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Freeze-Etch Study of *Pseudomonas aeruginosa*: Localization Within the Cell Wall of an Ethylenediaminetetraacetate-Extractable Component

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A freeze-etch study of normal cells of *Pseudomonas aeruginosa* and of cells after incubation with ethylenediaminetetraacetate (EDTA) and tris(hydroxymethyl)aminomethane (Tris) was performed. When cells were freeze-etched without a cryoprotective agent, a smooth outer cell wall layer, which showed a regular array of subunits, and the presence of flagella and pili were observed. These features were not observed in cells freeze-etched after cryoprotection with glycerol. Four fracture surfaces, which resulted from splitting down the center of the outer wall membrane and of the inner cytoplasmic membrane, were revealed in freeze-etched glycerol-protected cells. The murein layer was seen in profile between the outer cell wall membrane and the cytoplasmic membrane. Spherical units and small rods composed of the spherical units were observed in the inner layer of the outer cell wall membrane. These spherical units appeared to be attached to, or embedded in, the inner face of the outer layer of the outer cell wall membrane. These spherical units were removed from cells on exposure to EDTA-Tris, resulting in cells that were osmotically fragile. The spherical units were detected via electron microscopy of negatively stained preparations in the supernatant fluid of cellular suspensions treated with EDTA-Tris. Upon addition of Mg^{2+}, the spherical units were reaggregated into the inner layer of the outer cell wall membrane and the cells were restored to osmotic stability. The spherical units were shown to consist primarily of protein. These data are thought to represent the first ultrastructural demonstration of reaggregation of cell wall components within a living cell system.

Cell surfaces of gram-negative bacteria are damaged by exposure of the cells to ethylenediaminetetraacetate (EDTA). EDTA is particularly active against *Pseudomonas aeruginosa*, causing rapid cellular lysis (9, 12). Treatment of *P. aeruginosa* with EDTA in hypertonic sucrose produces osmotically fragile cells, termed osmoplasts, and osmotic stability is restored by the addition of multivalent cations (1, 2). Extraction of functional divalent cations is considered to be the primary action of EDTA (1, 2, 20). Moreover, treatment of isolated cell envelopes of *P. aeruginosa* with EDTA releases a protein-lipopolysaccharide complex and phospholipid (20, 21). The ultrastructural appearance of the protein-lipopolysaccharide complex as revealed by electron microscopy is that of rodlets 20 to 25 nm in length which in turn are made up of spherical units 7 ± 1 nm in diameter (21).

Electron microscopy of bacterial cells after freeze-etching has been shown to reveal much three-dimensional detail about the ultrastructure of the cell envelope. This technique appeared to lend itself to a study of the ultrastructural changes conferred on the cell envelope of *P. aeruginosa* on exposure to EDTA. The investigations described in this paper were undertaken, therefore, to elucidate the effect of EDTA on the ultrastructure of the cell envelope of *P. aeruginosa*.

MATERIALS AND METHODS

Cultivation of organism. *P. aeruginosa* strain OSU

64 was cultivated for 12 to 14 hr at 30 C on a rotary shaker in 500-ml Erlenmeyer flasks each containing 100 ml of Tryptic soy broth (TSB; Difco). Cells were harvested by centrifuging at 7,700 × *g* for 15 min in a refrigerated centrifuge at 4 C and were washed once before use at room temperature in deionized water with agitation in a Waring blendor, except where otherwise indicated.

Preparation of osmoplasts. Osmoplasts were prepared from 14-hr-old cells of *P. aeruginosa* by incubation in 1 mM EDTA, 33 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, and 0.55 M sucrose as previously described (1, 2), with the modifications that a *p*H of 8.6 was used and incubation was for 45 min at 30 C on a reciprocal shaker. Production of osmoplasts was verified by determining their osmotic fragility as follows. The osmoplasts were harvested from 10 ml of suspension by centrifuging at 5,000 × *g* for 15 min, the pellet was then suspended in 10 ml of deionized water, and lysis was detected by observing the immediate reduction in optical density at 660 nm.

Preparation of restored cells. Osmoplasts were converted to osmotically stable cells (i.e., restored cells) by the technique previously reported (1, 2) but modified by adding 10 mM $MgCl_2$ (final concentration) to the suspension of osmoplasts and incubating for 45 min at 30 C on a reciprocal shaker. Production of restored cells was verified by determining their osmotic stability as follows. The restored cells were harvested from 10 ml of suspension by centrifuging at 5,000 × *g* for 15 min, the pellet was then suspended in 10 ml of deionized water, and absence of lysis was determined by observing no significant reduction in optical density at 660 nm.

Standard freeze-etch procedure. The preparation to be freeze-etched was harvested by centrifugation, and some of the pellet was placed into specimen holders. The preparation was immersed in liquid Freon for 10 sec and then plunged into liquid nitrogen. The freeze-etch procedure was performed in a Balzers BA 360M high-vacuum freeze-etch unit. After fracturing was completed, etching was performed for 2 min at −100 C. The carbon-platinum electrode was evaporated for 8 sec, after which the carbon-carbon electrode was evaporated for 10 sec. The specimen holders were recovered and placed into a commercial solution of sodium hypochlorite (Clorox)-deionized water (1:1, v/v) until the replica floated free. The replica was next floated into an undiluted Clorox solution for 10 to 15 min, after which it was washed in three or four changes of deionized water. Cleaning was accomplished by floating the replica onto 70% sulfuric acid for 4 hr, washing in deionized water through four changes for 5 min each, and floating into Clorox overnight in a refrigerator. The replica was then washed again with three or four changes of deionized water and picked up on uncovered 300-mesh copper grids. Observation of the replicas was performed by use of a Phillips 200 electron microscope with 80-kv accelerating voltage.

Freeze-etch procedure without a cryoprotective agent. For the study of cells freeze-etched without a cryoprotective agent, the cells were harvested by centrifuging after 12 hr of cultivation; then the pellet was placed immediately into specimen holders, and the standard freeze-etch procedure was carried out.

Glycerol as cryoprotective agent. In one experimental procedure, 12-hr-old cells were placed immediately after harvesting and without washing into TSB-glycerol (7:3, v/v), held at 4 C for 2 hr, and then freeze-etched according to the standard procedure. For another experimental procedure, the harvested cells were washed twice with gentle stirring with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2'-ethane sulfonic acid (HEPES) buffer, *p*H 7.4, prior to being placed in the TSB-glycerol mixture. In the third experimental procedure, the cells were washed in deionized water with agitation in a Waring Blendor prior to being placed in the TSB-glycerol mixture.

