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THIOCAPSA FLORIDANA; A CYTOLOGICAL, PHYSICAL AND CHEMICAL CHARACTERIZATION

I. CYTOLOGY OF WHOLE CELLS AND ISOLATED CHROMATOPHORE MEMBRANES*

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SUMMARY

The ultrastructure of the purple sulfur photosynthetic bacterium *Thiocapsa floridana* was examined by chemical fixation and freeze–etching. In addition, we describe two isolation and purification procedures for the chromatophore membranes of *T. floridana*.

During freeze–etching membranes are split along a weak interior zone, resulting in two asymmetric membrane faces.

Freeze–etch studies of both whole cells and of isolated membranes, in conjunction with the solubilization and reconstituting of isolated chromatophores, support a subunit membrane model in which protein complexes extend through the entire membrane.

INTRODUCTION

Some of the earliest information concerning the structure of the procaryotic photosynthetic apparatus was provided by physico-chemical investigations. LEVY *et al.*[1] suggested that in the purple sulfur phototroph, *Chromatium okenii*, chlorophyll might be associated with soluble proteins dispersed in the cytoplasm and FRENCH[2] was able to obtain a water-soluble protein-bound pigmented fraction from *Spirillum rubrum* which had the same absorption spectrum as the original cell suspension. PARDEE *et al.*[3] later demonstrated that the pigmented complex from cell-free extracts of *Rhodospirillum rubrum* could be sedimented by high speed centrifugation, with the resulting separation of the "chromatophore"[3,4].

The literature dealing with the chromatophore as the structural unit of procaryotic photosynthetic activity is vast, and has been extensively reviewed elsewhere[5–9].

For many years the majority of the cytological and physiological studies on the purple sulfur bacteria have been confined to *Chromatium*, since it was the only

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representative of this group available in pure culture. However, little work has been done to elucidate the fine organization of the photosynthetic membranes from organisms of this group.

In this report we present a cytological examination of whole cells and isolated and purified chromatophore membranes from the purple sulfur phototroph, *Thiocapsa floridana*. During the course of this investigation we attempted to correlate the cytological views with chemical analysis (see ref. 40) in the hope of obtaining not only a better understanding of the architecture of the photochemical apparatus in *T. floridana*, but also to offer an effective approach to the investigation of the structural organization of biological membranes in general.

MATERIALS AND METHODS

Organism and growth

Thiocapsa floridana, Strain 9314, used in this investigation was isolated by Dr. Hans Trüper (Universität Göttingen) from Clarke Reservation Lake, in New York State. Cultures were grown at 30° in 500-ml screw-capped bottles, containing either PFENNIG'S medium[10] (P medium) or our modified PFENNIG'S medium (MP medium). Cells were grown autotrophically with bicarbonate as the sole carbon source and sulfide as the electron donor. MP medium was prepared in the following way: 30 g of $NaHCO_3$ were dissolved in 6 l of distilled water and sparged with CO_2 for 30 min. To this saturated CO_2 solution were added: 3 g each of $CaCl_2 \cdot 2H_2O$, NH_4Cl, $MgSO_4$, KCl; 1 g K_2HPO_4; 1.5 g KH_2PO_4; 12 ml of a 0.002% Vitamin B_{12} solution and 120 ml of PFENNIG'S trace elements[10]. The pH was adjusted to 6.8–6.9 with conc. NH_4OH, and the medium was transferred to a Millipore stainless steel pressure vessel and filter sterilized through a 142 mm (pore size 0.22 μm) Millipore filter, using positive CO_2 pressure into 500-ml culture bottles. Each bottle received approx. 475 ml of medium and 24 ml of neutralized, sterile 1.5% $Na_2S \cdot 9\ H_2O$ (ref. 10). The final pH of the medium depended on the amount of sterile, 1 M H_2SO_4 which was added to the $Na_2S \cdot 9\ H_2O$ solution, but was maintained between 6.9 and 7.1.

Photosynthetic conditions were maintained by using banks of fluorescent lamps. The light intensity incident on the bottles was maintained at approx. 300 ft-candles.

Harvesting of cells

Exponentially growing cultures were harvested by centrifugation (15000 rev./min) at 4°, washed with cold Tris–HCl buffer (Tris–Mg^{2+} buffer: 0.02 M Tris, 0.01 M $MgSO_4$, pH 8.0), and resuspended in the same buffer to a concentration of approx. 100 mg cellular dry wt. per ml. This cell suspension was either used immediately or kept frozen at −20°. Growth rates were determined by actual cell counts in a Petroff–Hausser bacterial counting chamber and by determining the increase in protein content.

Isolation and purification of chromatophore membranes

The scheme outlined in Fig. 1 was followed for the isolation of the chromatophore membranes from *T. floridana*. The final preparative step in membrane purification involved either RbCl isopycnic density gradient centrifugation or Sepharose column chromatography. For chromatography, the 3000 × *g* pigmented supernatant

(Fig. 1) was placed on top of either a Sepharose 4 B or 6 B column. Fractionation was achieved by continuous elution with Tris–Mg^{2+} buffer and the fractions examined for purity with the electron microscope.

Equilibrium density determinations

Linear sucrose density gradients were prepared from 18–58% (w/v) sucrose in either Tris–Mg^{2+} buffer or in one of the dialysis buffers, depending on the sample to be centrifuged. Samples were carefully layered on top of the gradients and centrifuged at 25000 rev./min (63000 × *g*) at 4° for 38–40 h. Bands were removed either

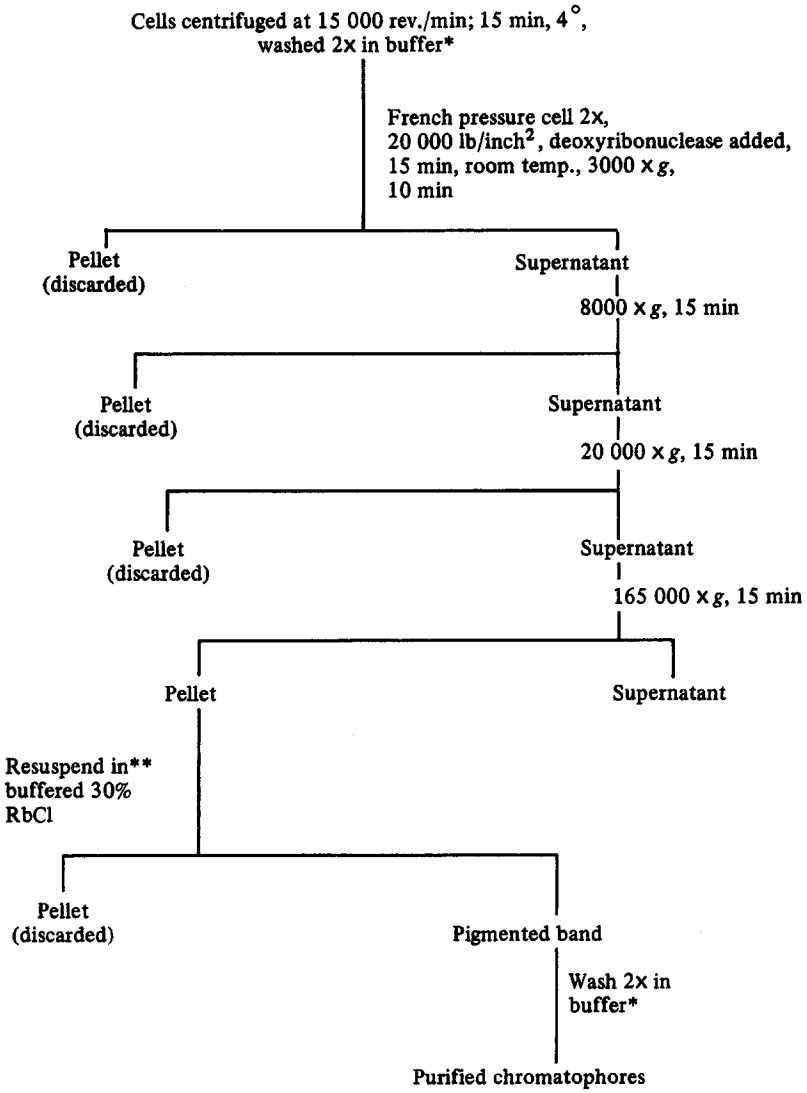


Fig. 1. Scheme for the isolation and purification of chromatophores from *T. floridana*.
* Buffer, 0.02 M Tris–HCl; 0.01 M $MgSO_4$ (pH 8.0).
** 30% RbCl in 0.1 M Tris–HCl; 0.05 M $MgSO_4$ (pH 8.0).

from the side of the tube with a syringe, or by collecting fractions through a hole punctured at the bottom of the tube. Densities were determined using tared one ml specific gravity bottles.

