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Demonstration by Freeze-Etching of a Single Cleavage Plane in the Cell Wall of a Gram-Negative Bacterium

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In the examination of protoplasts of a gram-negative bacterium classified as a *Pseudomonas* sp. by freeze-etching, we found a smooth external surface which is not seen if the preparations are not "etched." This external structure is seen as a sleeve surrounding and connecting the cells in unetched preparations, and we present evidence that it is a eutectic formed during the freezing of the specimen. In the system used in this study, the four layers of the cell wall of a gram-negative bacterium can be removed from the cell. The single cell wall cleavage plane is not affected by the removal of the loosely bound outer layer or of the peptidoglycan layer, but it is lost when the outer double track layer and the underlying soluble layer are simultaneously removed. Thus, we conclude that it is one of these two layers which is responsible for the cleavage plane which exposes variable areas of a smooth surface in the cell wall. This cell wall cleavage plane is more likely to deflect the actual cleavage of the frozen cell when cells are relatively old or when they are suspended in sucrose.

The refinement of the freeze-etching technique has led to its widespread use in the examination of biological systems (16, 20, 25), and it has added materially to our overall knowledge of cell structure. Paradoxically, although freeze-etching reveals the topography of the cellular membranes very effectively, the precise location of the cleavage plane in relation to the membrane remains has only recently been resolved (6, 17, 19). Until this time, there was no agreement on whether the actual cleavage follows the surface of the membrane (2) or a postulated hydrophobic median layer (12), or whether it alternates between the two (4), but it was clear that it does follow the contour of the membrane at some level.

The surfaces exposed by freeze-etching in the cell envelope of gram-negative bacteria were interpreted variously by different authors. They all described a cleavage at the level of the cytoplasmic membrane which exposes a surface covered with 5- to 12-nm particles in which particle-free smooth patches are seen to occur (2, 12, 18). External to the cytoplasmic membrane, from one (12) to four (18) relatively smooth surfaces were described and interpreted as resulting from cleavage in specific cell wall layers such as the "lipoprotein-lipopolysaccharide" layer (2). "Etching" revealed the presence of certain highly ordered structures on the surface of gram-negative bacteria (22, 28) but there is no evidence to suggest that these arrays of globular subunits constitute a cleavage plane.

Methods developed in this laboratory (5, 8, 10) enabled us to remove four cell wall layers, sequentially, from the cells of a marine pseudomonad and to produce true protoplasts of this organism. This system allowed us to study the effect of the removal of specific cell wall layers on the cleavage pattern produced by freeze-etching in the cell envelopes of these gram-negative cells.

MATERIALS AND METHODS

Organism. The organism used and designated marine pseudomonad B-16 was originally isolated from a marine clam and was classified as a *Pseudomonas* species type IV. It was deposited in the American Type Culture Collection (ATCC 19855) and in the National Collection of Marine Bacteria, Aberdeen, Scotland (NCMB 19). Studies on the metabolism and ultrastructure of this organism were reported in some detail in previous communications (8, 26).

Medium and growth conditions. The media used for the maintenance of stock cultures and for growth were previously described (5). The culture was maintained by monthly transfer on slants, and exponential cultures

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were produced by the method detailed in DeVoe et al. (8). These cultures were harvested by centrifugation, both for the preparation of specimens for freeze-etching and for the preparation of modified cells.

Preparation of modified cells. Spheroplasts were prepared by the method of Costerton et al. (5), cells lacking their loosely bound outer layers were prepared by the method of Forsberg et al. (10), and mureinoplasts were prepared by the methods of Forsberg et al. (10) and of DeVoe et al. (8). "Sucrose protoplasts" were prepared by centrifuging (16,000 × *g* at 4 C) mureinoplasts from the 0.5 M sucrose in which they were formed and suspending them (20 mg, dry weight, of cells per ml) in a solution containing 0.5 M sucrose, 100 μg of lysozyme per ml, and 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (Trizma-hydrochloride) at *p*H 8.0. "Complete salts protoplasts" were prepared by suspending the mureinoplasts (6 mg, dry weight, of cells per ml) in a solution containing 0.3 M NaCl, 0.05 M $MgSO_4$, 0.01 M KCl, 150 μg of lysozyme per ml, and 1 mM Tris buffer (Trizma-hydrochloride) at *p*H 7.5.

Electron microscopy. Specimens were prepared for freeze-etching by immersing discs bearing droplets of the thick cell pellets into liquid Freon 22. The discs bearing the specimens were placed on the specimen table of a Balzer's 510M unit at −100 C and cleaved with a cold knife (−168 C) until a large evenly chipped face was obtained. In preparations where "etching" was desired, the final chipped face of the specimen was held for 1 min at −100 C approximately 2 mm under the base of the knife holder (17). In preparations made without etching, the specimens were chipped until a smooth face was obtained, the knife was then lowered 36 nm and passed through the specimen, and the platinum shadow-coating was carried out immediately thereafter. Most preparations made by this method are virtually unetched, but some replicas contain small areas where depressions in the previous chipped face form a part of the final replicated face, and these areas are very highly etched and are easily distinguishable from the unetched areas.

All preparations were examined by means of an AEI EM-6B electron microscope using 60-kv electron accelerating voltage. Electron micrographs were taken by using Ilford N-50 plates and were printed in reverse to facilitate interpretation.

RESULTS

Protoplasts. Sucrose protoplasts are less stable than complete salts protoplasts and they do not show the intrusions of the cytoplasmic membrane and the peripheral vesicles seen in the latter (8). The treatment with lysozyme causes rounding of the mureinoplasts (8), which have already lost their outer cell wall layers (10); the protoplasts formed contain only traces of hexosamine (10), which indicates that the peptidoglycan layer has also been completely removed.

