

THE ANATOMY OF THE CELL ENVELOPE OF A MARINE VIBRIO  
EXAMINED BY FREEZE-ETCHING

By

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## KEY TO SYMBOLS

CM	cytoplasmic membrane
O	outer membrane
$\widehat{CM}$	cytoplasmic membrane fracture face, convex
$\smile CM$	cytoplasmic membrane fracture face, concave
$\widehat{R}$	rigid layer fracture face, convex
$\smile G$	globular layer fracture face, concave
$\widehat{O}$	outer membrane fracture face, convex
$\smile O$	outer membrane fracture face, concave
$O \widehat{sur}$	actual outer surface of the outer membrane
$O \smile sur$	actual inner surface of the outer membrane
cyto	cytoplasm
$\blacktriangle$	direction of shadow

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The structure of the cell envelope of the marine vibrio MW40 was examined by freeze-etching and other techniques. In thin section the organism was similar to other gram-negative bacteria. The cell envelope appeared as two double-track layers. No intermediate dense layer was observed.

Purified peptidoglycan was prepared from cells during the exponential phase of growth by treating cells with hot sodium dodecyl sulfate. The peptidoglycan had a typical amino acid and amino sugar composition and did not have a covalently linked lipoprotein. The material appeared fibrous when observed in the electron microscope.

Purified lipopolysaccharide (LPS) was extracted by the hot phenol-water procedure. Its composition and appearance was typical. The LPS was used to determine the specificity of anti-LPS antiserum.

For freeze-etching, cells were suspended in a salt solution containing 0.22M NaCl, 0.026M MgCl<sub>2</sub>, and 0.01M KCl. In some cases 20% glycerol was added as a cryoprotective agent. The cell envelope of this organism freeze-fractured in three planes. One fracture split the

cytoplasmic membrane along its hydrophobic center revealing particle-studded fracture faces similar to those seen in other bacteria. The convex face was more densely covered with particles than the concave face. Large particle-free areas were observed on both faces. The appearance of the faces was the same with and without glycerol.

A second fracture revealed smooth particle-free concave and convex faces. This fracture occurred primarily without glycerol, but was occasionally seen in glycerol-treated cells.

The third fracture produced a rough convex face and a concave face composed of subunits approximately 10 nm in diameter. In areas where the subunit layer was incomplete it was observed that the subunits were globular and were backed by a smooth surface. This fracture occurred in cells freeze-etched with glycerol, and occasionally the globular surface was seen without glycerol.

The outer surface of the cell was exposed by etching in preparations without glycerol. The surface appeared smooth or finely granular, but may have been obscured by a thin eutectic layer.

Complementary replicas were prepared and it was demonstrated that the three pairs of fracture faces were in fact apposed and were produced by three fractures.

Isolated cell envelopes were prepared by lysing cells in a French pressure cell. The appearance of these envelopes when freeze-etched was similar to whole cells. It was observed, however, that the globular surface could be exposed by etching alone indicating that it had separated from the rough surface before freezing.

Crude outer membrane material was prepared by washing the cells

with NaCl and sucrose solutions. This material freeze-fractured producing smooth concave and convex faces.

Specific rabbit anti-LPS antiserum was prepared and used to label the LPS on the cell surface. This antibody was then labelled with ferritin-conjugated anti-rabbit immunoglobulin antiserum. In thin sections these cells appeared coated with a band of ferritin along the outer double-track, but separated from it by an electron transparent space. For freeze-etching the cells were suspended in 0.05M  $MgCl_2$ . The preparations were deep etched and the presence of the ferritin on the etch face proved that the outer surface of the cell was revealed. The smooth convex fracture face was immediately adjacent to this ferritin coated surface.

It was concluded that the envelope fractured at three levels. One fracture split the cytoplasmic membrane. A second split the outer membrane along its hydrophobic center revealing smooth fracture faces. The third fracture exposed the rough convex surface of the rigid layer and a globular layer which separated the rigid layer from the outer membrane.

## INTRODUCTION

The cell envelopes of gram-negative bacteria are multilayered structures of complex composition. Numerous studies have been reported which dealt with the chemical composition, structure, and biosynthesis of the various components of the envelope. The organization of these components into the functional cell envelope has also been extensively studied. Related work has focused on the antigenic specificity of the envelope and its role in pathogenicity. The result has been the formulation of a generally accepted, though not particularly detailed, model of the gram-negative cell envelope (16).