Electron microscopy

(*1*) *Thin-sectioning*

(*a*) *Osmium fixation.* The samples were prefixed in 1% (w/v) OsO_4 in KELLENBERGER'S buffer[16] for 1 h at 4°, centrifuged, and then fixed with fresh 1% (w/v) OsO_4 overnight at 4°. Samples were then processed as previously described[17].

(*b*) *Glutaraldehyde fixation.* Pelleted samples were resuspended in 4% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 6.8) and fixed for 2 h at 4° (ref. 18), and postfixed for 2 h with 1 %OsO_4 in (in 0.1 M phosphate buffer), dehydrated and embedded as described[17]. All sections were obtained with a Porter–Blum MT ultramicrotome, using a diamond knife.

(*2*) *Negative staining*

For negative staining, 1% (w/v) phosphotungstic acid, adjusted to pH 6.8 with NaOH, 0.5% (w/v) uranyl acetate (pH 4.4), or 1% (w/v) ammonium molybdate (in 2% ammonium acetate buffer, pH 6.8) were used. Samples were stained as described by HOLT *et al.*[12].

(*3*) *Freeze–etching*

Samples for freeze–etching were prepared either directly in the medium, in buffer, or in 20% glycerin as described previously[12].

(*4*) *Shadowing*

Heavy metal shadowing was done with carbon–platinum pellets in a Denton DV-502 vacuum evaporator. All samples for electron microscopy were examined in a Philips EM-200 electron microscope[17].

RESULTS

Growth

The specific growth rate of *T. floridana*, grown in Pfennig's medium without

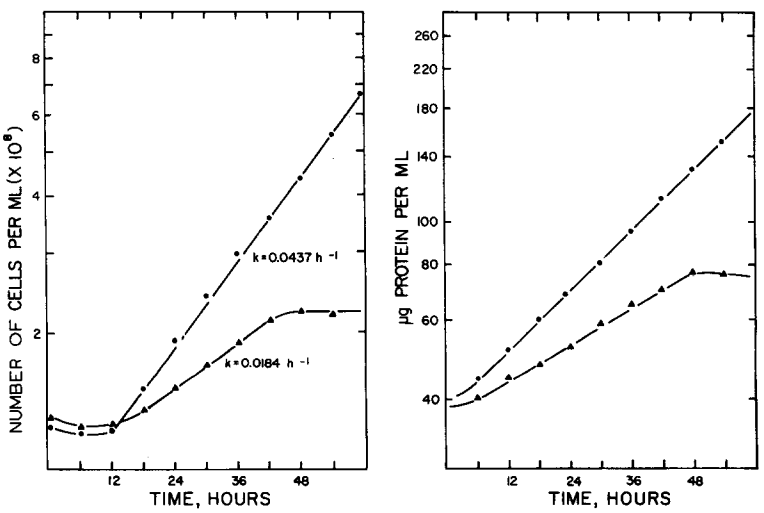


Fig. 2. Specific growth rate of *T. floridana* as a function of growth medium.

the subsequent addition of neutralized $Na_2S \cdot 9H_2O$ was 0.0184 h^{-1} (generation time = 34 h) while cells grown in the modified medium had a specific growth rate of 0.0437 h^{-1} (generation time = 16 h) (Fig. 2). In addition, cells grown in P medium reached the stationary phase in approx. 42 h, while cells which were grown in the modified medium became stationary at approx. 72 h post inoculation. At the end of exponen-

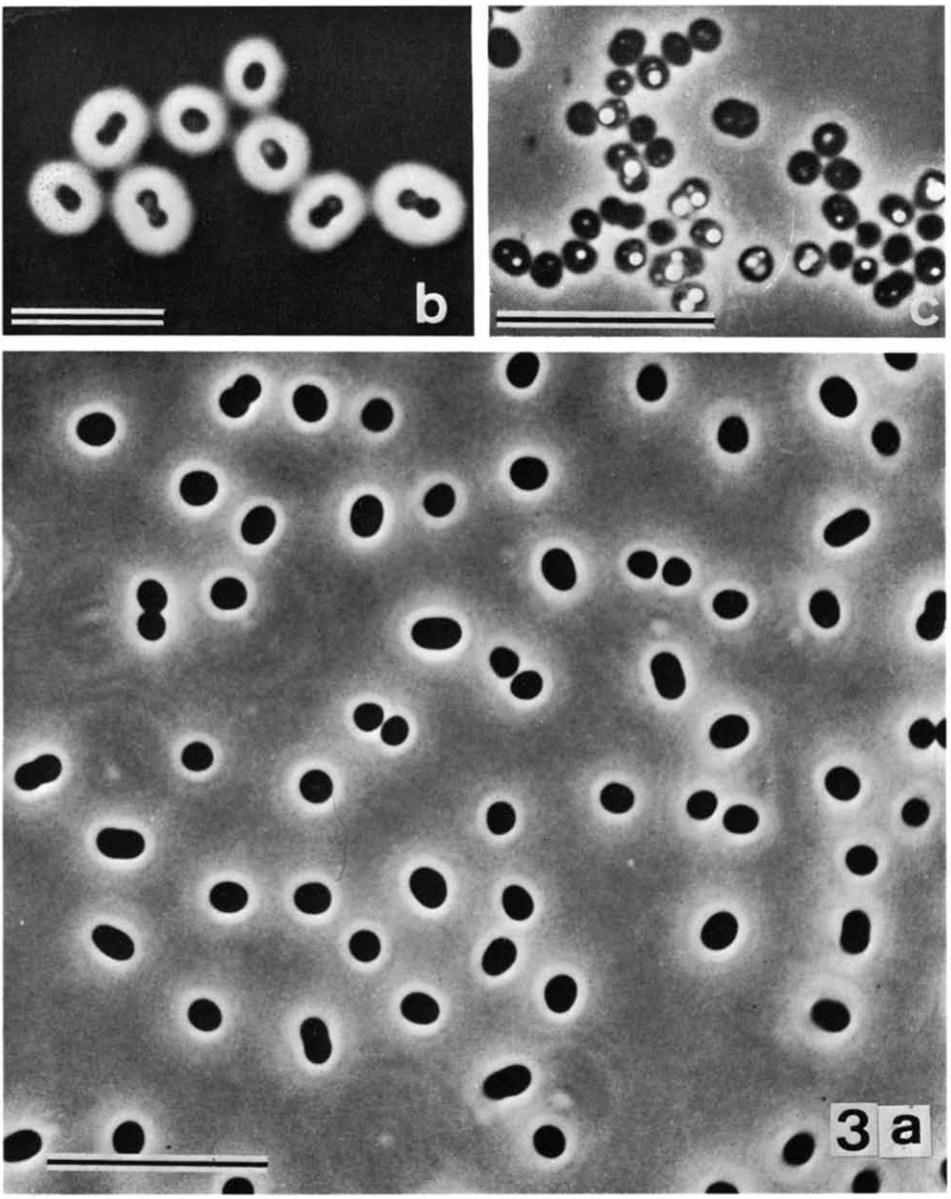


Fig. 3. Phase-contrast micrographs of *T. floridana*, (a) before, and (c) after "feeding". (b) represents an India ink stained preparation. Bars represent 10 μm.