When the protoplasts are freeze-etched, the surface which is exposed is largely covered with 5- to 12-nm particles which are either spherical or rod-shaped (Fig. 1). Smooth patches can be seen in certain areas where these particles are absent (Fig. 1, 2, 4, and 7). Such surface features are common in the cytoplasmic membrane cleavage plane of most cells studied to date. Both in the case of protoplasts prepared and frozen in sucrose and of those prepared and frozen in complete salts, a smooth "etched" surface was seen external to the cytoplasmic membrane (Fig. 1, arrow). In some instances, the etched surface joined the cleaved surface at a thin collar ca. 10 nm in height (Fig. 1, A), but more often a thick collar (15 to 100 nm) was formed (Fig. 1, B) where these surfaces joined.

When protoplasts were cleaved and not etched only the particle-studded cytoplasmic membrane plane was exposed (Fig. 2). The particles on this surface are larger (10 to 18 nm) than those seen in etched preparations, and the rod-shaped particles are not seen (Fig. 2). The particles in the cross-cleaved cytoplasm (16) are also larger and more discrete than those in etched preparations (compare circled areas in Fig. 1 and 2). The unetched preparations do not show a smooth layer external to the cytoplasmic membrane but a low sleeve-like structure of variable thickness (Fig. 2, arrows) can be seen to surround the cells and occasionally to form bridges between them. In cases in which the actual cleavage had passed under the cell, to give a concave depression in the ice, the surface exposed by the cytoplasmic membrane cleavage plane bears the familiar widely separated particles (Fig. 2), and a sleeve-like structure (Fig. 2, arrows) can be seen between the edges of this layer and the ice surface.

These preparations of protoplasts of a gram-negative cell have shown that etching reveals, or produces, a smooth layer external to the well known particle-studded surface exposed by the cytoplasmic membrane cleavage plane.

Mureinoplasts. The cleavage pattern of mureinoplasts (Fig. 3) is the same as that of protoplasts, and we see that a particle-studded surface is exposed by cleavage, whereas etching reveals a smooth outer layer (Fig. 3, arrow). Previous studies showed that these cells lost the outer layers of their cell wall but that their peptidoglycan layer is still intact (10). The fact that the etched surface of these cells is the same as that of the protoplasts may indicate either that the peptidoglycan is too fine to be resolved by this method, or that etching does not reveal the true surface of the cell.

Spheroplasts. The actual cleavage was seen to follow two cleavage planes in the envelopes of these cells (Fig. 4). Cleavage along the innermost plane exposed the familiar particle-studded surface associated with the cytoplasmic membrane, whereas cleavage along the outer plane exposed patches of a smooth surface, and etching re-