In the study reported here the structure of the cell envelope of a marine vibrio was examined, primarily by freeze-etching. The purpose of this investigation was first to compare the structure of this organism with that of more commonly studied gram-negative bacteria such as Escherichia coli. The second and main objective was to determine if the technique of freeze-etching could be used to obtain additional information on the structure of the cell envelope of gram-negative bacteria in general.

The cell envelope of gram-negative bacteria consists of at least three layers, the cytoplasmic membrane, the rigid layer, and the outer layer or membrane. The latter two layers comprise the cell wall, although this term is often used to designate the rigid layer alone. The cytoplasmic membrane appears to be a typical membrane composed of

phospholipid and protein, and is comparable in structure and function to the more easily studied cytoplasmic membrane of gram-positive bacteria (54).

The rigid layer is composed of peptidoglycan and associated proteins. It is in the form of a "bagshaped macromolecule" which gives the cell shape and strength (74). The outer membrane contains phospholipid, lipopolysaccharide, and proteins or lipoproteins, and is unique to gram-negative bacteria. The term "membrane" is used to denote its appearance when thin-sectioned and viewed in the electron microscope, and does not imply that it has the functions of other membranes.

In addition to this basic structure, certain bacteria have layers external to the outer membrane. These extra layers are usually found in halophilic, photosynthetic, and other more unusual bacteria. There are a number of excellent reviews on the structure and composition of the gram-negative cell envelope (14, 25, 27, 30, 34, 35, 47, 53).

Chemical composition and structure of envelope components.--Peptidoglycan has been isolated from a variety of gram-negative bacteria by various methods, usually involving phenol, hot detergents, or other harsh treatments, combined with enzymatic digestions. Peptidoglycan consists of a glycan backbone of alternating N-acetyl glucosamine and N-acetyl muramic acid residues with a peptide moiety linked to the carboxyl group of muramic acid. In E. coli the peptide is composed of L-alanine, D-glutamic acid, meso-diaminopimelic acid, and D-alanine. Some of the peptide chains are cross-linked from the amino group of diaminopimelic acid to the carboxyl group of D-alanine.

This basic structure appears to be universal for gram-negative bacteria, although in many cases all that is known about the peptidoglycan is the amino acid content (56). The amount of peptidoglycan varies from about 10% of the cell wall to none in the case of certain halophiles.

Braun and coworkers (10, 12, 13) have purified a peptidoglycan-lipoprotein complex from E. coli and shown that the lipoprotein is covalently linked to the carboxyl group of diaminopimelic acid. This linkage is specifically split by trypsin allowing the lipoprotein to be solubilized with hot sodium dodecyl sulfate (SDS). The amino acid sequence of the protein has been determined, and it was found that the lipid is covalently bound (8). The molecular weight of the lipoprotein is about 10,000.

These workers have also reported that a similar lipoprotein is attached to the peptidoglycan of several strains of Salmonella and Serratia marcescens, but is not found in Pseudomonas fluorescens or Proteus mirabilis (11). They later found that if the peptidoglycan of P. mirabilis is isolated from stationary rather than exponential phase cultures, a lipoprotein is attached (see ref. 36). No lipoprotein was found in the marine pseudomonad studied by MacLeod's group (24).

Weidel et al. (73) studied metal-shadowed preparations of isolated rigid layers of E. coli and found cell-shaped granular structures with globular units on their surface. Digestion with proteolytic enzymes removed these globules. Recently, Martin et al. (36) examined negatively stained preparations of purified peptidoglycan. All of the preparations appeared as granular cell-shaped structures. Layers from E. coli and P. mirabilis were covered with globular particles which were about 9 to

10 nm in diameter and about 20 nm apart. Preparations from Pseudomonas aeruginosa showed considerably fewer particles, and layers from Spirillum serpens were free of particles. The particles on the Pseudomonas peptidoglycan were readily removed by proteolytic enzymes.

Although it would be convenient to associate these particles with the lipoprotein studied by Braun, they are simply too large for a molecule with a molecular weight of 10,000. It is possible, however, that the lipoprotein molecules occur in groups and aggregate to form larger units.