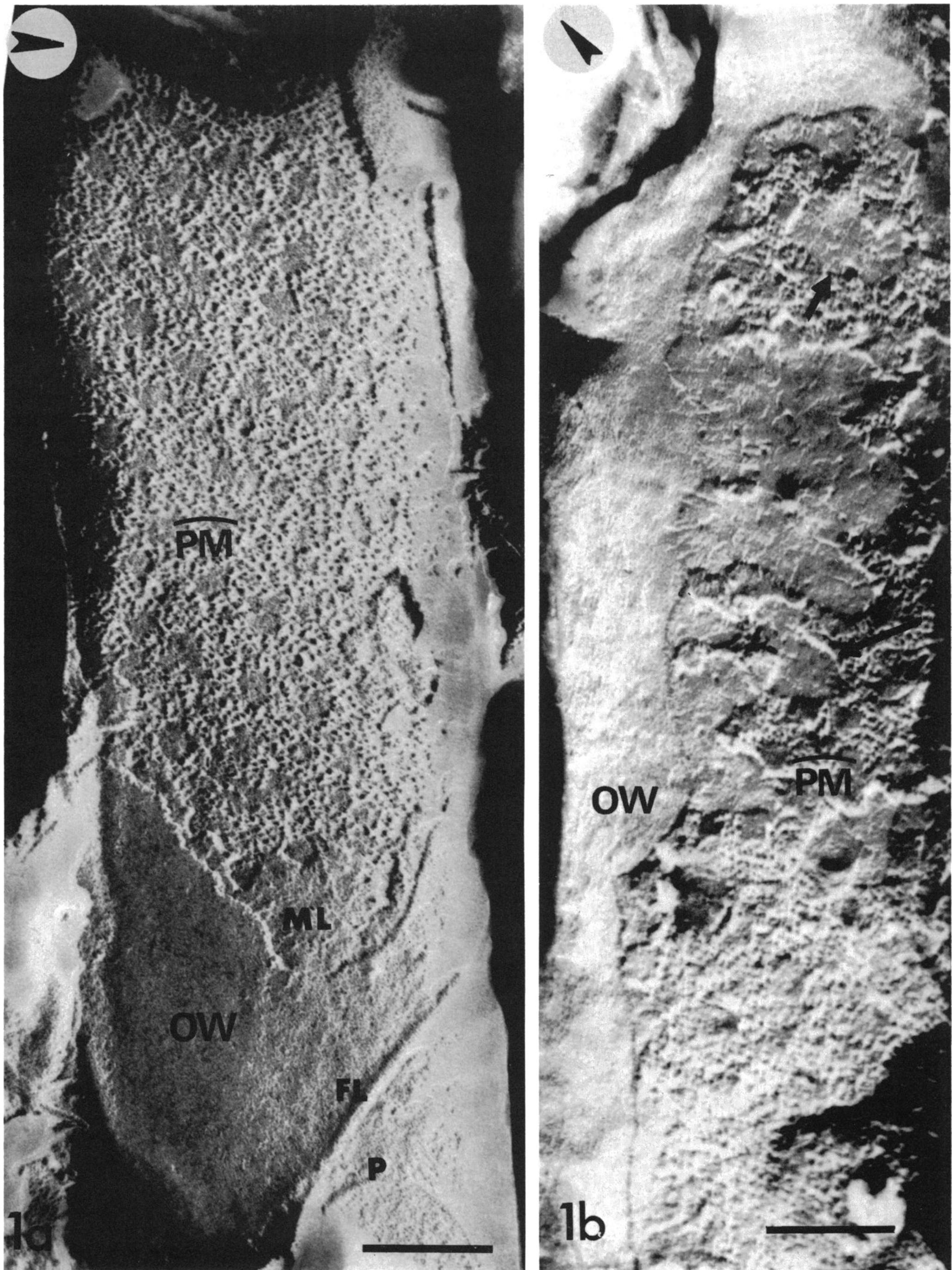
Freeze-etching of osmoplasts and of restored cells. Osmoplasts were harvested by centrifuging at 5,000 × *g* for 15 min, and were then suspended in 0.55 M sucrose containing TSB-glycerol. The osmoplasts were held in this mixture for 2 to 3 hr at 4 C, harvested by centrifuging, and freeze-etched according to the standard procedure. Restored cells were handled in the same manner as osmoplasts.

Freeze-etching of control cells. Normal cells of *P. aeruginosa*, after harvesting and washing in deionized water, were placed in 0.55 M sucrose containing TSB-glycerol for 2 to 3 hr at 4 C and were freeze-etched according to the standard procedure.

Electron microscopy of the supernatant fraction from the osmoplast suspension. A sample of the lyophilized supernatant fraction was reconstituted in a drop of deionized water and then mixed with a drop of 2% phosphotungstic acid, *p*H 6.1. This suspension was picked up on a nitrocellulose-coated 300-mesh copper grid, and the excess fluid was drawn off with filter paper. The grid was allowed to air-dry and then was examined with an electron microscope.

Chemical analyses of supernatant fractions from suspensions of osmoplasts and restored cells. After removal of osmoplasts or restored cells from the EDTA-Tris-sucrose mixture for freeze-etching, the resulting supernatant fractions were centrifuged at 37,000 × *g* at 4 C for 1 hr. Samples of the supernatant fractions were dialyzed for 72 hr at 4 C against several changes of deionized water. The changes in volume were recorded. The preparations were assayed for protein by use of the Folin-Ciocalteau phenol reagent (15), with bovine serum albumin as the standard, and for carbohydrate by the anthrone method (3). The amount of material per milliliter was calculated, and the values were corrected for the changes in volume

FIG. 1. *Freeze-etched cells without use of a cryoprotective agent. The arrowhead in the upper left corner of this and of all of the following electron micrographs showing freeze-etched preparations indicates the direction from which the metal was evaporated in the production of the replica. The horizontal bar in all figures represents 200 nm. Abbreviations used in this and the following figures are as follows: OW, smooth outer cell wall layer; ML, middle cell wall layer;* $\widehat{PM}$*, convex cytoplasmic membrane surface;* $\check{PM}$*, concave cytoplasmic*



membrane surface; $\widehat{CW}$, convex cell wall layer; $\widecheck{CW}$, concave cell wall layer; FL, flagella; P, pili; F, fibrils; S, subunits; M, murein; C, cytoplasm. (a) This cell shows the smooth outer cell wall layer, the middle cell wall layer, and the cytoplasmic membrane convex surface with its netlike arrangement of granules. Also shown is the presence of flagella and pili. (b) The outer wall layer and convex cytoplasmic membrane surface are shown. Patchy areas of cell wall attached to the cytoplasmic membrane, indicated by arrows, are seen in the central portion of the micrograph.

which occurred during dialysis.

RESULTS

Cells freeze-etched without cryoprotection. Cells freeze-etched without a cryoprotective agent (Fig. 1a) revealed a smooth outer cell wall layer and the presence of flagella and pili. The identification of the latter two structures was based on their size and appearance, which compared favorably with that described for *Escherichia coli* (24). The cytoplasmic membrane fractured to show the cytoplasmic membrane convex surface (i.e., the inner surface of the cytoplasmic membrane which is split down the center hydrophobic layer is considered to be the convex cytoplasmic membrane). The convex cytoplasmic membrane contained a netlike arrangement of granules on the surface. A middle cell wall layer, which is thought to be the inside "track" of the outer cell wall membrane, was observed between the outer wall layer and the cytoplasmic membrane convex surface. Patchy areas where the cell wall remained attached to the cytoplasmic membrane were also evident (Fig. 1b). Similar areas of attachment have previously been demonstrated in *E. coli* (4). A regular arrangement of subunits in the outer cell wall layer was observed (Fig. 2). A similar fine substructure in the smooth outer cell wall layer of *E. coli* has also been observed previously (17). In Fig. 2, fibrils can be seen to lead from the cell wall onto the cytoplasmic membrane.

Cells freeze-etched with cryoprotection. No outer cell wall layer, flagella, or pili were observed in cells that were cryoprotected by glycerol (Fig. 3). Irrespective of the washing procedure that was used prior to the glycerol treatment, the appearance of the outer cell wall surface was identical in all electron micrographs of the cryoprotected cells, and outer wall layer, flagella, and pili were not observed. These results are interpreted to indicate that the outer cell wall "double-track" layer split to reveal the inner surfaces. Further evidence to support this interpretation is presented below.

Four fracture surfaces were evident in glycerol-protected cells (Fig. 3). These were identified as convex cell wall, concave cell wall, convex cytoplasmic membrane, and concave cytoplasmic membrane. The convex cell wall layer is considered to correspond to the middle cell wall layer seen in cells freeze-etched without cryoprotection. The concave cell wall is composed in part of a closely packed layer of spherical units approximately 6 to 7 nm in diameter. The spherical units appear to form rodlets, composed of three or four spherical units and approximately 20 to 25 nm long. In some instances, longer rodlets were noted, owing to their being composed of a greater number of spherical units. Fibrils were seen between the convex cell wall and the convex cytoplasmic membrane. However, fibrils were also seen extending from the convex cell wall layer into the surrounding medium, which suggests that fibrils may actually be artifacts.