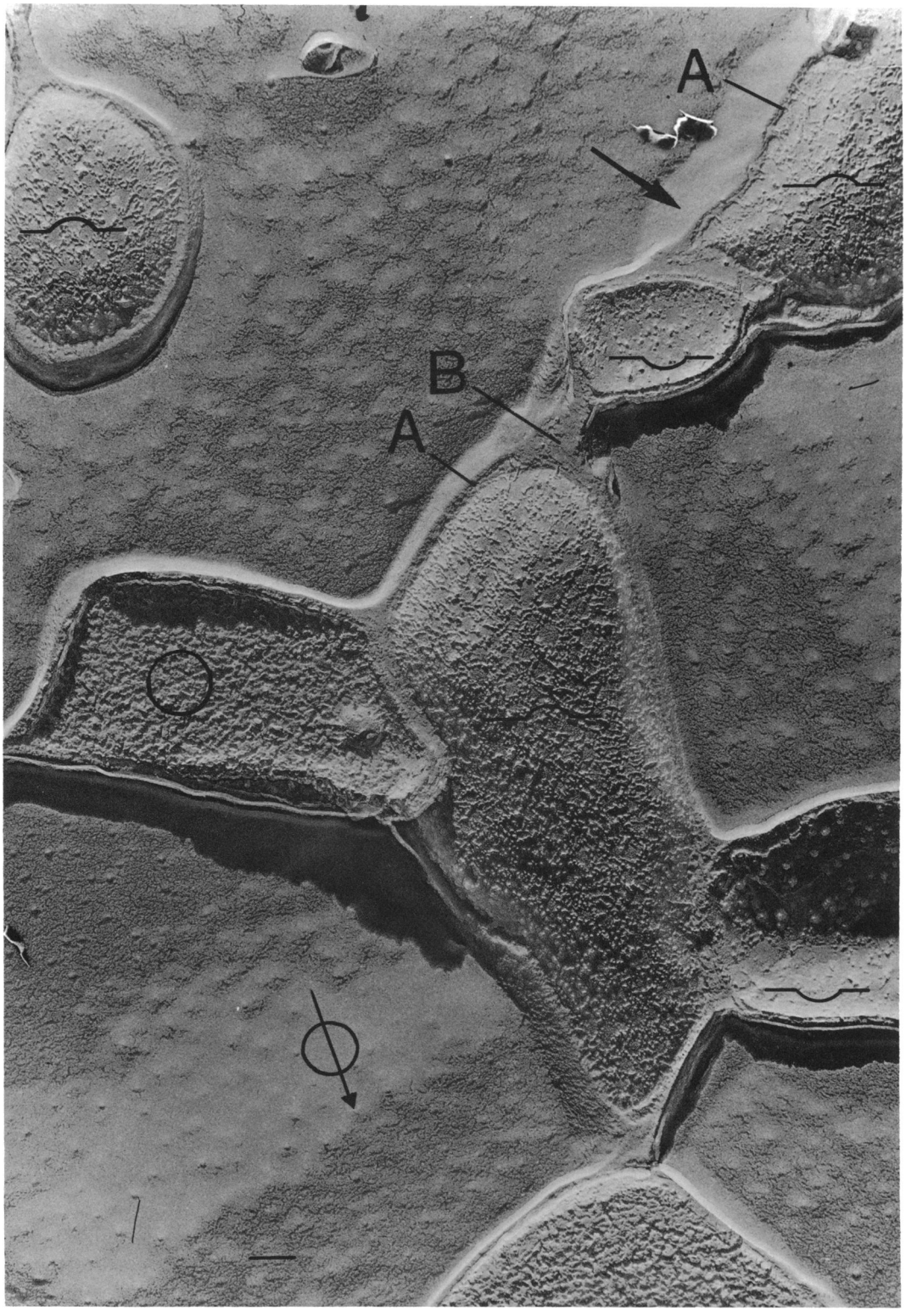

FIG. 1. *Replica of protoplasts prepared in sucrose after freeze-etching. Note that the smooth outer surface (arrow) is separated from the particle-studded surface exposed by the cytoplasmic membrane cleavage plane by a thin (A) or a thick (B) collar. The circled area shows the detail of the cross-cleaved cytoplasm. Symbols:* ⌒, *convex cleavage;* ⌣, *concave cleavage;* Ø, *direction of shadow. All illustrations were printed in reverse to aid interpretation. Bar indicates 0.1 μm in Fig. 1–7.*

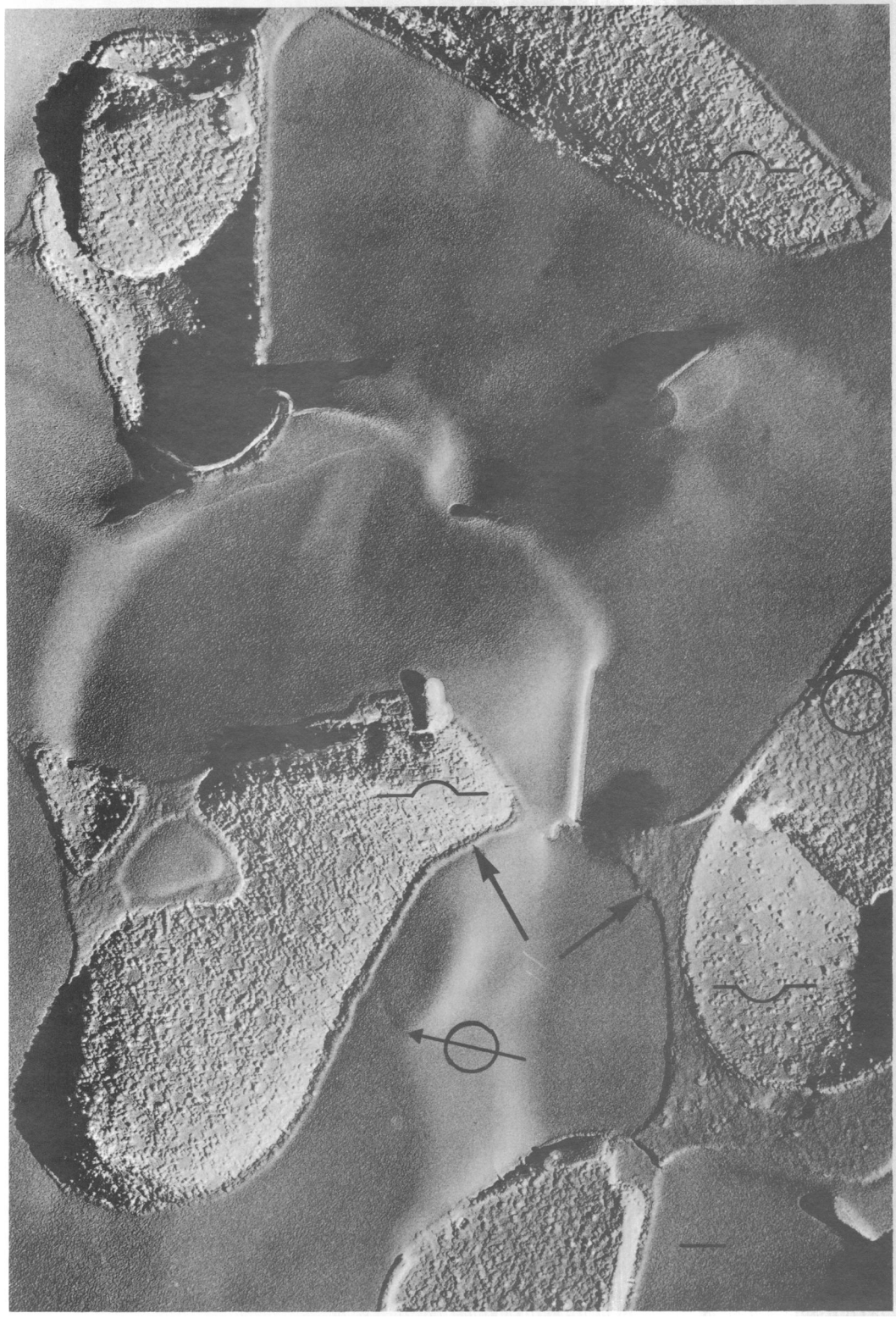

FIG. 2. *Replica of protoplasts which were cleaved but not etched. Note that the cells are connected and surrounded by a sleeve of variable thickness (arrows), which is distinguishable from the surrounding ice surface. The circled area shows the detail of the cross-cleaved cytoplasm.*