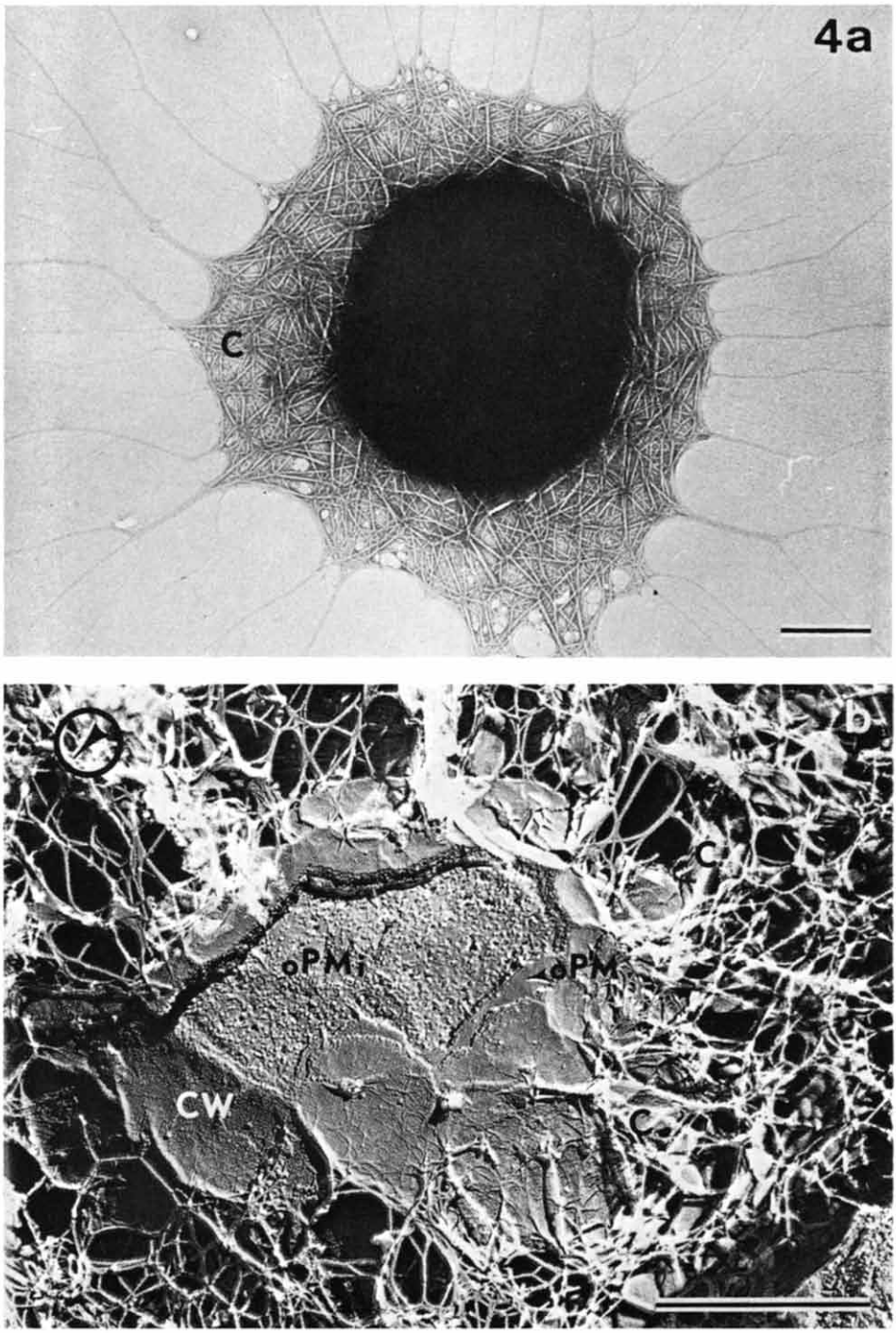


Fig. 4. a. Electron mirograph of a negatively stained cell of *T. floridana*. The preparation was stained with 1% phosphotungstic acid to show the filamentous network of the capsular material. (b) Frozen–etched preparation of *T. floridana*. Note the presence of the filamentous capsular network (c); cell wall (CW); surface of the plasma membrane (oPM); and the outer surface of the inner half of the plasma membrane (oPMi). Bars represent 500 nm.

tial growth, cultures grown in P medium displayed only a 2-fold increase in dry weight, while cultures grown in the modified medium showed a five and a half-fold increase.

Cytology

(*1*) *Phase contrast*

Phase-contrast microscopy of *T. floridana* grown in either P or MP medium revealed spherical cells 1–2 μm in diameter (Fig. 3a). The highly refractile granules (Fig. 3c), were intracellular sulfur.

(*2*) *Negative staining*

The halo surrounding the cells (Fig. 3) was due both to the usual phase microscopy ring and to capsular material. In the electron microscope the capsule was a filamentous network (Fig. 4a), which could be removed by 0.85% NaCl or with concentrated formamide. Beneath this thick capsule were the outer layers of the cell

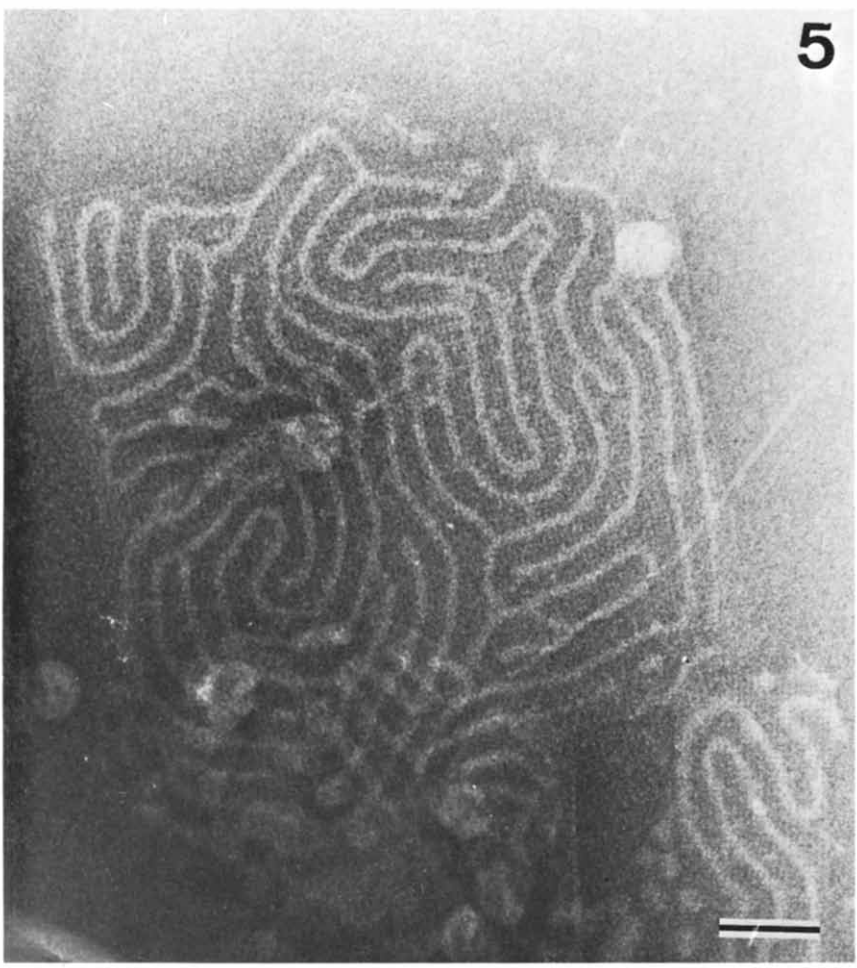


Fig. 5. Electron micrograph of the "maze" or "printed circuit" from cell wall fragments of *T. floridana*. Negatively stained with 1% (w/v) phosphotungstic acid. Bars represent 100 nm.