The convex cytoplasmic membrane displays a netlike pattern of granules, whereas the concave cytoplasmic membrane has the appearance of the corresponding depressions and smooth areas that would be expected to complement the granular surface of the convex cytoplasmic membrane (Fig. 3). This is interpreted as evidence that the cytoplasmic membrane has split down the center hydrophobic layer, exposing the complementary inner surfaces.

The convex cytoplasmic membrane surface is shown to a greater extent in Fig. 4a. The netlike pattern of granules is striking in its appearance. Fibrils can be seen between the convex cell wall and the convex cytoplasmic membrane. Convincing evidence is presented in Fig. 4b that the concave cytoplasmic membrane is the inner cytoplasmic membrane, because it conforms to the positioning of the cytoplasmic membrane separated from the cell wall profile in this slightly plasmolyzed cell. Also shown in this micrograph is another fracture of the concave cell wall, which reveals spherical units and rodlets lying on, or embedded in, an underlying smooth layer.

The location of all the layers in freeze-etched, glycerol-protected cells of *P. aeruginosa* is revealed within the single cell shown in Fig. 5. The convex and concave cell wall layers, the convex and concave cytoplasmic membranes, cytoplasm, and murein are all evident in this one cell. The murein is seen as a profile layer separating the concave cytoplasmic membrane and the concave cell wall layer. The identification of the murein layer was based on its location between these two layers and on the fact that profile layers in this same location have been previously identified as the murein layer (14, 17, 24). The convex cell wall appears to originate from the inner layer of the "double-track" outer membrane of the cell wall, as is revealed to best advantage at the area in Fig. 5 denoted by the unlabeled arrow.

Freeze-etch of control cells. Control cells were those that were cryoprotected before

FIG. 2. *Freeze-etched cell without a cryoprotective agent. The smooth outer cell wall layer, middle cell wall layer, convex cytoplasmic membrane surface, pili, fibrils, and, in the outer cell wall layer, a regular array of subunits are shown. Symbols are as defined in Fig. 1.*

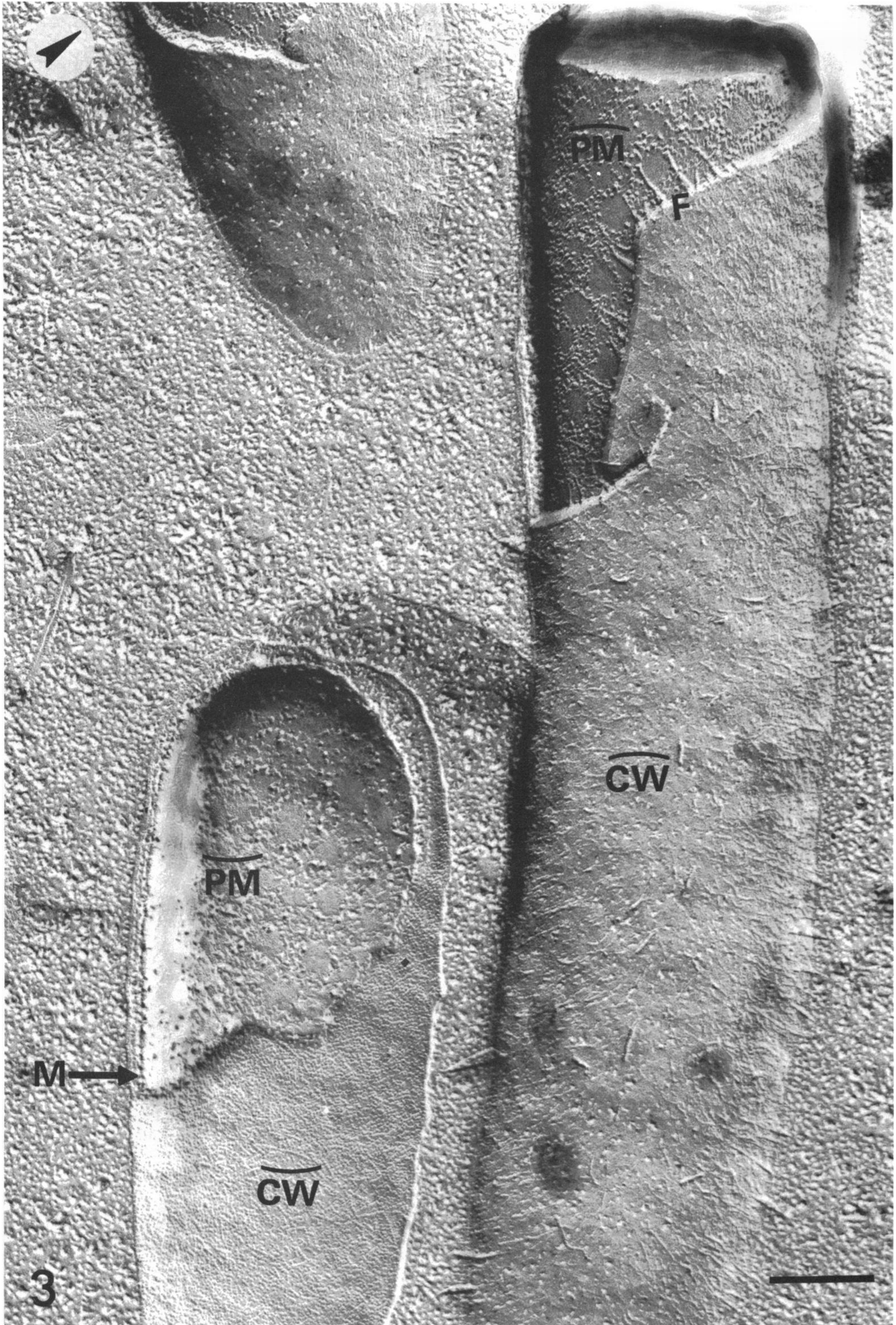


FIG. 3. *Freeze-etched cells after cryoprotection with glycerol. The cells shown here and in Fig. 4 and 5 were washed twice in HEPES buffer prior to glycerol treatment. The four fracture surfaces encountered in glycerol-protected cells are shown: convex cell wall layer, convex cytoplasmic membrane, concave cell wall layer, and concave cytoplasmic membrane. Fibrils can be seen running between the convex cell wall and the*

