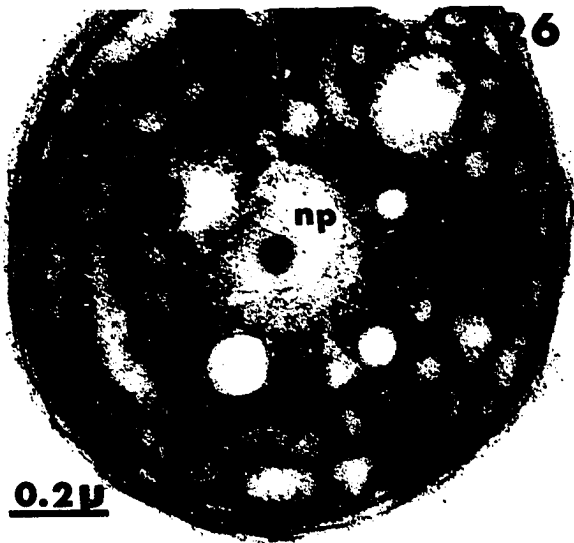(arrows).

Figure 23. Laser-light optical transform of Figure 22. The diffraction
pattern reveals a uniform spacing in six directions indicat-
ing a "perfect" hexagonal arrangement of subunits with a
point separation of 232 Å. Transform enlargement x 4.8.

Figure 24. Fragment from a disrupted cell of Rhodothece sp. negatively-
stained with PTA. Individual subunits arranged in a hex-
agonal configuration (arrows).

Figure 25. Laser-light optical transform of Figure 24. The diffraction
pattern indicates a nearly "perfect" hexagonal arrangement
of subunits having a point separation of 382 Å. Transform
enlargement x 4.8.

Figure 26. Median section of a cell Rhodothece sp. revealing virus-
            like particles (p) in the region of the nucleoplasm (np).
            Osmium-fixation with methanol uranyl acetate staining.

Figure 27. Profile of intact cell of Rhodothece sp. showing attach-
            ment of virus-like particle to the wall layers. Osmium-
            fixation with methanol uranyl acetate staining.

Figure 28. Profile of a disrupted cell of Rhodothece sp. showing virus-
            like particles attached to wall layers. Glutaraldehyde-
            fixation followed by osmium post-fixation with methanol
            uranyl acetate staining.

Figure 29. Disrupted cell of Rhodothece sp. negatively-stained with PTA.
            Structures resembling phage tails appear to be attached to
            elements of the wall (arrows).

Figure 30. Disrupted cell of Rhodothece sp. negatively-stained with PTA.
            Virus-like particles are intermingled with the tubular struc-
            tures of the photosynthetic apparatus (arrows).

Figure 31. Disrupted cell of Rhodothece sp. negatively-stained with PTA.
            Phage-like particles appear attached to the membranes of the
            tubular structures of the photosynthetic apparatus (arrows).

Figure 32. Wall fragment of Rhodothece sp. negatively-stained with PTA.
            Particles appear to have a longer tail core than is usual
            for attached phage (arrow).

Figure 33. Virus-like particles negatively-stained with PTA showing head
            (h), tail core (tc), contractile sheat (cs), and spikes (sp).

26

27

28

29

30

31

np

0.2 μ

50 mμ

0.2 μ

0.2 μ

0.2 μ

50 mμ

Figure 34. Phase-contrast photomicrograph of living cells of Chromatium
sp. Ps 668. Individual cells appear as short rods (arrow).
Highly refractile areas represent deposits of numerous in-
tracellular granules (g).

Figure 35. Cell of Chromatium sp. Ps 668 negatively-stained with PTA
revealing a polar flagellum (arrow).

Figure 36. Portion of a cell of Chromatium sp. Ps 668 showing the cell
wall consisting of encrustations (ec) upon the outer membrane
(om) and an inner layer (il). An electron transparent space
(arrows) exists in some areas between the slightly undulating
outer membrane and inner layer. A zone (v) corresponding to
the vestibule in cells of Rhodothece sp. exists between the
inner layer and plasmamlemma (pl). Osmium-fixation with
methanol uranyl acetate staining.

Figure 37. Portion of a cell of Chromatium sp. Ps 668 in which chrom-
atophores (ch) appear as discrete spherical structures
limited by unit membranes (arrow). Osmium-fixation with
methanol uranyl acetate staining.

Figure 38. Portion of a cell of Chromatium sp. Ps 668 revealing cylin-
drical structures (cl) near the cell periphery and intra-
cellular granular inclusions (g). Osmium-fixation with
methanol uranyl acetate staining.