The composition and structure of the lipopolysaccharides (LPS) of the outer membrane have also been extensively studied (34). They are readily isolated by the hot phenol-water extraction procedure (76) and have been characterized in a wide variety of bacteria. The lipid portion, lipid A, is covalently linked to a carbohydrate core containing the unusual sugars keto-deoxyoctanoic acid and glycerol-D-mannoheptose. Attached to the core are the O-antigenic side chains, the composition of which varies according to the strain of bacteria. In rough strains the side chains are short or absent. When purified LPS is observed in the electron microscope it appears to have the structure of membranes. Shands et al. (62) observed LPS positively stained with uranyl acetate and found various forms, all of which had areas which appeared membrane-like, that is as a trilaminar or double-track appearance. They also found that the dimensions of the double-track were the same in LPS from smooth and rough strains, indicating that the polysaccharide side chains are not stained and are not seen in thin sections. dePetris (16) observed that thin sectioned LPS also has a double-track appearance.

The cell envelope also contains a considerable amount of lipid,



with the major portion being phospholipid. The predominant fatty acids are C<sub>16</sub> and C<sub>18</sub> straight chain acids, and  $\beta$ -hydroxymyristate is found in the LPS. Sterols are absent.

In most descriptions of the outer membrane lipoproteins are listed as a major component. The work on these molecules has been very vague and no one has clearly shown that there are any true lipoproteins, that is covalently linked lipid and protein, in the outer membrane.

Recently, the proteins of the cell envelope of E. coli have been studied using SDS-polyacrylamide gel electrophoresis (57, 58). Schnaitman found from 20 to 30 bands of protein in cell envelope extracts, and one major protein possessing an apparent molecular weight of 44,000. By using sucrose gradient centrifugation he was able to partially separate the cell wall and cytoplasmic membrane. The major protein band was found to be localized in the cell wall and accounted for 70% of the wall protein. Although further work as indicated that the initial results may be oversimplified, the basic conclusion, that there are major structural proteins, is still valid (Schnaitman, personal communication). Comparable results have been obtained in E. coli (31) and Salmonella typhimurium (48).

Studies of proteins released from P. aeruginosa by ethylenediamine-tetraacetic acid (EDTA) treatment indicate that structural proteins exist in the cell wall (68). The location of these proteins will be discussed later.

Fine structure of the cell envelope.--When thin sections of gram-negative bacteria are examined in the electron microscope, the cell envelope appears to consist of a smooth inner membrane and a wavy outer membrane. Both of these membranes have a double-track appearance and

measure about 7.5 nm in width. In early work these were the only structures seen, but improved techniques have allowed the visualization of the intermediate rigid layer (43). This general anatomy has been observed in many gram-negative bacteria and no attempt will be made to review this literature (27).

Aside from its double-track appearance, the cytoplasmic membrane does not have any fine structure detectable in thin sections. Because of the difficulty in separating it from the cell wall, the membrane has not been thoroughly studied by negative staining, but available results indicate that it does not have a subunit structure (55).

The appearance of the intermediate dense layer, or rigid layer, varies according to the organism studied, the method of fixation, and the method of staining (27). In some bacteria this layer is seldom or never seen. This may be due to a thinner layer of peptidoglycan (24) or possibly to the lack of a covalently bound lipoprotein. The rigid layer generally follows the contours of the cytoplasmic membrane. It has a thickness of 3 to 8 nm.

Evidence that the peptidoglycan is associated with the dense layer seen in thin sections is provided by lysozyme digestion. Cells which have been treated with lysozyme and EDTA lack the intermediate layer, and this treatment allows the outer layer to separate from the cytoplasmic membrane (16, 43). It is not known whether the metal stain is localized in the peptidoglycan alone, or the dense layer represents another layer which is solubilized when the peptidoglycan is digested. Purified peptidoglycan "sacculi" appear as a dense layer in thin section, although they are thinner than the layer seen in whole cells (16, 29).

The space between the rigid layer and the cytoplasmic membrane is generally not stained, but is apparently not "empty" since the two layers are never in contact. In Nitrosocystis oceanus globular material was observed in this area (72).