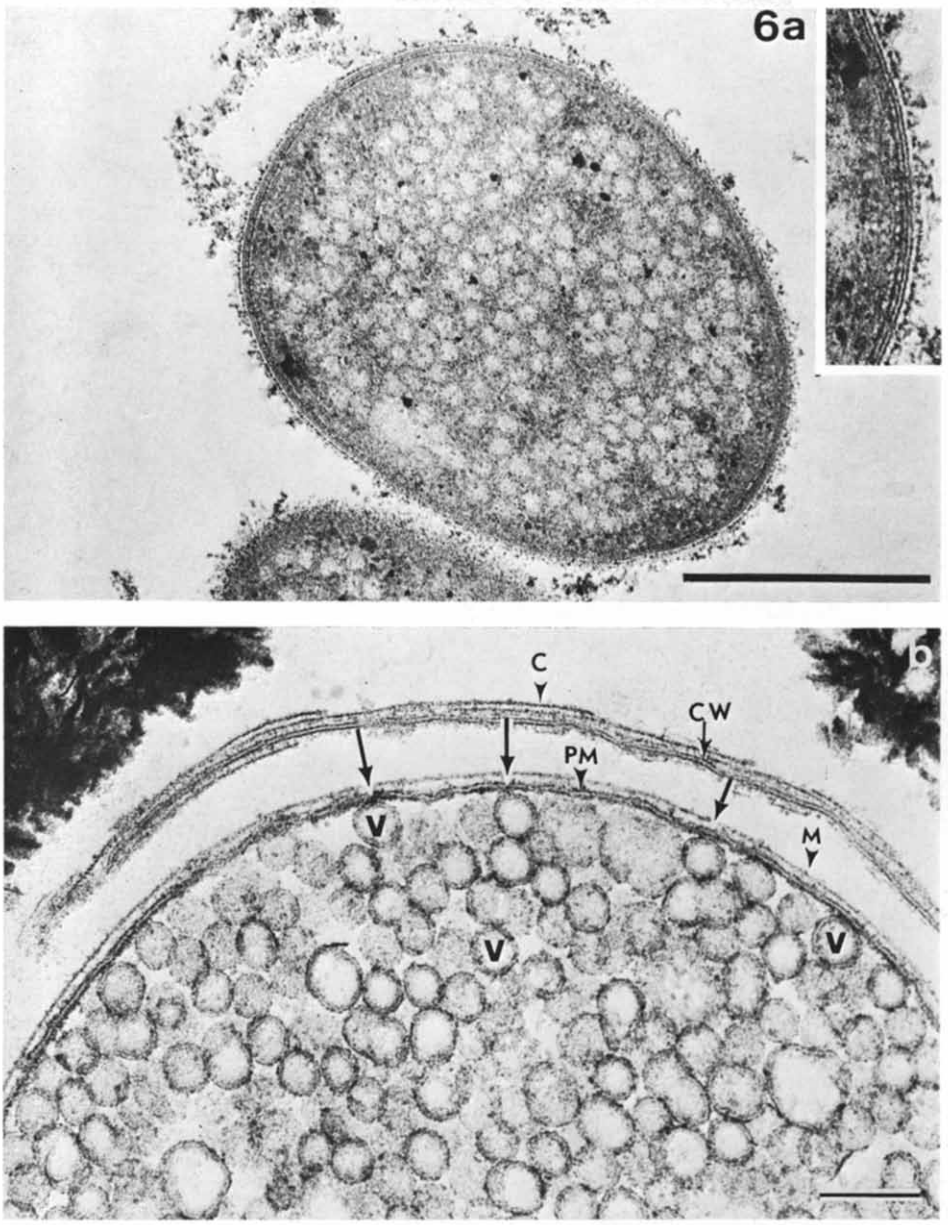


Fig. 6. a. Thin section of chemically fixed cell of *T. floridana*. Note that the photosynthetic vesicles are obscured due to the presence of the large array of densely stained ribosomes. (b) Thin section of osmotically shocked and chemically fixed cell of *T. floridana*. The release of the ribosomes provides a clearer view not only of the membrane bound vesicles (V) but also of their relation to the plasma membrane (arrows). Remnants of the capsule (c); the unit membrane-like layer of the cell wall (CW); mucolayer of the cell wall (M); and the plasma membrane (PM) are also resolved. Bar represents (a) 500 nm, (b) 100 nm.

envelope with its ordered macromolecular arrays, characteristic of many Gram-negative envelopes[19,20].

Negative staining of cell wall fragments revealed that sandwiched within the cell envelope was a layer which took on the appearance of a "maze" or "printed circuit" (Fig. 5), most probably representing the lipopolysaccharide layer of the cell wall. This material was reminiscent of negatively stained artificial lipid bilayers[21].

(3) *Thin sectioning*

Thin sections of chemically fixed cells of *T. floridana* (Fig. 6a), revealed a profile that was similar to that exhibited by other procaryotic phototrophs[11,22]. The outer most layer represents remnants of the capsular material. Underlying the capsule was the multilayered cell envelope, composed of the triple-layered cell wall, which includes the rigid or muco-layer. Beneath the outer envelope lies the triple-layered cytoplsmic membrane.

The cytoplasmic region was filled with an extensive system of membranes, similar to that found in *R. rubrum* and *Chromatium*[22]. The photosynthetic vesicles are somewhat obscured in Fig. 6a by the presence of the dense array of ribosomes. Removal of the latter by osmotic shock provided a clearer view of the membranes and their attachment to the plasma membrane (Fig. 6b arrows).

(4) *Freeze-etching*

Freeze-etch preparations of whole cells (Fig. 4b) confirmed the presence of the thick filamentous capsular network. The outer layer of the cell envelope was composed of an ordered array of particles, approx. 25 Å in diameter with a center-to-center spacing of 80 Å (Fig. 7).

In addition, both cross-fractured structures and the inner layers of the plasma membrane (Fig. 8) are apparent. Tangential fractures exposed surfaces which were most frequently covered with a random array of particles (60–80 Å in diameter) but occasionally a linear or paracrystalline array was present (Fig. 9a). The number of particles associated with convex surfaces was always greater than the number of particles present on concave surfaces (Fig. 9b). The linear pattern observable in Fig. 9b probably represents the structural arrangement that apposes the paracrystalline array of particles present in Fig. 9a.

Freeze-etching of glycerin impregnated samples of *T. floridana*, even after prolonged etching (10 min, −100°), only exposed cross-fractured, convex and concave inner membrane faces (Fig. 10). In contrast, when cells washed with saline were frozen in buffer, large areas of the outer cell wall surface were revealed after etching (Fig. 11).

Chromatophore membranes exposed by cross-fracture of a cell contained particles, especially at the external border of the membrane (Fig. 12a). These particles were more pronounced when isolated chromatophore membranes were suspended in buffer (Fig. 12b). Freeze-fracturing of glycerin-impregnated isolated chromatophores revealed membrane faces that were covered with 60–80 Å particles (Fig. 13), similar to those exposed by the tangential fracture of whole cells (Figs. 8 and 9a).

Isolation of chromatophore membranes

Sucrose density gradient centrifugation of chromatophore membranes from *T. floridana* did not yield the classical patterns of separation characteristic of *R. rubrum*[23], *Chromatium*[24] and *Rsp. spheroides*[25], for only a single pigmented band was

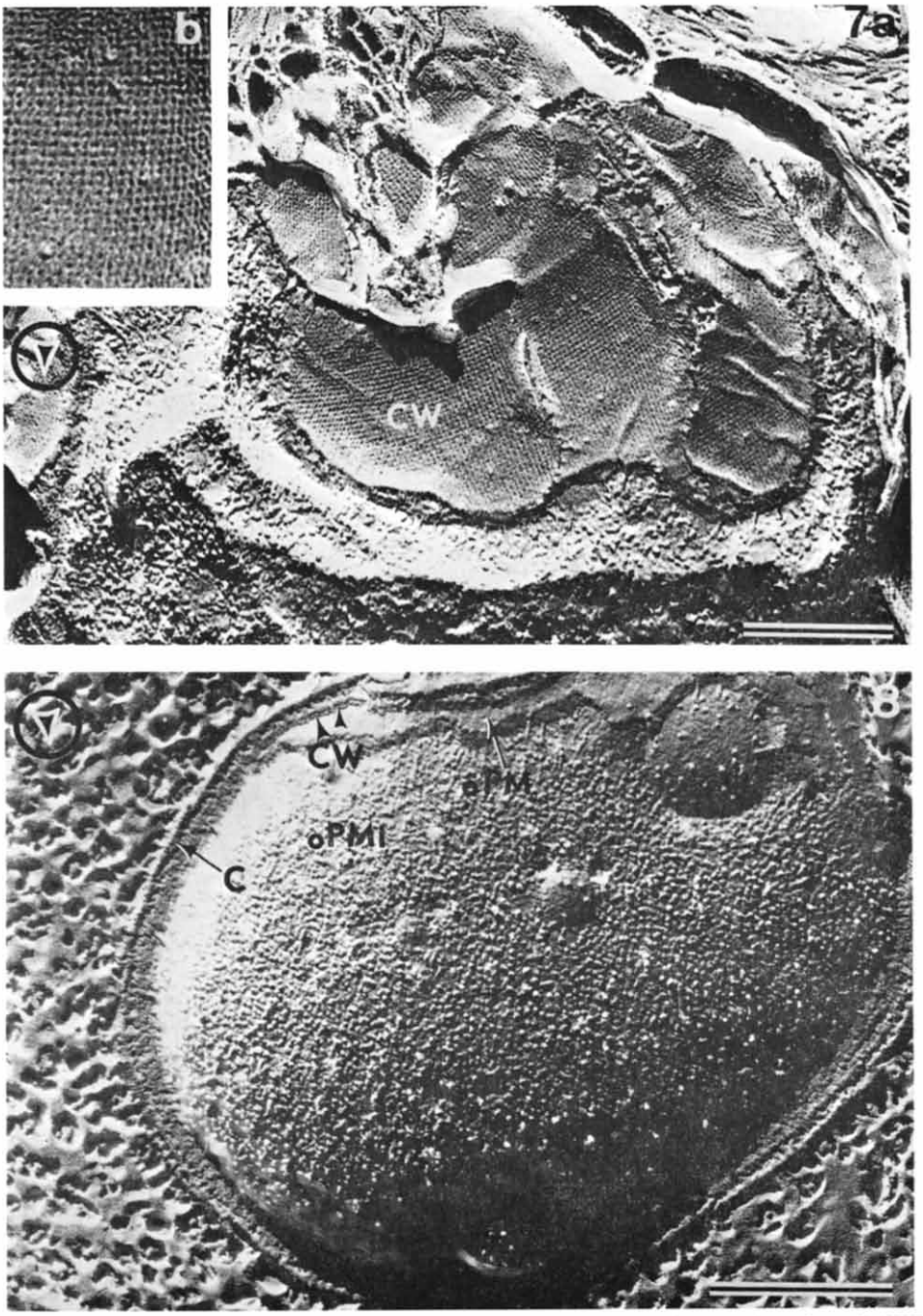


Fig. 7. a. Freeze–etch preparation of *T. floridana*. Note that the surface of the cell wall (CW) is composed of a highly ordered macromolecular mesh (b, insert), apparently made up of interwoven fibers (2.5 nm in diameter). Bar represents 250 nm.