Figure 39. Longitudinal section of a 4 month cell of Chromatium sp. Ps
668 revealing papillose encrustations projecting from the
outer membrane of the cell wall (arrows). Granules (g) and
membranous structures (ms) appear in the cytoplasm. Osmium-
fixation with methanol uranyl acetate staining.
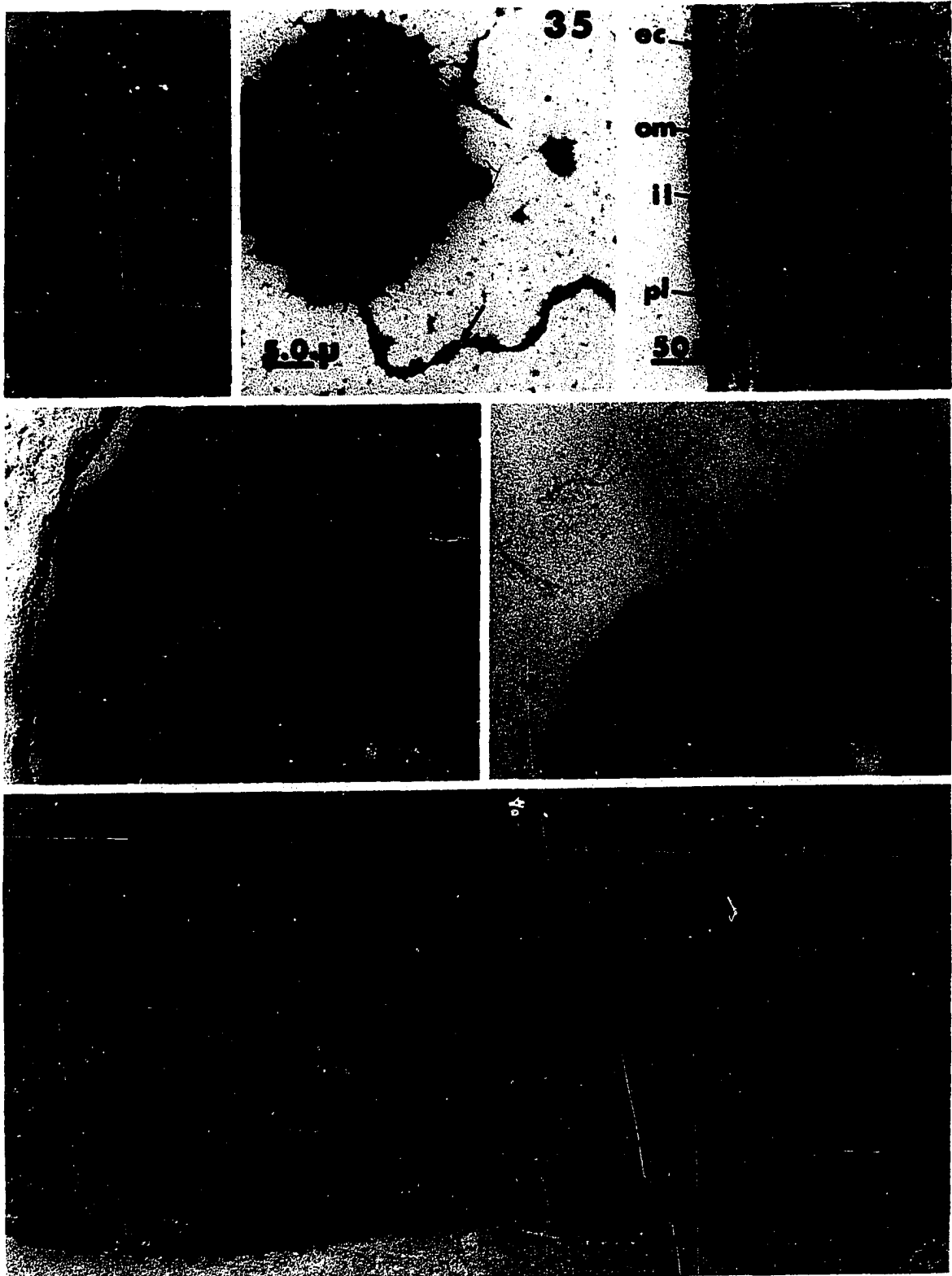
Figure 40. Dialysis experiment. Portion of a cell of _Chromatium_ sp.
            Ps 668 showing myelin-like figures (my) near the cell
            periphery, an elaborate lamellar system (la) and chrom-
            atophores (ch). Osmium-fixation with methanol uranyl
            acetate staining.

Figure 41. Dialysis experiment. Portion of a cell of _Chromatium_ sp.
            Ps 668 revealing cylindrical structures (cl) near the cell
            periphery. Note gross local evaginations of the cell wall.
            Osmium-fixation with methanol uranyl acetate staining.

Figure 42. Dialysis experiment. Portion of a cell of _Chromatium_ sp.
            Ps 668 showing membranous structures (ms) in the cytoplasm.
            Osmium-fixation with uranyl acetate staining.