There is also a space between the rigid layer and the outer membrane. In some organisms the material in this space is stained and the rigid layer seems to be associated with the outer layer (27). dePetris (15, 16) showed that this material sometimes appears globular. Digesting the cells with proteolytic enzymes eliminates this material and allows the outer membrane to separate from the rigid layer. Digestion of isolated E. coli cell envelopes with trypsin, a procedure which cleaves the lipoprotein from the peptidoglycan, also caused the envelope to separate, but in this case the rigid layer was not visible in the thin sections (10).

The outer double-track layer is identical in appearance to typical membranes and is believed to be a lipid-protein bilayer. The LPS and protein are probably arranged in a mosaic fashion, with the lipid portion of the LPS extending into a phospholipid bilayer. Extraction of cells with hot phenol-water removes the LPS and such cells are devoid of the outer membrane (5, 16). With a marine pseudomonad it is possible to remove the outer layer by washing the cells (22, 23). This procedure yields isolated membrane material which contains most of the LPS of the cells.

The O-antigenic side chains of the LPS extend out from the outer layer and are not stained in routine procedures. In thin sections of packed cells there is generally a clear space between the outer layers of adjacent cells, but with rough strains the outer membranes appear to fuse.

Shands (61) used ferritin-labelled antibody to directly demonstrate the presence of the LPS in the outer membrane of E. coli and Salmonella. He observed that the ferritin molecules extended for considerable distances from the outer double-track and concluded that there was a large amount of unstained polysaccharide on the cell surface. Similar results were obtained with Veillonella (37). It is not known if the LPS is also located on the inner surface of the outer membrane, but the apparent area required for the side chains would make such an arrangement unlikely.

Freeze-etching studies of gram-negative bacteria.--In recent years the new technique of freeze-etching has been used to study the structure of cells (39, 40). In this process a sample is flash frozen, placed in a vacuum chamber, and fractured. Ice is sublimed (etched) from the fracture surface, and the surface is shadowed with evaporated metal and replicated with evaporated carbon. The replica is cleaned of adhering cellular material and is ready for examination in the electron microscope. This procedure reveals the surface topography of the fracture faces and the faces exposed by etching. A modification of this method, double-recovery or complementary replica technique, allows the replication of both surfaces produced when the specimen is fractured (42, 67). By observing the same cell in both replicas, it is possible to directly demonstrate the association of the various fracture faces.

One aspect of freeze-etching that caused considerable confusion and controversy for a period of years is the concept of membrane splitting. When membranes are freeze-etched, two surfaces, one concave and one convex, are produced. These surfaces were originally thought

to be the inner and outer surfaces of the membrane. Branton (6) proposed an alternate interpretation, suggesting that membranes split along an internal hydrophobic plane revealing two complementary surfaces which do not represent the true surfaces of the membrane. Pinto da Silva and Branton (49) proved this by labelling the outer surface of red blood cells with ferritin and showing that the fracture face was unlabelled and the labelled surface was only exposed by etching. These results were verified by double-replica technique, by which it was demonstrated that the convex and concave faces are produced by the fracture of a single membrane in a particular cell (41).

The structure of the cell envelope of gram-negative bacteria has been examined by freeze-etching (50). In both gram-positive and gram-negative bacteria the cytoplasmic membrane fractures, producing particle covered convex and concave faces (45, 65, 71). The convex face is studded with a large number of particles, often arranged in a netlike array. The concave face is sparsely covered with particles and sometimes appears pitted. Between the particles the surface is smooth, and occasionally particle-free areas are present (44, 65). Fiil and Branton (19) observed that in magnesium starved E. coli the particles are arranged in regular arrays and there are large particle-free areas.

The nature of these particles, which are also seen in a variety of other freeze-etched membranes, has not been clearly established, but they probably represent proteins which are intercalated into the lipid bilayer (7, 64). Membranes which have little or no protein, such as myelin sheath or artificial lipid bilayers, do not have particles on their fracture faces (7).