Fig. 8. Freeze–etch preparation of *T. floridana*. Both cross-fractured surfaces and the less accessible inner layers of the plasma membrane are apparent. (C) represents the capsule; (CW) cell wall; (oPM) outer surface of the plasma membrane; (oPMi) outer surface of the inner half of the plasma membrane. Bar represents 250 nm.

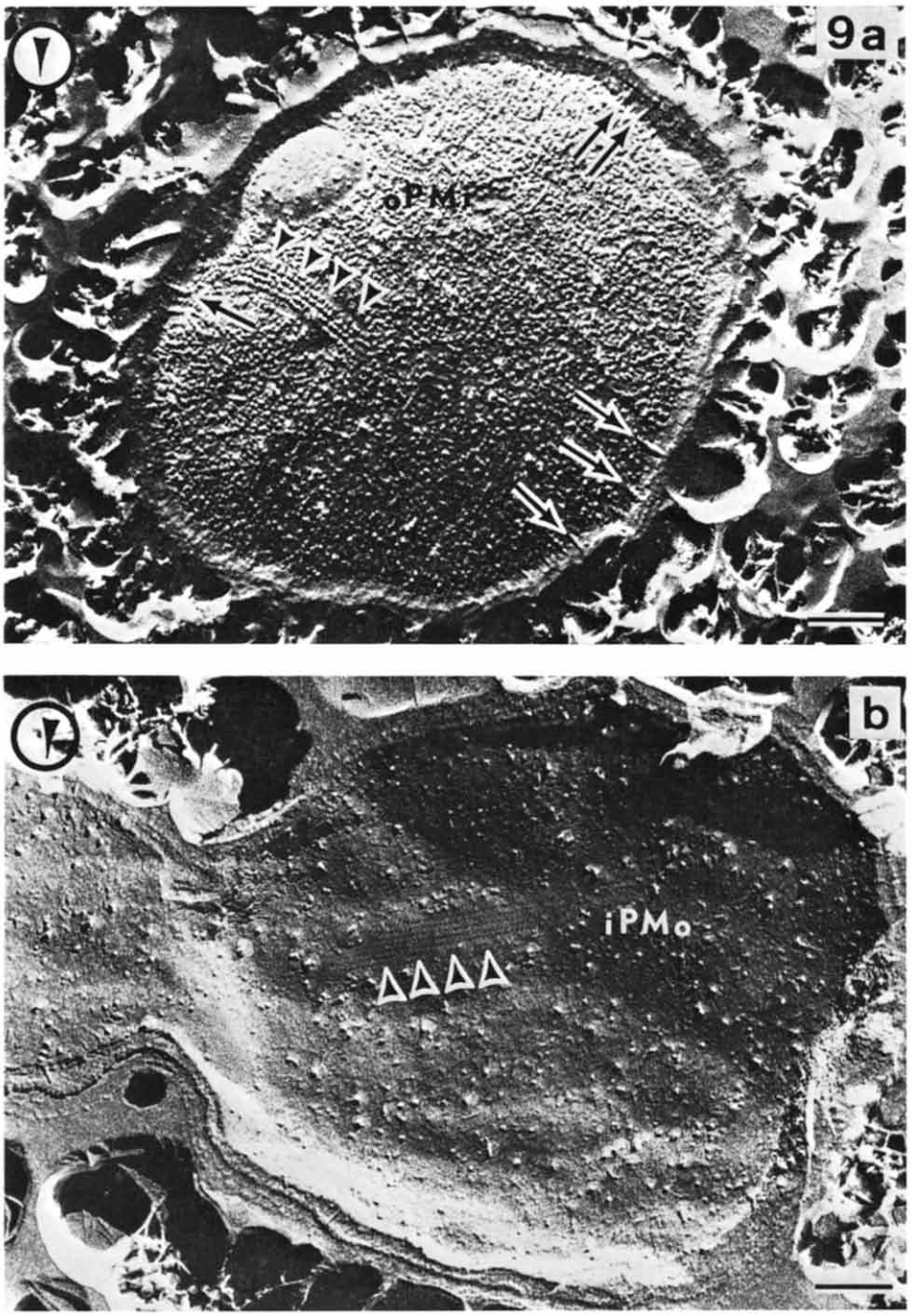
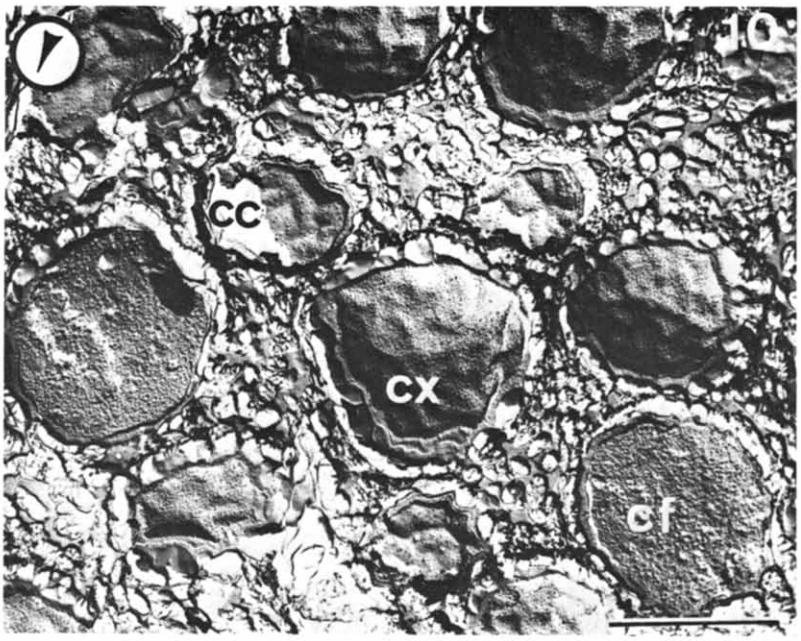


Fig. 9. Freeze–etch preparations of *T. floridana*. In (a) the fracture has removed part of the cell envelope, exposing the outer surface fo the inner half of the plasma membrane (oPMi) studded with randomly distributed and a paracrystalline array (arrows) of particles. In (b) the fracture has revealed the relatively smooth inner concave surface of the outer half of the plasma membrane. Bars represent 100 nm.



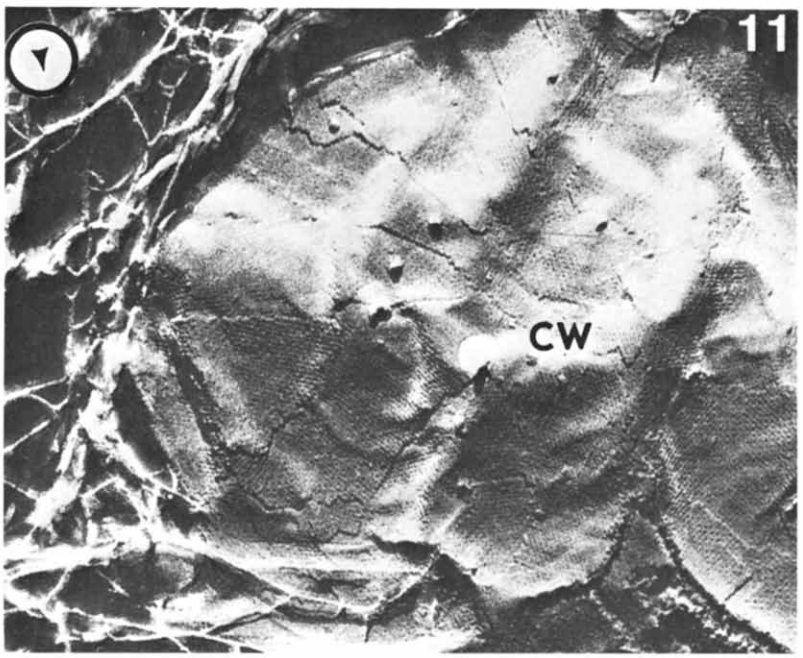


Fig. 10. Freeze-etch preparations of glycerin (20%) impregnated cells of *T. floridana*. Note that freeze-etching of these samples did not reveal any cell wall material; instead, cross-fractured (cf), convex (cx), and concave (cc) inner membrane faces are visible. Bars represent 1 μm.