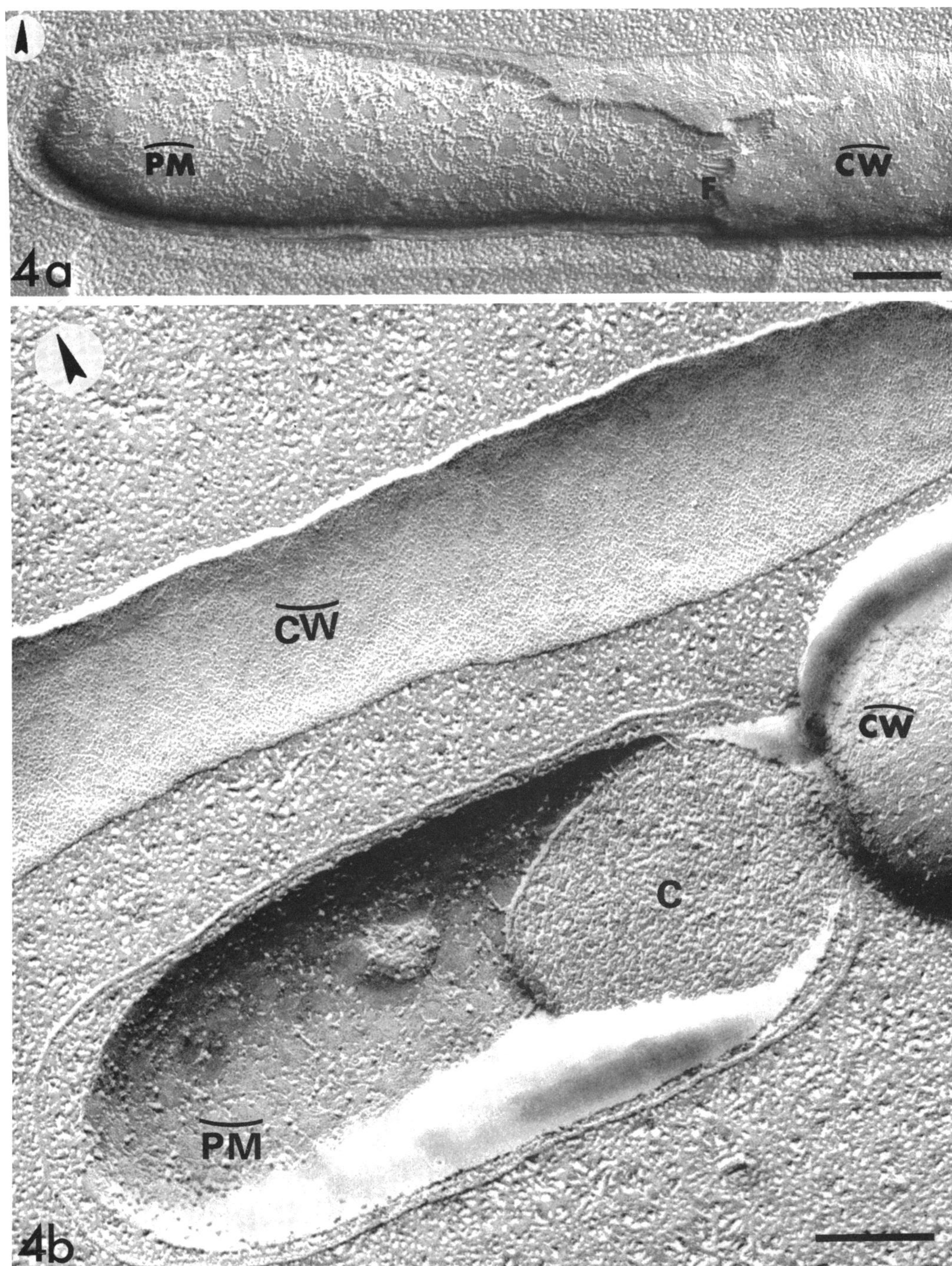


FIG. 4. *Cells freeze-etched after cryoprotection with glycerol. (a) The convex cytoplasmic membrane surface is clearly represented. Note the netlike arrangement of particles. Fibrils are shown to run between the convex cytoplasmic membrane and the convex cell wall. (b) The following layers are shown: convex cell wall, cytoplasm, concave cytoplasmic membrane and concave cell wall. The concave cytoplasmic membrane can be seen to conform to the limit of the cytoplasmic membrane in this slightly plasmolyzed cell. Spherical subunits can be seen on the concave cell wall surface. Symbols are as defined in Fig. 1.*

convex cytoplasmic membrane. A profile murein layer can be seen between the concave cell wall and cytoplasmic membrane layers. A closely packed layer of rodlets 20 to 25 nm in length, composed of spherules 6 nm in diameter, make up in part the concave cell wall. Symbols are as defined in Fig. 1.

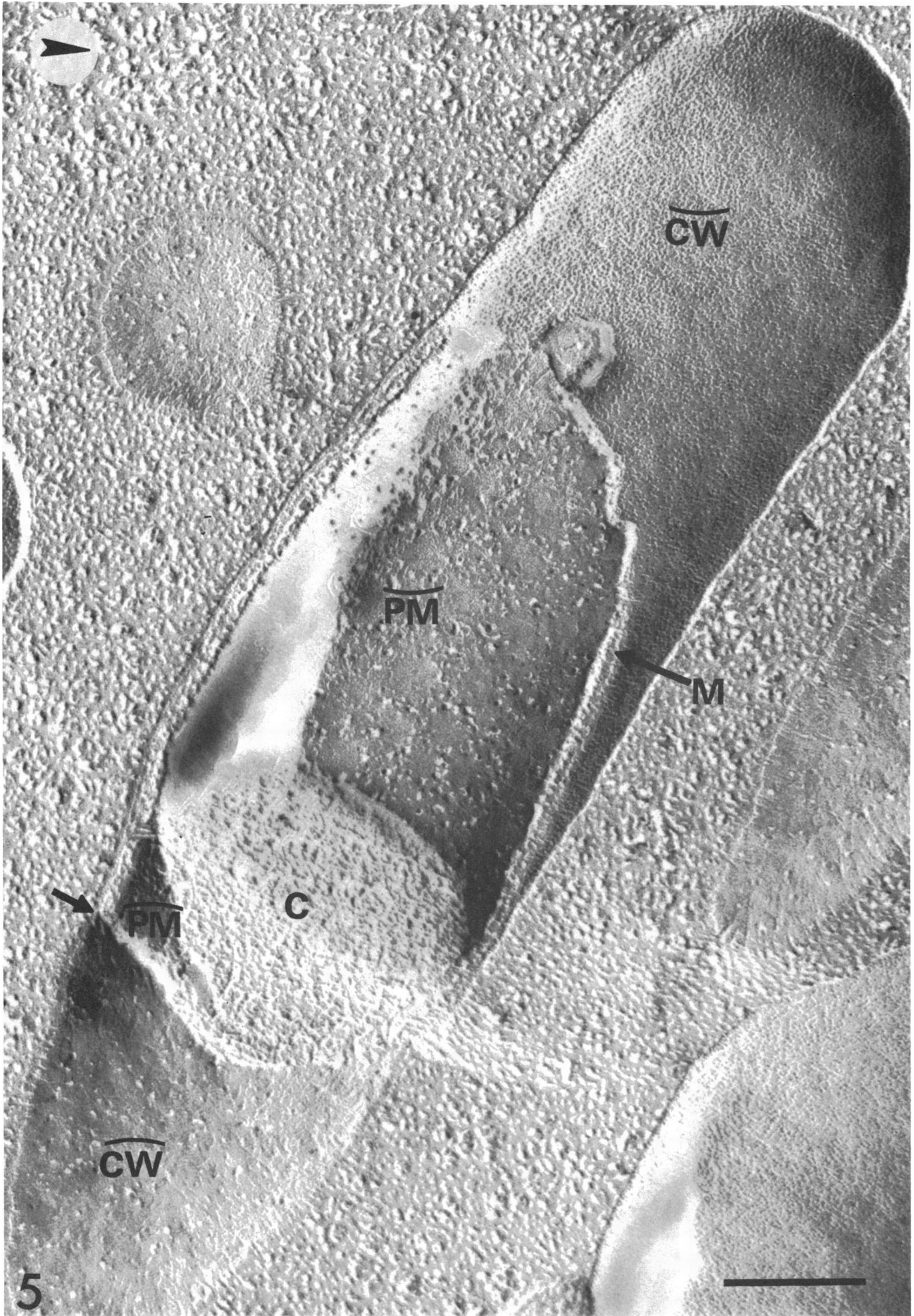


FIG. 5. *Freeze-etched cell of P. aeruginosa after cryoprotection with glycerol. This uniquely fractured cell shows all of the fractured surfaces seen in glycerol-protected cells: convex cell wall, convex cytoplasmic membrane, concave cell wall, concave cytoplasmic membrane, cytoplasm, and murein. At the unlabeled arrow,*

freeze-etching in 0.55 M sucrose containing TSB-glycerol. The concave cell wall layer in these control cells also contains closely packed spherical units which, in turn, form rodlets (Fig. 6a and b). Thus, the appearance of this layer in the control cells was identical to that in cells that were cryoprotected with glycerol alone. This demonstrates that the incorporation of the cryoprotective agent, glycerol, into 0.55 M sucrose did not alter the ultrastructure of the concave cell wall.

Freeze-etch of osmoplasts. Figure 7 shows the concave cytoplasmic membrane and cell wall layers and the murein layer of an osmoplast. The concave cell wall was altered ultrastructurally as compared with cells not treated with EDTA. The spherical units are less closely packed in the concave cell wall of osmoplasts than in that of cells not treated with EDTA. The other cell envelope layers of osmoplasts had the same appearance as in untreated cells. Other views of the concave cell wall are shown in Fig. 8a and b. Again, the loss of spherical units is evident.