The cell envelope of gram-negative bacteria fractures in at least

one other plane. Nanninga (44) observed that the cell wall of glycerol-treated E. coli fractures revealing a rough irregular convex face and a concave face which is composed of tightly packed flattened subunits approximately 10 nm in diameter. He suggested that the subunits represented a protein layer superimposed on the peptidoglycan. In a later study Van Gool and Nanninga (71) prepared complementary replicas and proved that the two faces are apposed. By this time the idea of membrane splitting had been established, and they concluded that this fracture split the outer membrane in a manner analogous to the splitting of the cytoplasmic membrane. This interpretation requires that the subunit layer be superimposed on the inner surface of the outer half of the outer membrane.

Gilleland et al. (26) demonstrated a similar fracture plane in glycerol-treated P. aeruginosa, and they also concluded that it was located in the outer membrane. The concave face in P. aeruginosa appears as a smooth surface partially covered with spherical units which are somewhat smaller than the subunits seen in E. coli. Extraction of the cells with EDTA solubilizes proteins and yields osmotically fragile cells. This treatment eliminates the spheres on the concave fracture face. The protein can be restored to the cell wall reversing the effect. P. aeruginosa was also studied by Lickfeld et al. (32). A similar fracture was observed and was again postulated to be a fracture of the outer membrane.

In the E. coli (71) and Pseudomonas (26) studies the cells were suspended in glycerol as a cryoprotective agent. When these cells were freeze-etched without glycerol the convex fracture face was smooth. Both groups suggested that the smooth surface was the same

as the rough convex face and its appearance was altered by glycerol. Neither study mentioned a concave fracture face in cells without glycerol. Similar results were observed in Nitrosomonas europaea (70).

DeVoe et al. (17) observed a smooth convex fracture face in a marine pseudomonad freeze-etched without glycerol. This surface was also seen in spheroplasts produced by lysozyme digestion. No cell wall fracture plane was present in cells which had been treated to remove the outer membrane.

Nitrosomonas oceanus, an organism with a complex cell wall, was examined by Watson and Remsen (72). They freeze-etched the bacteria in a salt solution without glycerol and observed a concave globular layer similar to the one seen in E. coli. According to their interpretation, the corresponding convex layer is another globular layer obscured by a thin rough layer. They concluded that the cell wall split at the level of the peptidoglycan and the globular concave layer separated the peptidoglycan from the outer membrane.

The outer surface of the cell envelope has been demonstrated by etching of preparations without glycerol. The surface is often smooth, but a fine subunit structure has been reported in E. coli and P. aeruginosa (4, 19, 26). Large subunit structures have been observed on the surfaces of a few bacteria, but these are clearly extra layers external to the outer membrane (50).

In this study it will be shown that the cell envelope of the marine vibrio MW40 is typical of gram-negative bacteria. When freeze-etched under a variety of conditions the cell envelope fractures in three planes. The location of the fracture planes and the nature of the fracture faces are discussed and a model is presented which is consistent

with the current understanding of the freeze-etch process and the structure of the cell envelope as determined by other techniques.



## MATERIALS AND METHODS

Organism and maintenance of cultures.--The organism used in this study was isolated in this laboratory from a sample of water from the Waccasassa River estuary on the west coast of Florida and was designated marine vibrio MW40. It is a marine organism requiring added salts for growth in ordinary culture media and lysing in distilled water. It is one of nine strains studied phenotypically in a preliminary study (unpublished results). It was found that the organism is a gram-negative polarly flagellated slightly curved rod, which is oxidase positive, fermentative on glucose, sensitive to the pteridine O/129, and has a GC ratio of approximately 43%. It produces an insoluble blue-black extra-cellular pigment which is probably indigoidine. Using the scheme of Shewan (63) the organism would be classified as a Vibrio. The organism has sheathed flagella and would be classified by Baumann as a member of the genus Beneckea (1). It appears to be similar to the organism he named Beneckea nigrapulchrituda (2).

Stock cultures were maintained on plates of a medium containing 0.5% gelatin (Difco Laboratories, Detroit, Mich.) and 2% agar, in a salt solution, hereafter designated "complete salts," consisting of 0.22M NaCl, 0.026M MgCl<sub>2</sub>, and 0.01M KCl.

Culture and harvest of cells.--Cells were cultured in a medium containing 0.5% trypticase (Baltimore Biological Laboratories, Baltimore, Md.) and 0.1% yeast extract (Difco) in complete salts. The trypticase

and yeast extract medium was prepared at twice the final concentration and adjusted to pH 7.6 with KOH before mixing with an equal volume of double strength complete salts.