Fig. 11. Freeze-etch preparations of *T. floridana*, washed with saline prior to freezing. Note that large areas of the outer surface of the cell wall (CW) are apparent, and the fibrous remnants of the capsular material. Bars represent 100 nm.

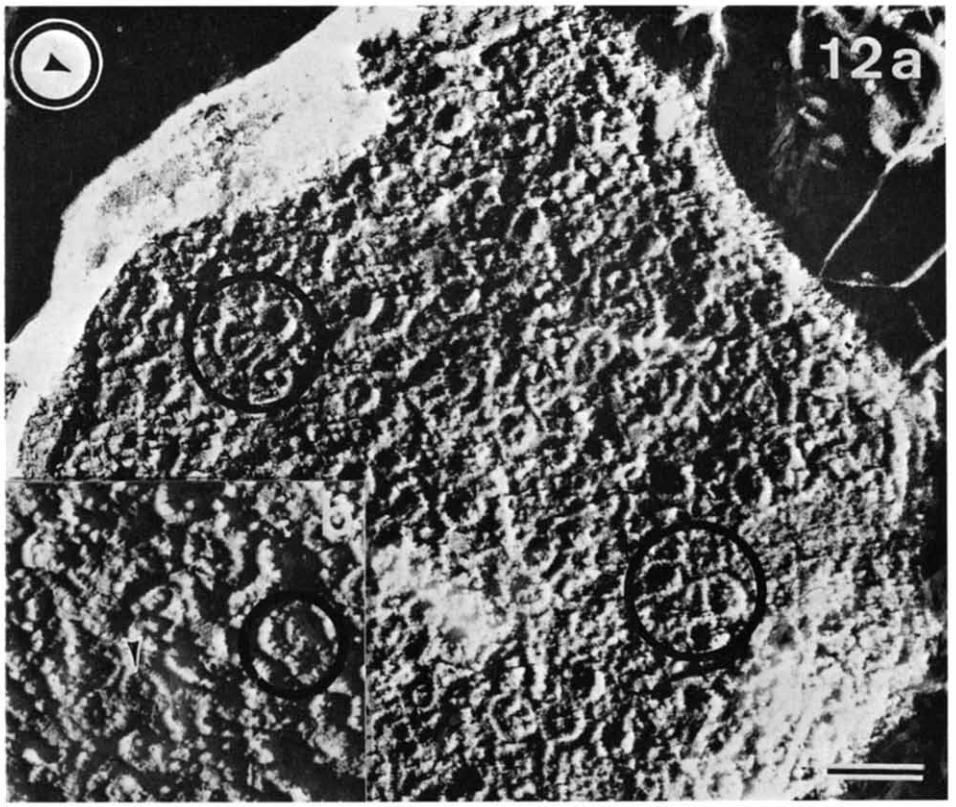


Fig. 12. Freeze–etch preparations of (a) whole cell and (b) isolated chromatophore membranes, prepared in buffer. Note that the particulate nature of the vesicles is evident in both the cross-fractured cell and in the isolated membrane preparation (circles). Bars represent 100 nm.

obtained which corresponded in density to the "light" fraction obtained from *Rsp. spheroides* (1.14 g/cm^3).

Sepharose column chromatographic isolation of *T. floridana* membranes (Fig. 14) aided membrane isolation and purification. Since materials are eluted from Sepharose in the order of decreasing molecular size, cell wall fragments were eluted first (Fig. 14b); followed by purified membranes (Fig. 14c); and finally by ribosomes and small membrane fragments (Fig. 14d).

Another method routinely used to isolate the chromatophore membranes from *T. floridana* involved the use of RbCl isopycnic density gradients. When the crude membrane preparation was centrifuged at 130000 $\times g$ for 3 h in 30% RbCl, most membrane floated to the top of the tube, while ribosomes and cell wall fragments sedimented to form a pellet. Fig. 15b shows the results of such a purification. The chromatophore membranes were homogeneous in size (approx. 650 Å in diameter) and free from ribosomal and cell wall contaminants. The flattened,"doughnut shape" of the membranes was probably due to dehydration by RbCl.

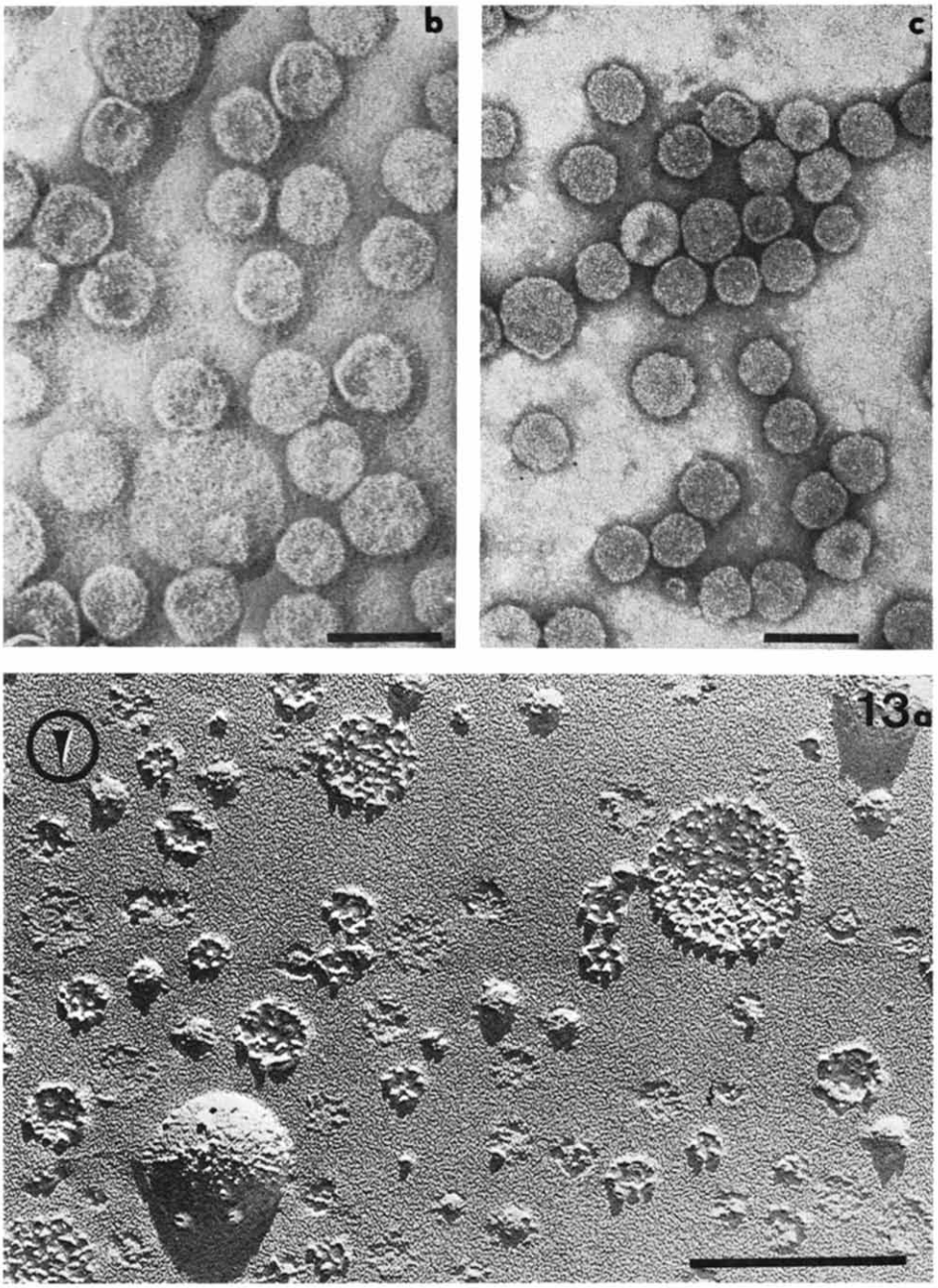


Fig. 13. (a) Freeze–etch preparation of chromatophore membranes prepared in 20% glycerin. The particulate nature of the vesicles is especially evident. Note that particles (6–8 nm in diameter) are associated with the concave surfaces, while the convex surfaces are relatively smooth. Similar particles are not revealed on the surface of the chromatophore membranes by either shadowing (b) or negatively staining (c). Bars represent 100 nm.