Electron microscopy of the supernatant fraction from an osmoplast preparation. Spherical units and rodlets composed of spherical units were demonstrated in the supernatant fluid from an osmoplast preparation (Fig. 9a and b). The spherical units were 7 ± 1 nm in diameter, and the rodlets averaged 7 nm wide by 25 nm long. These measurements closely approximate those of the spherical units in the concave cell wall of cells not treated with EDTA. Similarly, the appearance and measurements of the spherical units and rodlets observed in the supernatant fluid from an osmoplast preparation also closely agree with those previously reported for spherical units and rodlets that were released from isolated cell envelopes of *P. aeruginosa* on exposure to EDTA (21). No spherical units or rodlets were observed in the supernatant fraction from control cells that did not receive EDTA-Tris treatment.

Freeze-etch of restored cells. Reaggregation of the spherical units into the concave cell wall occurred upon the addition of Mg^{2+} to the suspension of osmoplasts, as evidenced by the appearance of a concave cell wall with closely compacted spherical units in restored cells (Fig. 10a and b).

The concave cell wall layer of restored cells has an altered ultrastructure as compared with the same layer of normal cells. It appears to be disorganized, as evidenced by closely compacted spherical units, so that individual spherical units and rodlets cannot be as clearly discerned. The restored cells are restored to osmotic stability but not to normal viability. This lack of normal physiological functioning is consistent with the disorganized appearance of the concave cell wall.

The concave cell wall layer observed in cells grown in a medium deficient in Mg^{2+} has an ultrastructural appearance similar to the same layer of restored cells. These cells also have a disorganized concave cell wall layer with closely compacted spherical units (*unpublished data*).

Evidence for the production of osmoplasts and restored cells. Evidence that the osmoplasts were osmotically fragile cells and that osmotic stability was conferred back to the restored cells upon addition of Mg^{2+} was shown by their respective reactions upon being suspended into deionized water. The osmoplasts suffered a significant drop in optical density at 660 nm (OD_{660}), whereas the restored cells did not. An osmoplast suspension having an OD_{660} of 0.46 after initial incubation yielded an immediate OD_{660} of 0.28 after suspension in an equal volume of deionized water. The restored cells, on the other hand, gave an OD_{660} reading of 0.45 immediately after suspension in deionized water and did not change after 10 min of incubation.

Osmoplasts, however, could not be restored to osmotically stable cells by Mg^{2+} if they were first harvested and then suspended in sucrose (i.e., in the absence of material released from the cells by the action of EDTA-Tris). This indicates that reaggregation of material from the supernatant fluid was necessary for restoration to take place.

Analyses of the supernatant fluids from osmoplast and restored-cell preparations. Supernatant fractions from osmoplast and restored-cell preparations were assayed for protein and carbohydrate. The supernatant fluid from the osmoplast preparation contained 95 ± 5 μg of protein per ml, whereas the supernatant fluid from the restored cells contained 12 ± 3 μg of protein per ml. There was no detectable difference between the carbohydrate content of the supernatant fractions of the osmoplast and restored-cell suspensions. Thus, these data show that, of the total protein that was released from cells of *P. aeruginosa* on exposure to

the convex cell wall appears to be originating from the inner track of the "double-tracked" outer cell wall membrane. Note the spherical units on the concave cell wall and the netlike arrangement of depressions on the concave cytoplasmic membrane. Symbols are as defined in Fig. 1.

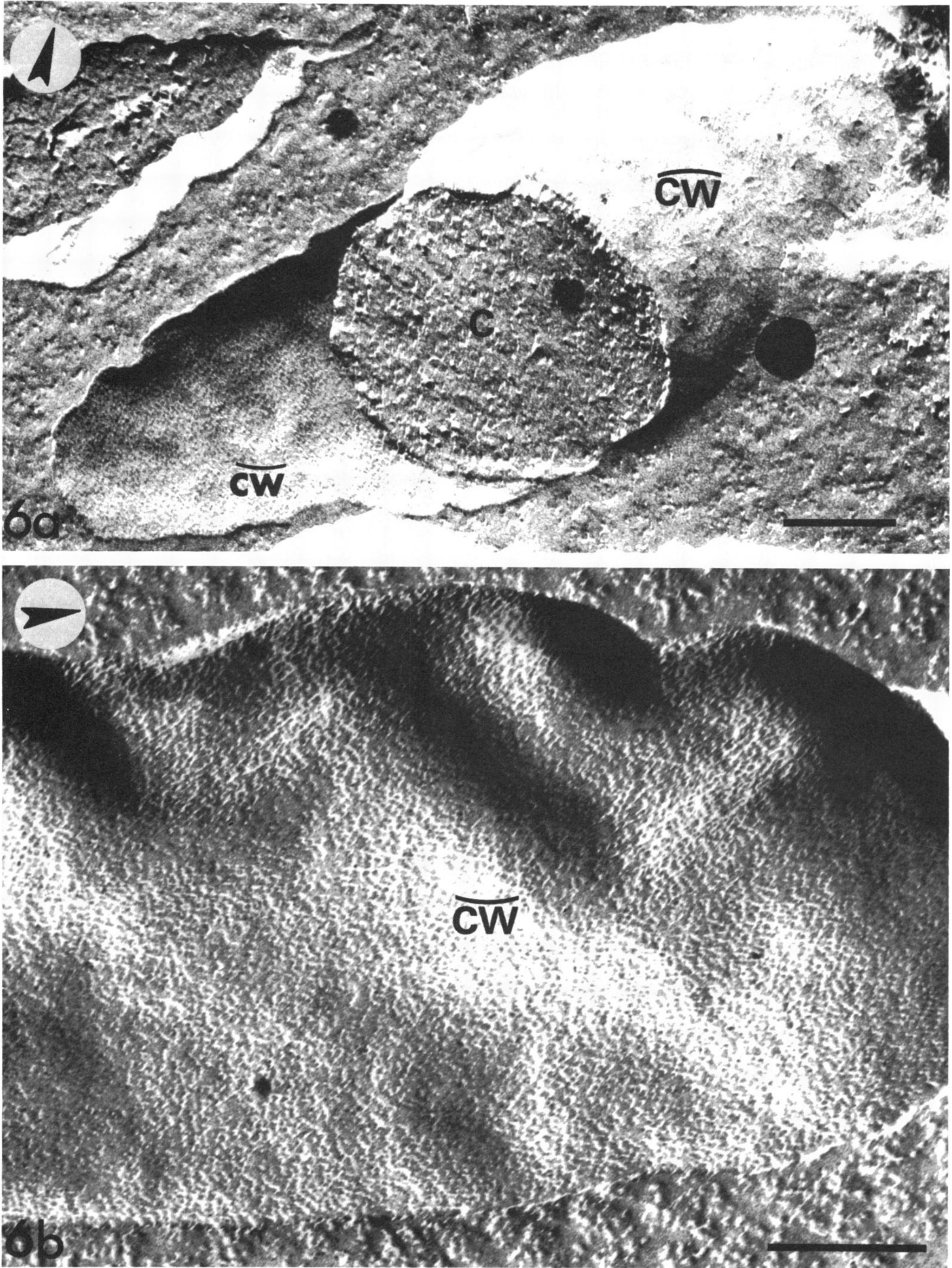


FIG. 6. *Freeze-etch of control cells cryoprotected with 30% glycerol in 0.55* M *sucrose. (a) This fracture shows convex cell wall, concave cell wall, and cytoplasm. Note that the concave cell wall has closely packed spherical units. (b) The concave cell wall can be seen to consist of closely packed spherical units and rodlets composed of these spherical units. Symbols are as defined in Fig. 1.*