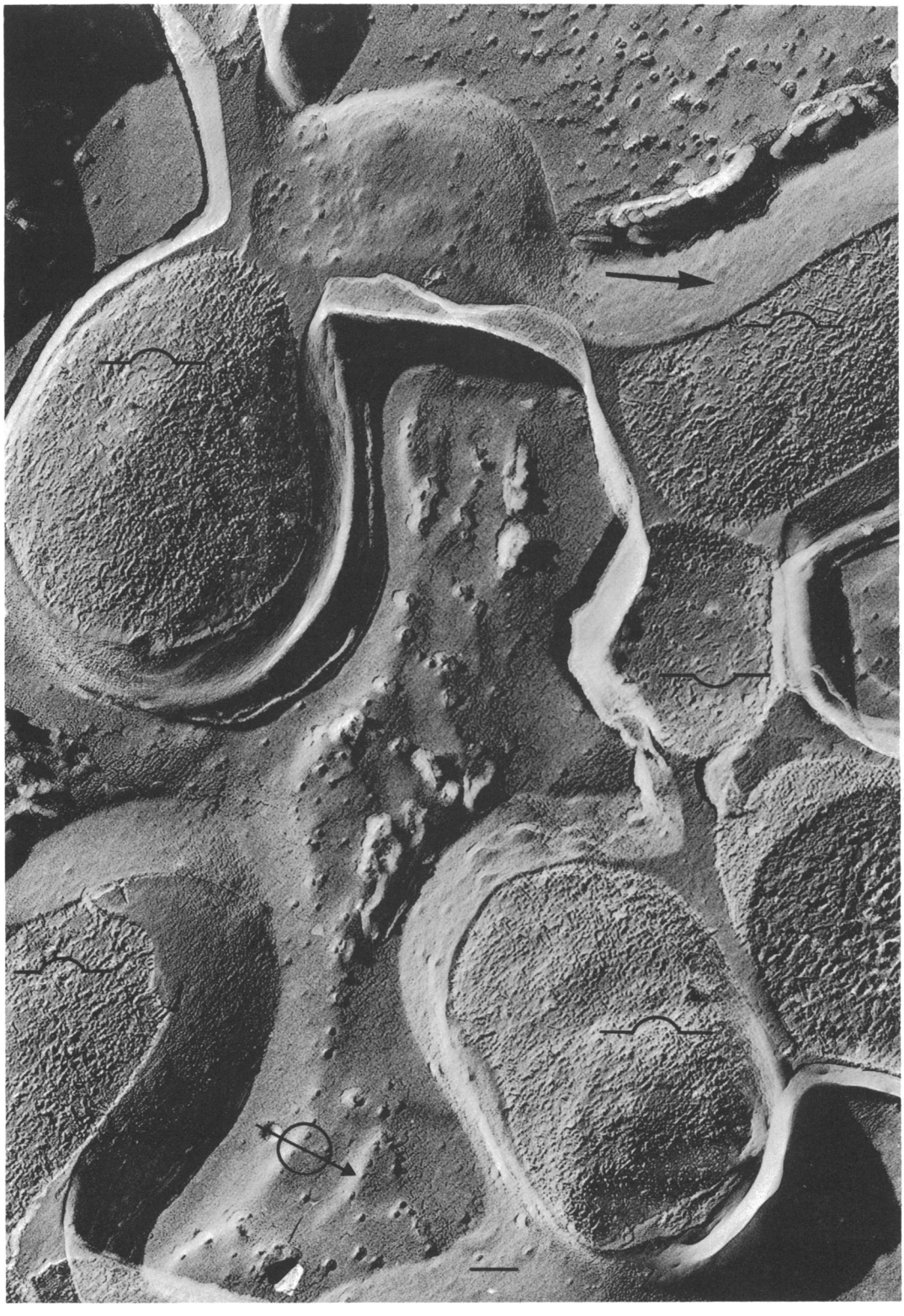

FIG. 3. *Replica of mureinoplasts after freeze-etching. Note the smooth outer layer (arrow) and the presence of a collar of variable thickness similar to that seen in the etched protoplast preparations.*