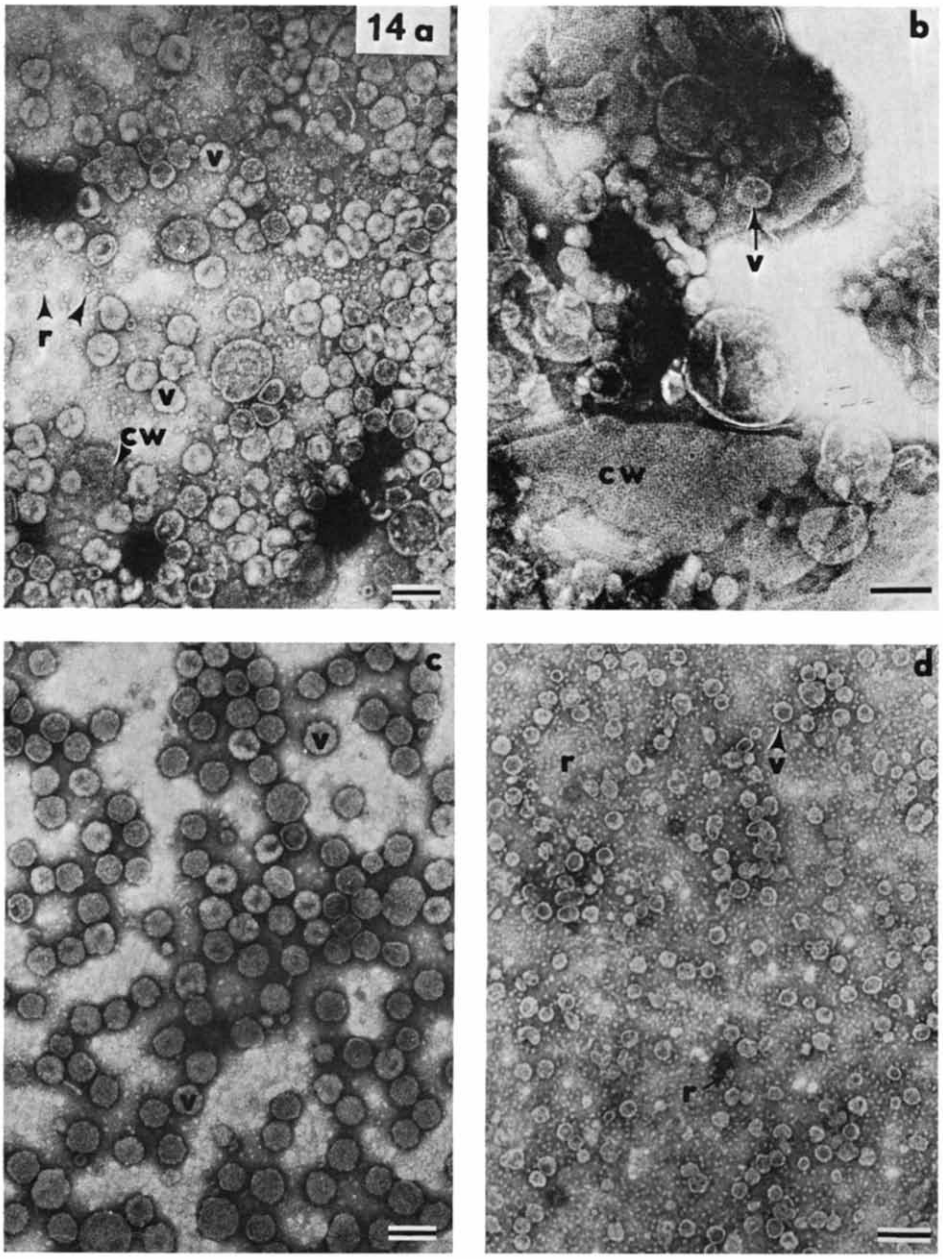


Fig. 14. Electron mirographs of negatively stained preparations obtained in the purification of chromatophore membranes through Sepharose column. (a) represents the crude preparation placed on top of the column. Cell wall fragments (CW) came through first (b); followed by purified membranes (c) and ribosomes (r) and small membrane fragments last (d). Negatively stained with 1% phosphotungstic acid (pH 6.8). Bars represent a–c = 100 nm; d = 200 mn.

DISCUSSION

Although COHEN-BAZIRE[11] examined thin sections of Thiocapsa in 1963, there has been no extensive investigation of the subcellular particles from this organism.

With respect to the isolation and purification of chromatophore membranes, it was our aim to obtain not only structures that were homogeneous in size and chemical composition, but also functional structures which contained measurable activities at different structural levels, and which could be measured by light-induced electron transfer reactions or by photosynthetic phosphorylation under a variety of controlled conditions. These expectations have been realized. We have described two reproducible isolation and purification procedures by which may be obtained from *T. floridana* chromatophore membranes which exhibit a remarkable degree of homogeneity both in shape and size (Fig. 15). The chromatophore membranes are considered purified on the basis of extensive washing, isopycnic banding, the observation of a single symmetrical Schlieren peak in the analytical ultracentrifuge[40] and by the absence of contaminating material as revealed by electron microscopy. In this and the succeeding paper[40] we have also described the fractionation of chromatophores into identifiable subunits (membrane solubilization) and the reassociation of these subunits into structures that resemble the original complex not only in morphology but also in their ability to carry out photophosphorylation.

Unlike subcellular particles from other purple bacteria, the chromatophore membranes from *T. floridana* do not separate into two bands by centrifugation through sucrose density gradients. The single pigmented band obtained corresponds in density to the "light" fraction obtained from *R. rubrum*[23] and *Rsp. spheroides*[25].

We have also examined the ultrastructure of both whole cells and purified chromatophores from *T. floridana* by thin sectioning and freeze–etching to bring more information to bear on the location of membrane fracture during freeze–etching, and also to clarify the structural organization of these membranes. Freeze–etching preparations of whole cells of *T. floridana* reveal "typical" surfaces seen previously by other investigators[12,26–29]. Removal of part of the cell envelope exposes a surface which is studded with particles of various sizes. The chemical nature, function, and location of similar membrane particles has been the subject of numerous reports[13–15,30–32].

Our data indicate that during freeze–etching membranes are split so as to expose inner faces. This conclusion is based on the observation that freeze–etching of glycerin impregnated samples of *T. floridana*, even after prolonged etching (10 min), did not reveal any cell wall material, and only convex and concave inner membrane faces were exposed (Fig. 10). In contrast, when cells were frozen directly in the medium, or in buffer, large areas of the cell wall surface were revealed (Fig. 11). Since glycerin is commonly used an an antifreeze agent in freeze–etching, and is non-etchable (since it forms an eutectic mixture with water), surfaces exposed during freeze–etching of a glycerated sample should be those that were exposed directly by freeze-fracturing. Therefore, our finding is in agreement with the hypothesis that freeze–fracturing actually splits the membrane to expose inner membrane faces. Furthermore, chromatophores exposed by cross-fracture of a cell, are particulate, especially at the external border of the membrane (Fig. 12), and this is especially pronounced when glycerin impregnated isolated chromatophores are examined (Fig. 13).