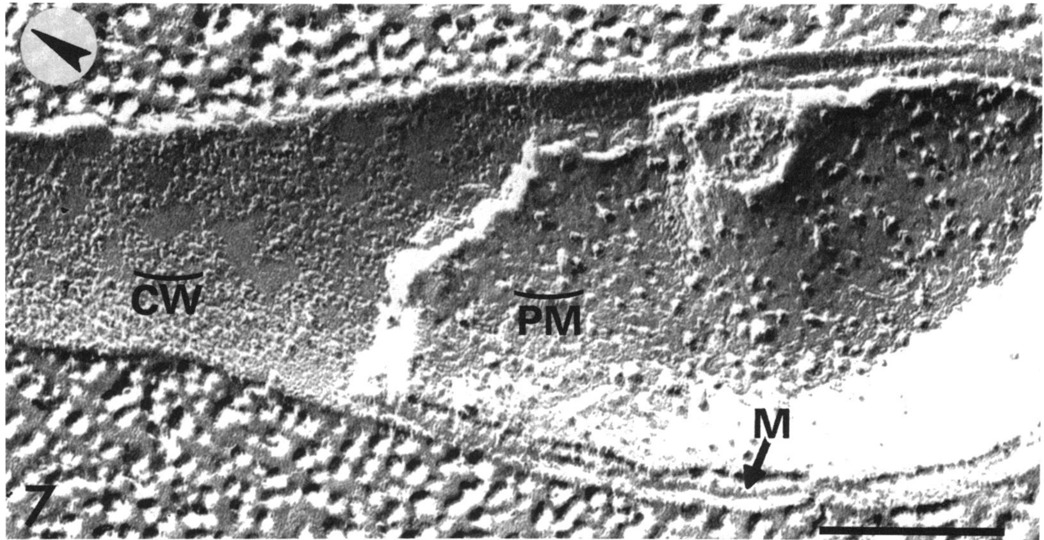


FIG. 7. *Freeze-etch of osmoplasts. Concave cytoplasmic membrane, concave cell wall, and murein are shown. Symbols are as defined in Fig. 1.*

EDTA with the formation of osmoplasts, 85% was reaggregated into the restored cells. On the other hand, 10 to 15 μg of protein per ml was consistently released when cells were suspended in sucrose plus Tris but in the absence of EDTA. This is the same concentration of protein that remained in the supernatant fluid after osmoplasts were restored. These data suggest that all of the protein that was released as a direct consequence of EDTA action was reaggregated into the restored cells. Moreover, the change in the solubilized protein is consistent with the results of electron microscopy, which showed the reaggregation of spherical units into the concave cell wall layer upon restoration of osmoplasts to osmotic stability.

DISCUSSION

The ultrastructure of the cell envelope of normal cells of *P. aeruginosa* and of cells incubated with EDTA-Tris, as revealed by electron microscopy after freeze-etching, was studied. The various freeze-etch fractures of the cell envelope of *P. aeruginosa*, as interpreted from the results of these studies, are represented diagrammatically in Fig. 11.

P. aeruginosa was shown to possess a smooth outer cell wall layer, and a regular arrangement of subunits was evident in some cases, as revealed by freeze-etching of cells not protected by a cryoprotective agent; however, this smooth outer cell wall layer was not observed in freeze-etched preparations of cells cryoprotected with glycerol. Moreover, flagella and pili were also not observed in glycerol-protected cells. Similar findings have been reported for *E. coli* (17, 24). These data suggest that, in glycerol-protected cells, the convex cell wall surface that was revealed is a cell wall layer which lies below the outermost layer of the cell wall. It is believed that the convex cell wall represents L3 of the cell wall profile as given in Fig. 11. This indicates that the outer membrane of the cell wall splits down the middle in glycerol-protected cells. The proposal that the outer membrane of the cell wall of *E. coli* (24) and of *P. aeruginosa* (14) splits in this manner to reveal the inner layers has been previously proposed. Whether the regular array of subunits of the smooth outer cell wall layer constitutes the outer surface of L1 (Fig. 11) or whether the subunits constitute a separate layer superficial to L1 cannot be discerned from the present studies.

The next structure encountered after the convex cell wall as the cell is viewed from the outside going toward the cytoplasm is the convex cytoplasmic membrane. The granules associated with this layer are believed to be L8, and the underlying supporting layer is thought to be L9 (Fig. 11). The netlike arrangement of granules in this layer is similar in appearance to that previously observed in *E. coli* (10). These data suggest that the cytoplasmic membrane splits down its center to reveal the inner layers, as has been suggested by other workers (5-7, 18, 23, 24).

The murein layer, L5 (Fig. 11), was seen in

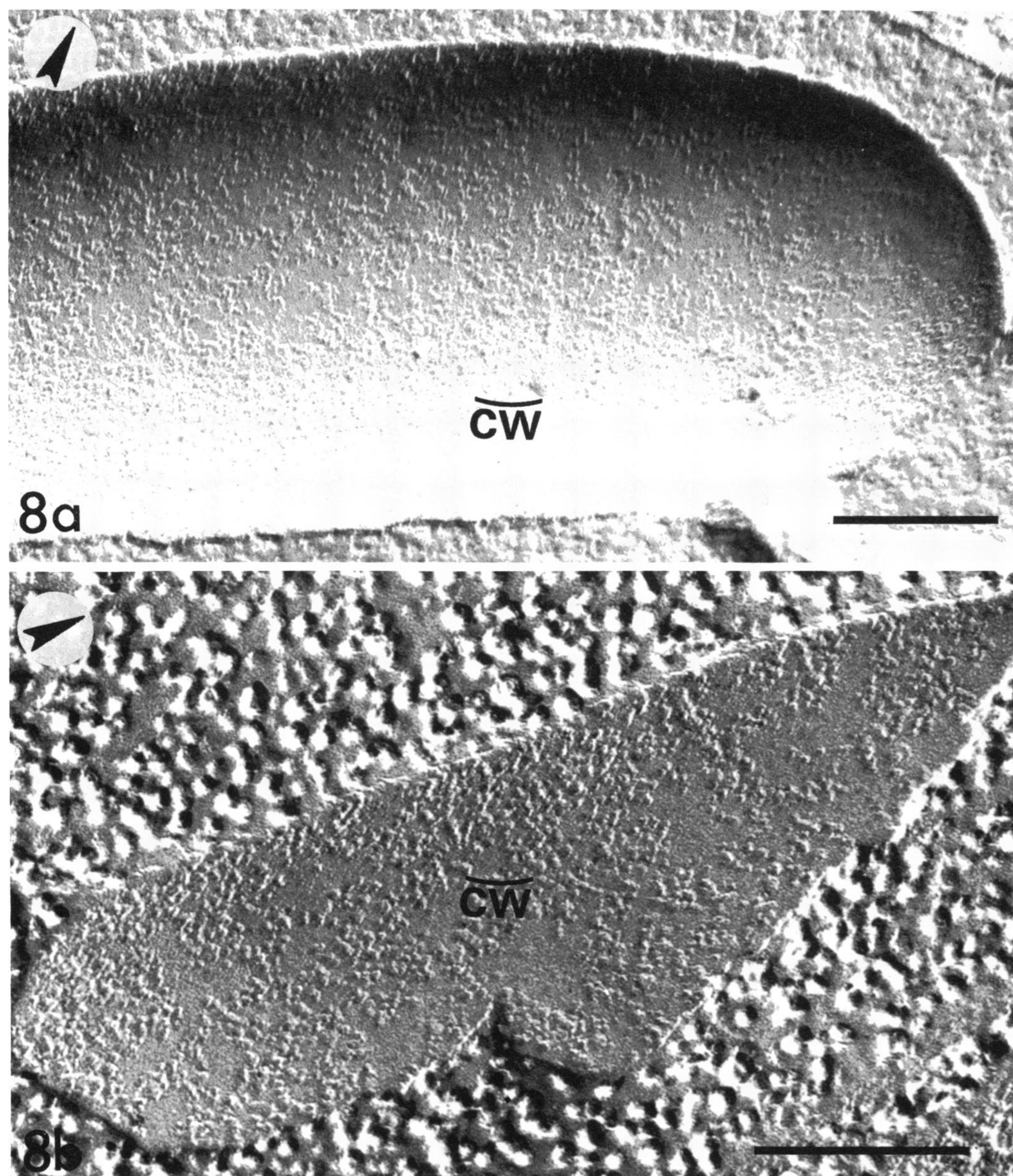


FIG. 8. *Freeze-etch of osmoplasts. (a) The concave cell wall is shown to have lost many of the spherical units. (b) Another concave cell wall fracture is shown with the loss many of the spherical units being evident. Symbols are as defined in Fig. 1.*

profile between the concave cytoplasmic membrane and cell wall layers. Fibrils between the convex cell wall and cytoplasmic membrane layers were also observed; these may be composed of murein, as has been proposed by some investigators (19), or they may be artifacts, as suggested both herein and by other workers (10).