For routine work cells were cultured in 50 ml of medium in a 250 ml flask, or in 250 ml of medium in a 1000 ml flask, incubated on a rotary shaker overnight at 25 C. Cells were harvested by centrifugation at 3000 x g for 10 min. Except where otherwise noted, all centrifugation was performed at 1-4 C in a Sorvall RC-28 refrigerated centrifuge using an SS-34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.).

Large quantities of cells were cultured in 11 l of medium in a 20 l carboy incubated at 25 C with aeration until late exponential phase of growth, giving an approximate cell concentration of  $1.5 \times 10^9$  cells/ml. The culture was cooled to 0 C by addition of 4 l of ice and sufficient NaCl (50 g) to maintain the salt concentration. Cells were harvested in a precooled Delaval cream separator (Delaval Separator Co., Poughkeepsie, N. Y.).

Preparation of peptidoglycan.--Cells from an 11 l batch culture were suspended to 200 ml in complete salts at 0 C. The suspension was rapidly heated to 60 C by adding 1 l of complete salts preheated to 70 C, and placing in a water bath at 60 C for 10 min. The cells were lysed by addition of 200 ml of a 28% (w/v) solution of Triton X-100. The mixture was stirred for 10 min and then transferred to an ice-water bath and cooled to about 4 C. When the temperature had fallen to 37 C, 500 ug of deoxyribonuclease (DNase) were added to reduce the viscosity. The suspension was centrifuged at 10,000 x g for 90 min in a GSA rotor.

The pellets were washed once in 1200 ml of 0.1M NaCl and divided into two equal portions. One half of the material was resuspended to

200 ml in 0.01M tris (hydroxymethyl)-amino methane (Tris) buffer, pH 8.2, containing 40 mg of trypsin, and shaken for 30 min at 25 C. A 120 ml volume of a solution of sodium dodecyl sulfate (SDS) was added to give a final concentration of 4%. The mixture was shaken for 30 min at 25 C, and then centrifuged at 27,000 x g for 1 hr at 20 C. The other half of the material was suspended to 200 ml in 0.01M Tris buffer, pH 7.2, without trypsin, mixed with SDS, and centrifuged as described. The subsequent treatment was the same for both pellets.

The pellet was suspended in 50 ml of 4% SDS and added slowly with stirring to 250 ml of boiling 4% SDS. After boiling for 5 min the suspension was cooled slightly, shaken for 2 hr, and left overnight at room temperature. The peptidoglycan was pelleted by centrifugation at 27,000 x g for 1 hr at 20 C, and washed once in 0.02M NaHCO<sub>3</sub>. It was dialyzed for 48 hr against three changes of 0.02M NaHCO<sub>3</sub> and two changes of distilled water, and then lyophilized.

Peptidoglycan was also prepared by lysing cells in hot SDS. A 600 ml volume of culture was removed from the shaker, poured into 600 ml of boiling 4% SDS, and placed in a water bath at 60 C for 30 min. The suspension was cooled to 20 C and centrifuged at 16,000 x g for 1 hr in a GSA rotor. The pellet was treated with boiling SDS, dialyzed, and lyophilized as previously described. This procedure produced only a small quantity of material, but it appeared to be whiter and purer than the peptidoglycan prepared from the batch cultures.

Preparation of LPS.--LPS was extracted by a modification of the hot phenol-water procedure (76). As suggested by O'Leary et al. (46) a solution of MgCl<sub>2</sub> was substituted for distilled water in this procedure. Cells from an 11 l batch culture were washed once in complete salts and

resuspended in 200 ml of 0.05M  $MgCl_2$ . The suspension was mixed with an equal volume of 90% phenol held in a water bath at 60 C, and the mixture was shaken vigorously for 30 min. The mixture was cooled to 20 C and centrifuged at 1500 x g for 30 min. The top aqueous phase containing the LPS was removed and saved, and the phenol phase and the interface were reextracted with an equal volume of fresh 0.05M  $MgCl_2$ .

The aqueous phases were combined and centrifuged at 27,000 x g to remove debris. The supernatant was dialyzed for 36 hr against several changes of 0.01M  $MgCl_2$  and then lyophilized.