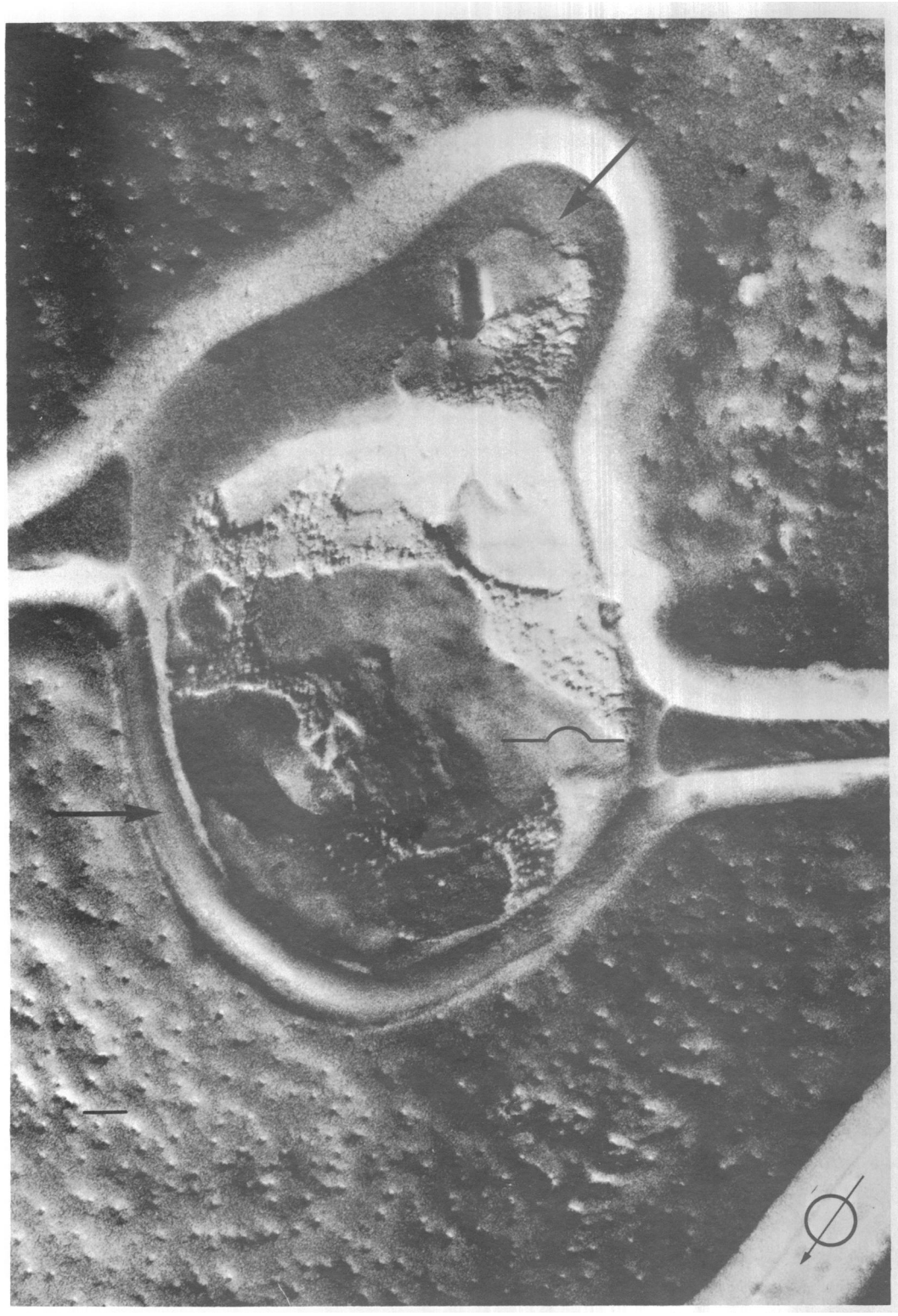

FIG. 4. *Replica of a spheroplast after freeze-etching. Note the presence of patches of a smooth surface lying outside the particle-studded surface exposed by the cytoplasmic membrane cleavage plane and inside the collar (arrows) revealed by etching.*

vealed another smooth layer lying external to these patches (Fig. 4, arrows). Electron microscopy of sectioned material (5) showed that the outer double track layer of the cell wall is retained in these cells.

Whole cells. Cleavage of frozen whole cells exposed the same two surfaces seen in cleaved spheroplasts. A particle-studded surface was seen at the level of the cytoplasmic membrane (Fig. 5–7) and patches of a smooth surface were seen in the cell wall. The latter plane was not strong enough, however, to deflect the actual cleavage in all cells and the smooth surface in the cell wall is not exposed in some cells (Fig. 6). Etching revealed a smooth layer external to these described above (Fig. 5 and 6, arrows) but this layer was not seen when the preparations were not etched. As in the case of the protoplasts. the cytoplasmic particles and membrane particles appear larger in unetched preparations, and no "rodlets" are seen on the membrane (Fig. 7).

When the actual cleavage followed a concave pattern, so as to produce a depression in the cleaved surface, the familiar sparsely studded "inside" (24) surface of the cytoplasmic membrane was exposed (Fig. 6 and 7); the weaker cleavage plane in the cell wall exposed variable amounts of a smooth surface external to the membrane (Fig. 7). As in the unetched protoplasts, there was a "sleeve" of material surrounding the cells and occasionally forming bridges between them (Fig. 7, arrows).

These preparations have shown that, in spheroplasts and in whole cells, there is a single cleavage plane that lies external to that of the cytoplasmic membrane and internal to the surface exposed by etching. In whole cells, this cell wall cleavage plane is not as effective in deflecting the actual cleavage as is that of the cytoplasmic membrane, but it is more effective in older cells (24- and 48-hr cultures) and in spheroplasts.

Cells lacking the loosely bound outer layer. Cells were suspended in 0.5 M NaCl to remove the outermost layer of the cell wall, which comprises 4.7% of the dry weight of these cells (11), but no change was seen in the cleavage pattern as compared with whole cells. Although morphological units discrete enough to be seen by negative staining were removed by this procedure, no changes are seen in the smooth external layer revealed by etching.

Diagramatic representation of the cleavage planes. Figure 8a shows how the actual cleavage of frozen protoplasts follows the cytoplasmic membrane and how etching reveals the "extra" layer outside the membrane. The deflection of the actual cleavage by both the cytoplasmic membrane and cell wall cleavage planes of the whole cell is illustrated in Fig. 8b, as well as the way in which the presence of a eutectic layer produces an external smooth surface on etching. The median cleavage pattern illustrated in the diagram was recently established by da Silva and Branton (6) and by Nanninga (*in press*).

DISCUSSION

In this study, we were concerned with the number of cleavage planes in the cell envelopes of whole cells and of protoplasts rather than with the very controversial subject (17) of the location of the planes themselves within the membrane and the specific wall layer. A cleavage plane is a zone of least resistance to cleavage and, whether the plane lies along the hydrophobic median layer (6; Nanninga, *in press*), the polar-hydrophobic interface (13), or whether it alternates between these two levels (4, 23), it is clear that it is associated with the organized phospholipid molecules of the membrane. On this basis, it can be argued that the existence of a cleavage plane, which significantly deflects the actual cleavage of a cell, indicates the presence of an organized zone of high lipid concentration.