The concave cytoplasmic membrane is the first layer encountered as glycerol-protected, freeze-etched cells are viewed from the cytoplasm going toward the environment. This represents L7 in Fig. 11. It is the side of the cytoplasmic membrane which splits away from L8 and L9 when the membrane fractures down the center. Thus, it has a netlike pattern of

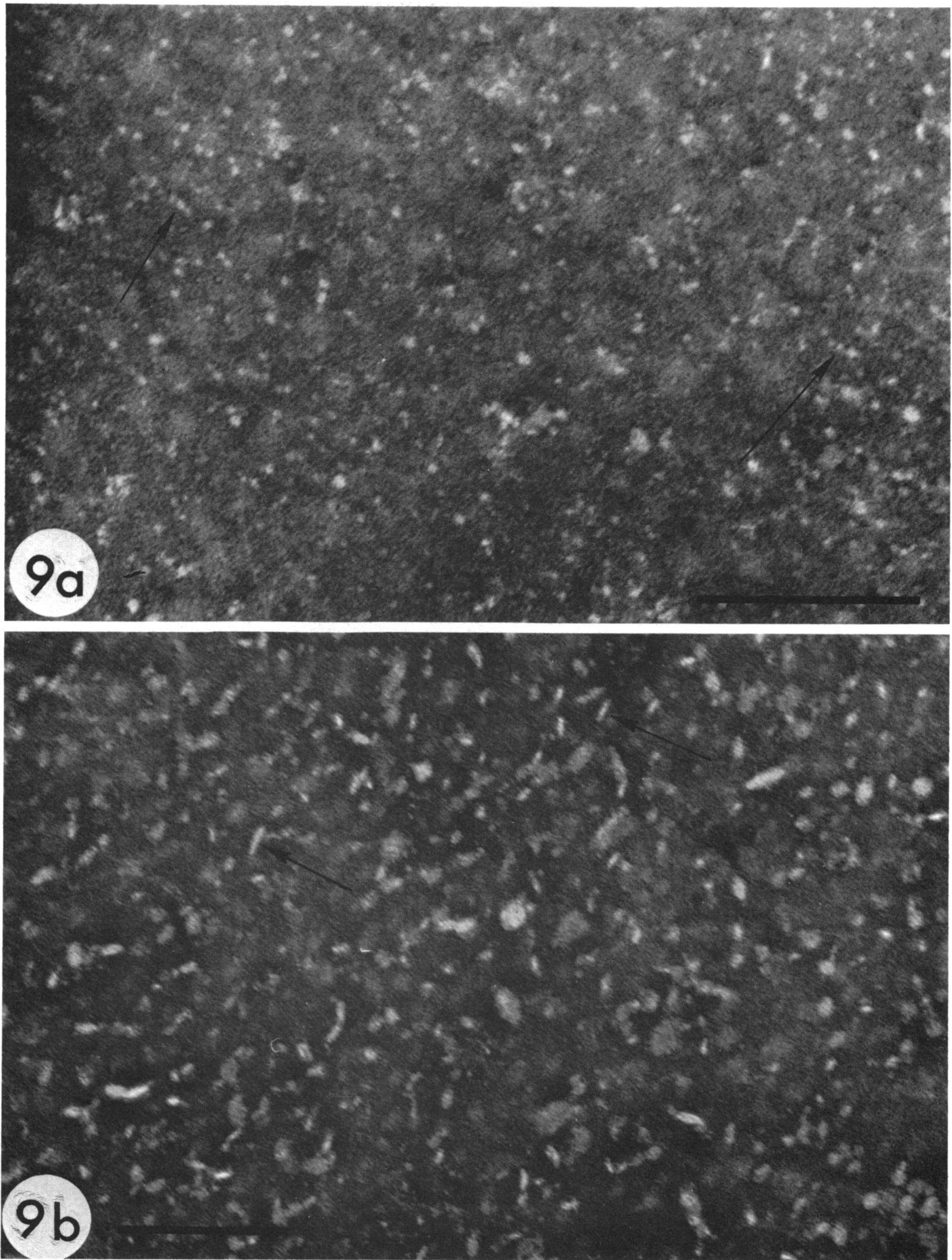


FIG. 9. *Negatively stained (phosphotungstic acid) preparation of the supernatant fraction from an osmoplast suspension. (a) Spherical units and rodlets (arrows) are shown. The rodlets can be seen to be composed of three spheres in a chainlike fashion. (b) Another supernatant preparation from an osmoplast suspension with numerous rodlets shown.*