Figure 43. Dialysis experiment. Portion of a cell of _Chromatium_ sp.
            Ps 668 showing membranous structure (ms) located between
            the plamemalemma (pl) and inner layer (il) of the cell
            wall. Osmium-fixation with methanol uranyl acetate stain-
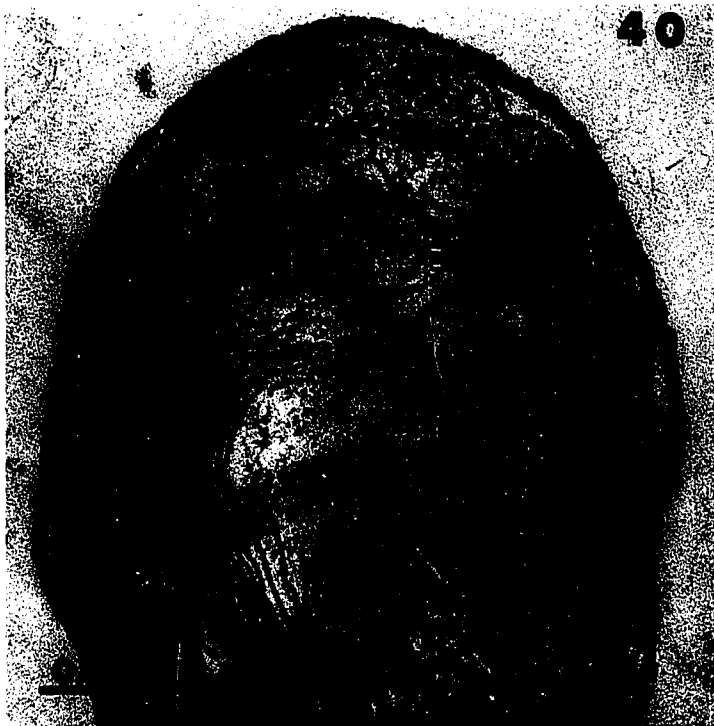   ing.
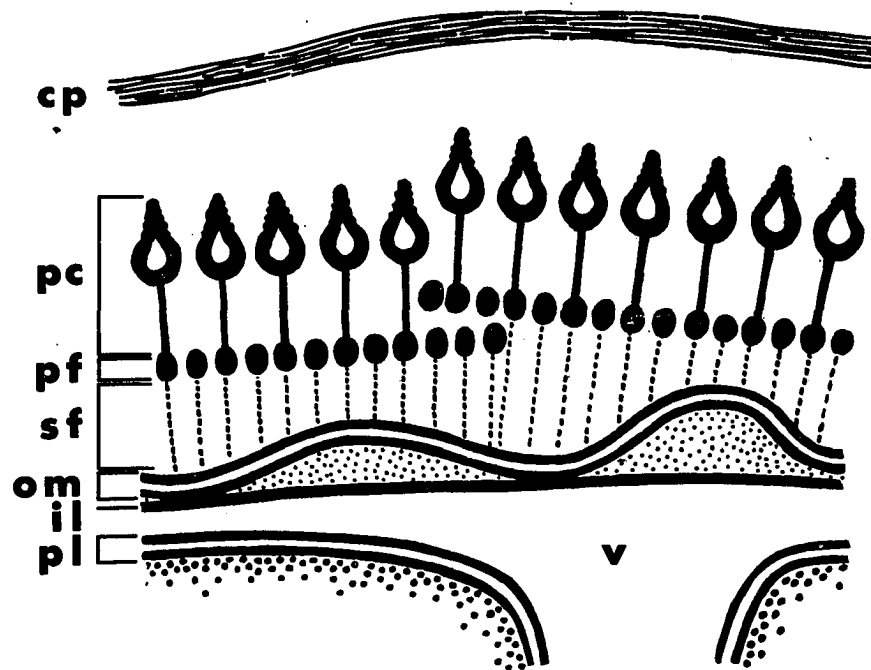
Figure 44.   Schematic representation illustrating the cell envelope of
             Rhodothece sp. The capsule (cp) surrounds the punctate layer
             (pc) composed of spines whose stalks penetrate the perforate
             layer (pf) forming the structural elements in the sub-perfor-
             ate region (sf). The cell wall proper consists of an outer
             membrane (om) and a discrete inner layer (el) surrounding the
             plasmalemma (pl). The vestibule (v) represents the intra-
             tubular space between the inner layer of the cell wall and
             the plasmalemma.

Figure 45.   Schematic representation illustrating the cell envelope of
             young cells of Chromatium sp. Ps 668. An encrustation (ec)
             surrounds the surface of the cell wall which consists of an
             outer membrane (om) and a discrete electron-dense inner layer
             (il) adjacent to the plasmalemma (pl).
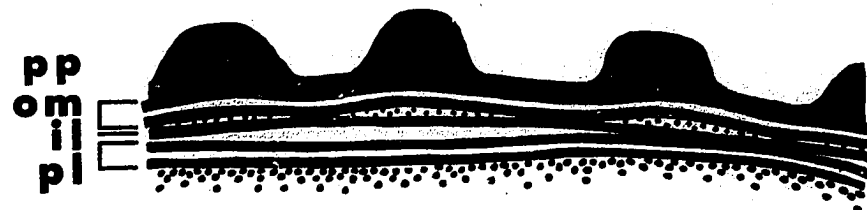
Figure 46.   Schematic representation illustrating the cell envelope of
             aged cells of Chromatium sp. Ps 668. Papillae (pp) formed
             from the encrustation project from the surface of the cell
             wall. The wall is similar to that of young cells consisting
             of an outer membrane (om) and an inner layer (il) adjacent
             to the plasmalemma (pl).

cp

pc

pf

sf

om

il

pl

v

44

ec

om

il

pl

45

pp

om

il

pl

46