Freeze-fracturing of the plasma membrane always exposes two types of surfaces

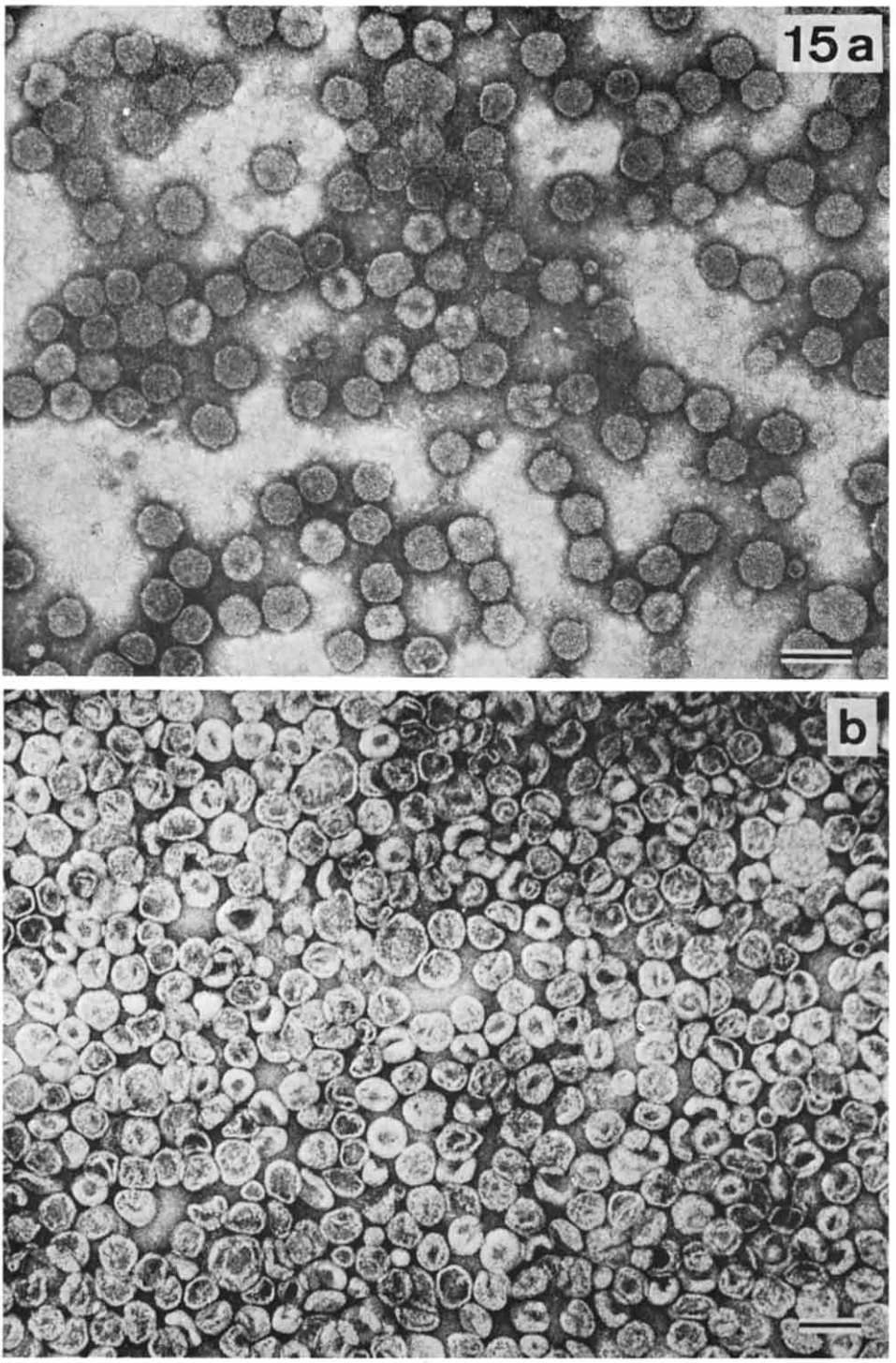


Fig. 15. Electron micrographs of negatively stained (1% phosphotungstic acid (pH 6.8)) chromatophore membranes from *T. floridana* (a) purified through Sepharose column, and (b) purified by isopycnic banding on RbCl density gradient. Bars represent 100 nm.

— a convex surface that is studded with a large number of particles, and a concave surface studded only with a few particulate units. This relatively smooth layer represents the inner surface of the outer half of the plasma membrane (Fig. 16). Since the chromatophore membranes are formed by the infolding of the plasma membrane,

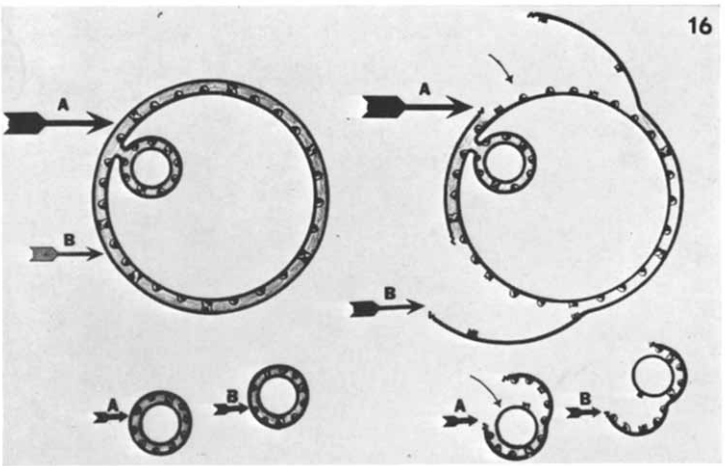


Fig. 16. Schematic representation of possible fracture planes of tangentially fractured membranes Fracture (A) exposes the outer surface of the inner half of the plasma membrane (arrow); (B) exposes the inner surface of the outer half of the plasma membrane (arrow). Note that fracture (A) of the plasma membrane exposes a convex surface that is covered with numerous particles; (B) exposes a relatively smooth, concave surface. Freeze -fracturing of the chromatophore membrane on the other hand results in a smooth convex and a particulate concave surface.

the outer surface of the plasma membrane comprises the inner surface of the chromatophore. This reversal should also be observable in the type of association of the particles, that is, freeze–etching of isolated chromatophores should give rise to concave structures with numerous particles, and to convex structures with relatively few particles (Fig. 13). Since intact membranes when examined by negative staining or shadowing (inserts Fig. 13) do not display a similarly particulate surface, we conclude that freeze–etching splits the membrane exposing inner membrane faces.

The particulate units exposed by freeze-fracturing of biological membranes, represent globular protein structures lying within the hydrocarbon portion of the lipid bilayer[31,32]. Examination of tangentially fractured cells, reveals that along the entire fractured cell wall layer, the inner half of the plasma membrane remains attached to it by strandlike material (Fig. 9a, also Figs. 3 and 4 in ref. 29). Similar fibers or strands have been noted in a large number of organisms[12,27,29,33,34]. These strands most probably originate within the plasma membrane and may penetrate the entire thickness of the cell wall. It is possible, therefore, that some of the globular structures observed in the matrix of freeze-fractured membranes actually represent the "stubs" left behind by the shearing of these strands. BAYER[35] has observed that in plasmolyzed *Escherichia coli* the cytoplasmic membrane remains attached to the cell wall at numerous locations by "ductlike extensions", and that bacteriophages T1 to T7 adsorb almost exclusively to the surface of the wall in juxtaposition to these ducts[36]. Some bacteriophage receptors are also shared by certain colicins. Since coli-

cins remain on the bacterial surface of susceptible cells and exert their inhibitory activities from there[37,38] and since in protoplasts the bactericidal effect of colicins is instantaneous and not reversible by trypsin[39], part of the plasma membrane must extrude through the cell wall. The most likely candidates are structures such as the "strands" observed in Fig. 9a.

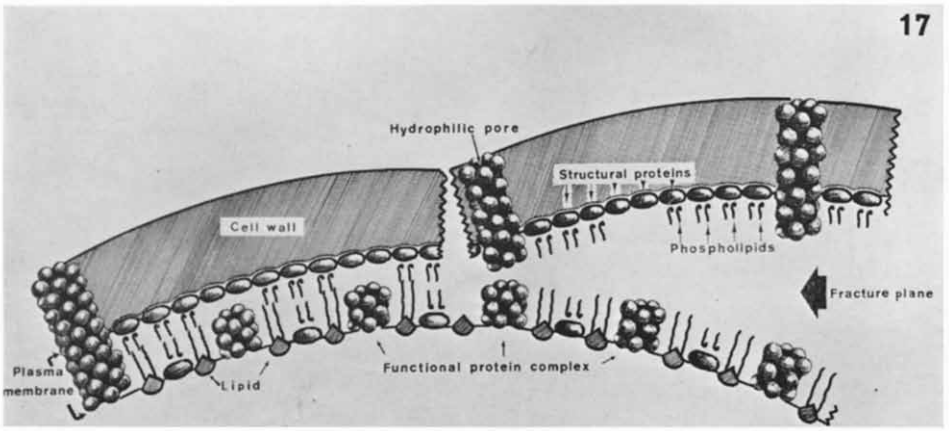


Fig. 17. Schematic representation of the architecture of the plasma membrane and of the possible fracture plane during freeze-fracturing. Note that during freeze-fracturing the plasma membrane is split through a "weak interior zone", resulting in two assymmetric membrane faces; one exhibiting numerous particles (inner half) the other with fewer particulate units (outer half).

The solubilization and reconstitution studies of isolated chromatophores[40], and the freeze–etch studies of both whole cells and of isolated membranes support a subunit membrane model in which protein complexes extend through the entire membrane (Fig. 17). These protein complexes probably comprise the basic framework of the membrane, some of them extending through the cell wall. A similar membrane model was recently proposed by MEYER AND WINKELMANN[41]. It does not, however, account for several of the features suggested by Fig. 17. The model presented in Fig. 17, although highly schematic, might explain that during freeze-fracturing membranes are split along a "weak zone", through the interior, resulting in two asymmetric membrane faces, one exhibiting numerous particles (inner half) and the other with fewer particulate units on its surface (outer half). The model also accounts for the strands which penetrate the cell envelope and which are observable along the cross-fractured cell wall in tangentially fractured cells.

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