We previously reported that the outer double track layer of the cell wall of the marine pseudomonad used in this study can be removed from the cells by a simple manipulation of the cation concentration (5), and that this layer is composed largely of proteins and phospholipids (11). Preliminary X-ray diffraction studies of this detached outer double track layer (Forge and Costerton, *unpublished data*) show a radial pattern in a region that indicates the presence of highly organized alkyl chains of the phospholipid much like that proposed by Engleman (9). The presence of a layer containing oriented phospholipids would have considerable functional significance if its organized structure allowed it to act as the "molecular sieve" layer (15) which acts as the outer boundary of the periplasmic space (3).

In the etched preparation of protoplasts presented in this study (Fig. 1), we showed that the particle-studded surface exposed by the cytoplasmic membrane cleavage plane is surrounded by a smooth surface which extends from the membrane surface to the ice. The absence of this smooth surface in unetched preparations (Fig. 2) shows that it is revealed by the etching and not exposed by cleavage. Because these protoplasts are essentially free of cell wall constituents (11), there are only two possible explanations for these smooth "collars" around the protoplasts. If we accept recent evidence (6; Nanninga, *in press*) supporting median cleavage, the smooth layer could be the outer half of the cytoplasmic mem-

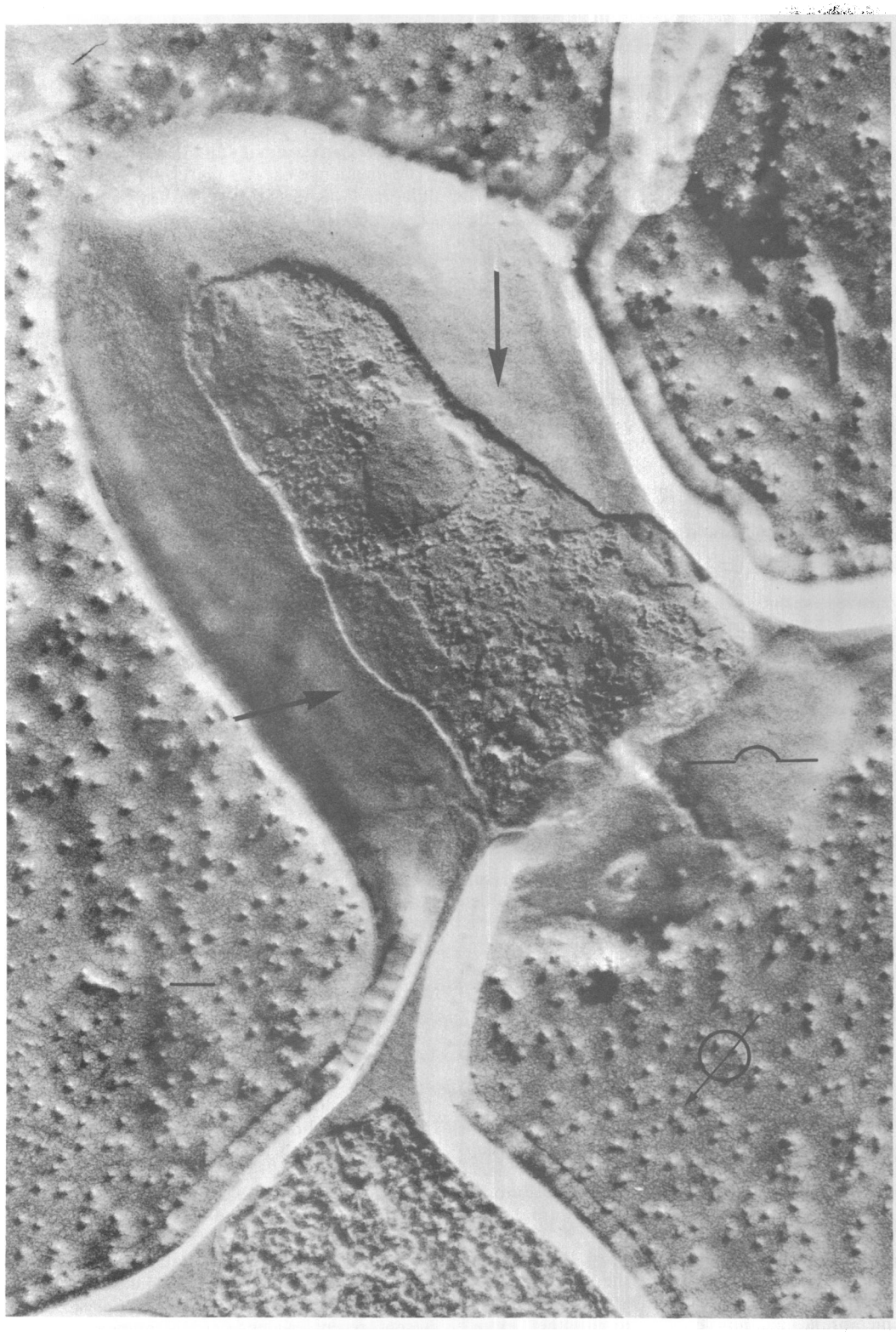

FIG. 5. *Replica of an untreated cell after freeze-etching. Note the presence of patches of a smooth surface outside the particle-studded surface and inside the collar (arrows) revealed by etching.*