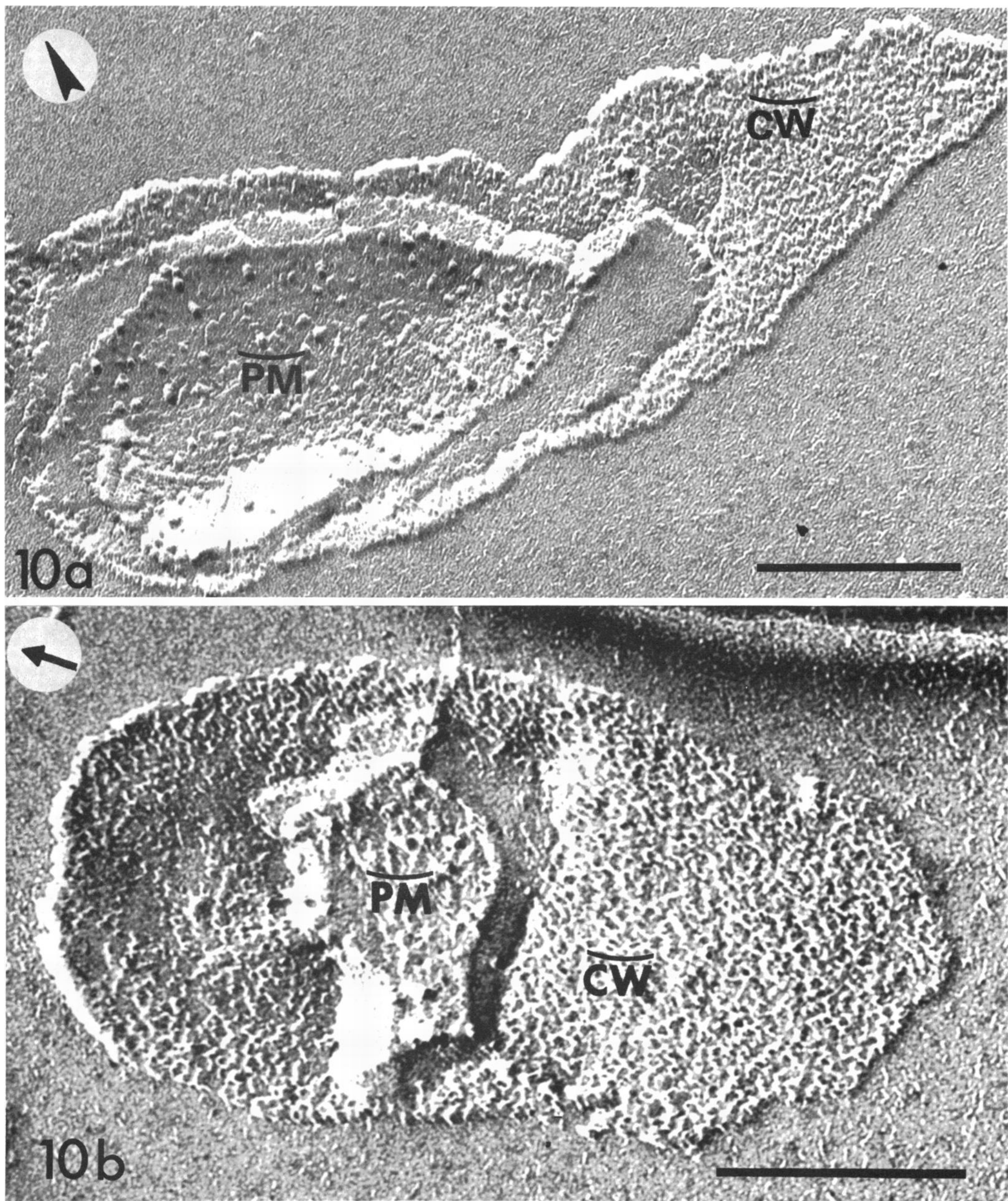


FIG. 10. *Freeze-etch of restored cells. (a) This cell shows the concave cytoplasmic membrane and the concave cell wall. The concave cell wall can be seen to be closely packed with spherical units. (b) Another view of the concave cell wall which shows it to be closely packed with spherical units. Symbols are as defined in Fig. 1.*

depressions and smooth spaces complementary to the netlike pattern of granules and smooth spaces associated with the convex cytoplasmic membrane.

The granules of L8 are assumed to be protein (11, 16). It is interesting to speculate, therefore, that L7 and L9 may be lipid layers. Hence, the cytoplasmic membrane could be viewed as a lipid-protein-lipid structure with protein units embedded more deeply in the lipid layer represented by L9 than in L7. Thus, when the membrane fractures down the middle, the gran-

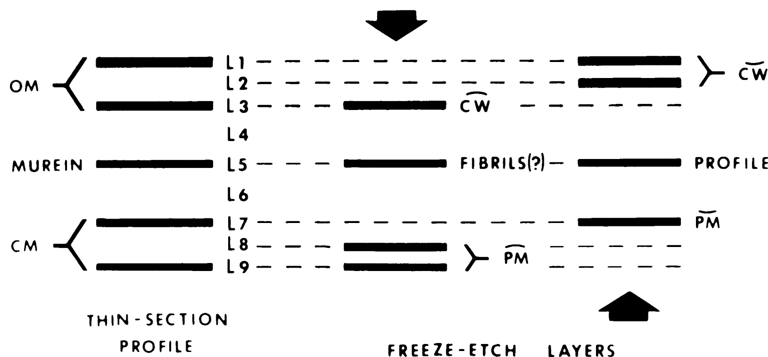


FIG. 11. *Diagrammatic representation of the layers (L1-L9) of the cell wall of P. aeruginosa as revealed by thin-sectioning and by freeze-etching techniques employing glycerol cryoprotection. In the thin-sectional profile, the following symbols are used: outer membrane (OM), murein, and cytoplasmic membrane (CM). The electron-transparent space between the outer membrane and murein is represented by L4, and the periplasmic space, by L6. In the freeze-etch layers, the following symbols are used: concave cell wall (CW), convex cell wall (CW), concave cytoplasmic membrane (PM), and convex cytoplasmic membrane (PM). When viewed from the direction denoted by the arrow, the layers represented by dark, solid lines are the layers which would be revealed by the fracture, whereas the dashed lines represent the layers not revealed.*

ules remain in the lipid layer designated L9, and complementary depressions can be seen in L7. This concept is consistent with similar interpretations as discussed by other workers (6, 13), and it is also consistent with the fluid mosaic membrane model (11, 22).

The concave cell wall layer exposed upon freeze-etching is believed to correspond to L1 and L2, with the convex cell wall, or L3, being complementary to L1-L2 (Fig. 11). L2 consists mainly of spherical units which are considered to be composed mainly of protein. The spherical units form small rods. The data suggest that L1 is the backing on which the spherical units rest or in which they are embedded. The outer cell wall membrane of gram-negative bacteria is thought to be a "simple" biological membrane consisting of both lipid and one or two major proteins in addition to lipopolysaccharide. Moreover, the lipid content of the outer cell wall membrane and the cytoplasmic membrane has been shown to be quantitatively similar in *E. coli* (25). Finally, the "smoothness" of L1 is similar in appearance to the smooth faces of other freeze-cleaved biological membranes which have been interpreted to consist of lipid, probably representing the central plane of a bilayer (26). Thus, consistent with current concepts, L1 is considered to be rich in lipids. Lickfeld et al. (14) also demonstrated an inner cell wall with the same appearance as our concave cell wall, and these workers also identified this layer as L1 and L2. Other investigators, moreover, have similarly postulated that the cell wall outer membrane splits down the middle to reveal a protein layer (24). The cell wall outer membrane, therefore, may also be a lipid-protein-lipid membrane comparable to the cytoplasmic membrane.

The outer smooth layer with the regular array of subunits seen in micrographs of cell walls of cells freeze-etched without a cryoprotective agent is considered to be on the outside surface of L1. This was not revealed in glycerol-protected, freeze-etched cells owing to the fracturing of the cell wall outer membrane down the middle. This would also explain why flagella and pili were not seen in glycerol-protected freeze-etched cells.

Freeze-etching of osmoplasts shows that the spherical units and rodlets of the concave cell wall are extracted by the EDTA-Tris treatment. The components were demonstrable in the supernatant fluid of the osmoplast preparation. Upon the addition of Mg^{2+}, the concave cell wall can be seen once again to be closely packed with the spherical units, and the osmoplasts were shown to be restored to osmotically stable cells. Restoration of these spherical units to the concave cell wall involves the removal from the supernatant fluid of 85% of the protein that was released from the cells on exposure to EDTA-Tris. These data indicate that the spherical units are composed of protein. These results are consistent with previous work which showed that protein and lipopolysaccharide were released from isolated cell envelopes of *P. aeruginosa* on exposure to EDTA-Tris (20, 21).

These present results suggest that an EDTA-Tris-sensitive site in the cell wall of *P.*

aeruginosa resides in the layer designated as the concave cell wall (i.e., L2, Fig. 11). The EDTA-extractable material, which is composed mostly of protein, is revealed as spherical units. These units can be reaggregated onto the concave cell wall upon the addition of Mg^{2+}, and osmotic stability is restored to these cells. The protein units were not characterized further in the present work, but their nature is now under investigation.

Attempts to freeze-etch osmoplasts without cryoprotection were unsuccessful owing to their osmotic fragility. Thus, it could not be discerned from these studies whether the smooth outer cell wall layer and the regular array of subunits were affected by EDTA-Tris.

The osmotically stable restored cells that result upon the addition of Mg^{2+} to the osmoplast suspension containing EDTA-Tris respire normally but have a low percentage of viability, as evidenced by their failure to multiply when transferred to a favorable medium. This phenomenon has been reported previously (1, 2), and it was reconfirmed during these investigations. Whether EDTA-Tris affects the cell membrane, causing impaired permeability or a loss of cytoplasmic pools of essential metabolites, or whether the cell wall-associated protein has essential enzymatic activity that is impaired upon reaggregation cannot be discerned from these experiments.

In vitro reaggregation of components of outer membrane vesicles isolated from *E. coli* has been demonstrated (8). The present report, however, is believed to be the first ultrastructural demonstration of bacterial cell wall components reaggregating onto the cell wall in a living cell system.

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