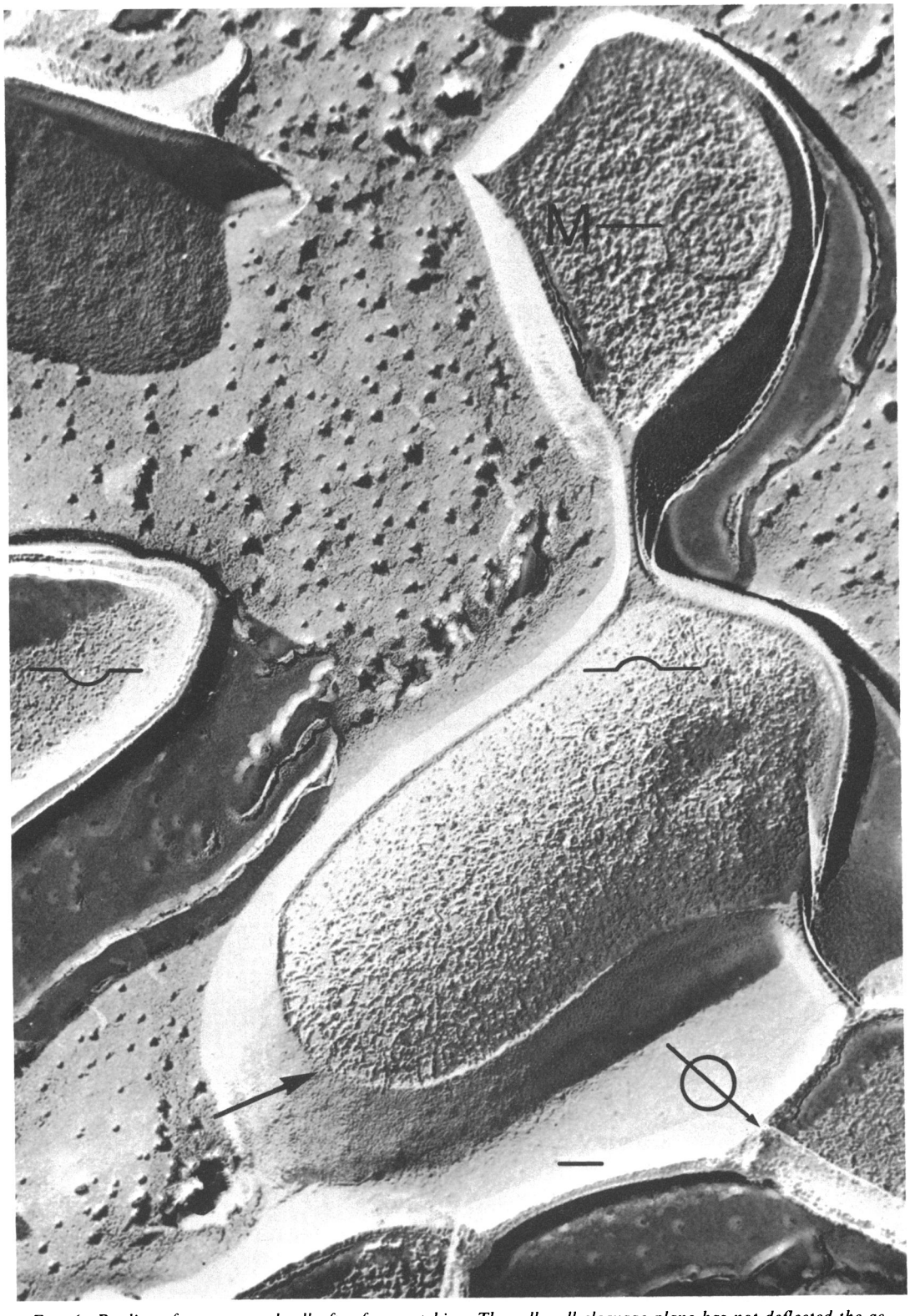

FIG. 6. *Replica of an untreated cell after freeze-etching. The cell wall cleavage plane has not deflected the actual cleavage in this instance, and only the particle-studded surface and the collar (arrow) are seen. Note the mesosome (M) in the cross-cleaved cell.*

FIG. 7. *Replica of untreated cells which were cleaved but not etched. The cells are surrounded and connected by a sleeve (arrow) similar to that seen in Fig. 3.*

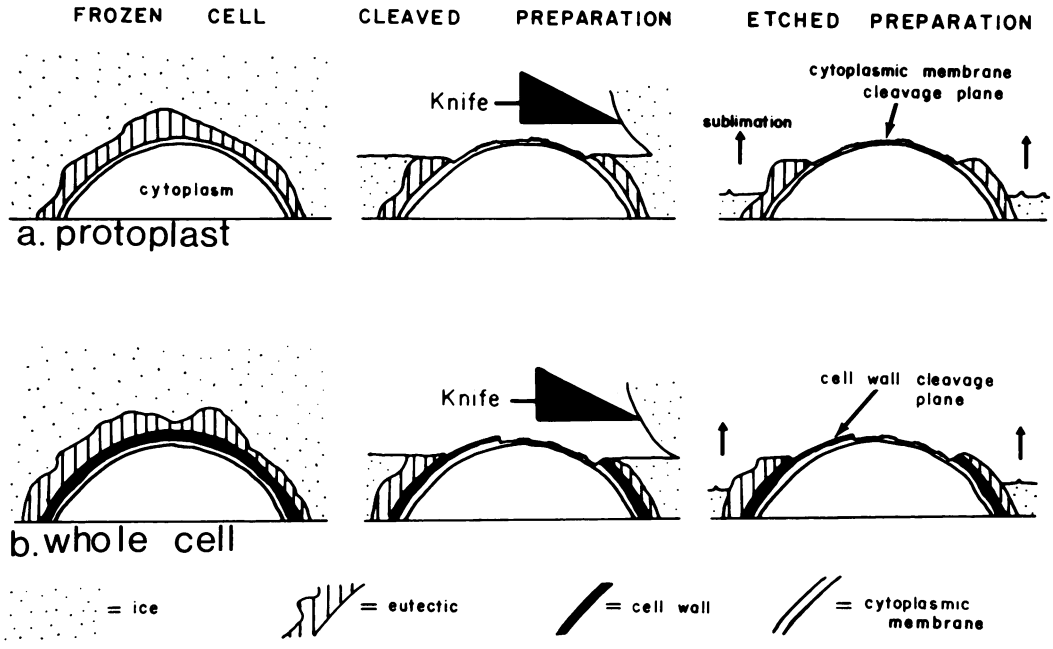

FIG. 8. *Diagrammatic representation of the stages in freeze-etching (a) protoplasts and (b) whole cells. The unetched preparations presented in Fig. 2 and 7 were replicated at the "cleaved preparation" stage, whereas the other preparations were replicated after etching.*

brane, but the "collar" seen in Fig. 1 is much thicker than the 3 to 4 nm expected, and we think it is unlikely that this layer could be comprised of the outer half of the membrane alone. On the other hand, Nečas et al. (19) showed that yeast cells are surrounded by a eutectic layer, when they are frozen in medium, and that this eutectic appears as an extra structure on the cell surface after etching. Because the smooth surface outside of the cytoplasmic membrane of the protoplasts studied herein does not form if the preparations are not etched, and because of its considerable thickness, we suggest that it is a eutectic layer surrounding the cell.

Unetched preparations (Fig. 2 and 7) show that the intercellular menstruum forms two phases on freezing so that one phase surrounds the cells in a sleeve of variable thickness and also forms bridges between cells, and the other fills the intervening spaces and is lowered by the etching process. Because of the presence of this eutectic sleeve, etching would not reveal the true surface of the cell; this would explain why we cannot see any trace of surface structures such as the peptidoglycan layer and the loosely bound outer layer at the cell surface. Etching revealed certain structures at the surface of bacterial cells (14, 22, 28), but these structures are highly ordered and so large (5 to 15 nm) that they are partially seen through the eutectic layer.

The preparations described here show evidence of only one cleavage plane in the cell wall, between the cytoplasmic membrane and the external eutectic shell. Other cell wall layers are irregularly seen in the "step" between the cleavage plane of the cytoplasmic membrane and that of the cell wall but they do not significantly deflect the actual cleavage in the cell envelope. Fiil and Branton (12) saw a similar pattern in *Escherichia coli*, and their Fig. 3 shows the relationship between the surface exposed by the cytoplasmic membrane and the cell wall cleavage planes, as well as the eutectic shell revealed by the etching procedure. Our observation that the cell wall cleavage plane in the marine pseudomonad is not especially strong in whole cells is supported by Fiil and Branton's (12) Fig. 9 and 11. They show that some cells of *E. coli* also cleave without following the cell wall cleavage plane to any significant extent. The cell wall cleavage plane exposes a very small surface in *Ectothiorhodospira mobilis* (22) and in the *E. coli* examined by Bayer and Remsen (2), but large surfaces are exposed by cleavage in this plane in cells of *Nitrocystis oceanus* (21), *Ferrobacillus ferrooxidans* (20), and *E. coli* studied by Nanninga (18). Bayer and Remsen (2) and van Gool et al. (27) state that pretreatment of gram-negative bacterial cells with sucrose and low concentrations of OsO_4, respectively, increases the

tendency of the actual cleavage to follow the cell wall cleavage plane; we noted an increased "strength" of this plane in the spheroplasts prepared and frozen in sucrose. This suggests that the efficacy of this layer in deflecting the actual cleavage is dependent on its physical and chemical state.

Surfaces other than that exposed by the main cell wall cleavage plane were seen in freeze-etched preparations of gram-negative cells (cf. the CWI layer of Nanninga, 18), and small areas of the surfaces are sometimes exposed (Fig. 7), but there appears to be only one cleavage plane in the cell wall which is "strong" enough to deflect the actual cleavage over a significant distance.

The identification of the cell wall layer which is responsible for the production of the cell wall cleavage plane is of interest to us. Fiil and Branton (12) suggested that the cell wall of *E. coli* cleaves in the "soft" or "L" layer (7), which is the double-track structure seen in electron micrographs of sectioned material. Forsberg et al. (10) identified four layers in the cell wall of the marine pseudomonad used in this study. We showed that the outermost of these layers can be removed without the loss of the cell wall cleavage plane, but that this plane is lost when the outer double-track layer and the underlying soluble layer are removed to produce mureinoplasts. Because these latter two layers cannot be removed separately, we do not know which one is responsible for the cell wall cleavage plane. The cleavage pattern of the mureinoplasts, which are surrounded by the complete peptidoglycan layer, is the same as that of protoplasts; thus, we may conclude that this cell wall layer is not involved in cleavage plane formation. Because the outer double-track layer contains a relatively high proportion of protein and phospholipid (11), we expect that this layer constitutes the cell wall cleavage plane, and freeze-etching studies of the isolated layer are in progress.

The "rodlets" seen among the particles on the surface exposed by the cytoplasmic membrane cleavage plane (20) are not seen in unetched preparations and may therefore be artifacts, as was suggested by Fiil and Branton (12). In unetched preparations, the particles on the surface exposed by the cytoplasmic membrane cleavage plane and those seen in the cytoplasm of cross-cleaved cells are larger than those seen in etched preparations, which suggests that their size is reduced by loss of water by sublimitation during etching.

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