The crude LPS was purified by a modification of the method of Romeo et al. (52). The LPS, 2 mg of ribonuclease (RNase), and 50 ug of DNase were suspended in 60 ml of a solution consisting of 0.01M Trisacetate buffer, pH 7.5, 0.1M NaCl, 0.01M  $MgCl_2$ , and 0.01M sodium azide. The suspension was incubated with stirring for 24 hr at 37 C, and then dialyzed for 4 hr against 3 l of the same salt solution minus the NaCl. A 1 mg quantity of protease was added to the suspension in the dialysis bag, and it was incubated for 24 hr at 37 C. The solution was diluted to 100 ml with 0.01M  $MgCl_2$  and centrifuged at 96,000 x g for 8 hr in a Beckman Model L-2 ultracentrifuge using a Ti50 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The pellets were resuspended to 40 ml in 0.01M  $MgCl_2$ , 160 ml of 95% ethanol were added, and the mixture was stored overnight at 4 C to allow complete precipitation of the LPS. The LPS was collected by centrifugation at 10,000 x g for 30 min in a GSA rotor, resuspended in double-distilled water, and lyophilized.

#### Preparation of cell envelopes using the French pressure cells.--

Except where otherwise noted, all of the manipulations involved in

preparing cell envelopes, cell walls, and isolated outer membranes were performed at 0-4 C, and the final products were stored at 0 C until processed for electron microscopy.

Cells from a 250 ml culture were suspended in 35 ml of 0.05M  $MgCl_2$  containing 50 ug of RNase and 50 ug of DNase. The cells were lysed by passing the suspension thru a French pressure cell (American Instrument Co., Silver Springs, Md.) operated at a pressure of approximately 16,000 psi. The lysate was diluted with an equal volume of distilled water and centrifuged at 17,000 x g for 1 hr. The pellet was re-suspended in 0.01M  $MgCl_2$  and centrifuged at 3,000 x g for 15 min to remove whole cells. The supernatant was decanted and centrifuged at 17,000 x g for 1 hr to pellet the cell envelopes.

Preparation of cell walls using Triton X-100.--Cells from a 250 ml culture were resuspended in 250 ml of complete salts containing 50 ug of RNase and 50 ug of DNase. The cells were lysed by the addition of 5 ml of a 50% (w/v) solution of Triton X-100 and the mixture was shaken for 10 min. The cell walls were pelleted by centrifuging at 17,000 x g for 1 hr, and washed once in a solution containing 0.05M  $MgCl_2$  and 0.01M Tris buffer, pH 7.5. The pellet was resuspended in 40 ml of the Tris-Mg solution and divided into two portions. A 0.5 mg quantity of lysozyme was added to one of the suspensions and both were incubated for 5 min at 37 C in a water bath. The suspensions were cooled and centrifuged at 17,000 x g for 1 hr.

Preparation of crude outer membranes.--Crude outer membranes were released from the cells by a modification of the procedures of Forsberg et al. (22). Cells from a 250 ml culture were washed once with complete salts and three times with 0.5M NaCl by resuspending them in 200 ml

volumes of the wash solution and centrifuging at  $3000 \times g$  for 10 min. The final pellet was resuspended in 100 ml of 0.5M sucrose and placed in a rotary shaker for 30 min at 20 C. The cells were pelleted by centrifuging at  $7500 \times g$  for 15 min. The supernatant was carefully removed and recentrifuged to remove any remaining whole cells.  $MgCl_2$  was added to the suspension to give a final concentration of 0.01M, and the cell wall material was pelleted by centrifuging at  $96,000 \times g$  for 2 hr. The pellets were resuspended, pooled in one tube, and centrifuged as before.

Preparation of anti-LPS antiserum.--Three antigen preparations were used: (i) whole cells treated briefly in a blender to remove flagella and suspended in complete salts; (ii) cell walls prepared in Triton X-100 and suspended at a concentration of 1 mg/ml in 0.9% NaCl; (iii) partially purified peptidoglycan prepared by treating cell walls with warm SDS and suspended at a concentration of 1 mg/ml in 0.9% NaCl.

New Zealand white rabbits were injected subcutaneously with 1 ml of antigen. Four injections were given at two-week intervals, and the animals were bled two weeks after the final injection. Serum was heated at 56 C for 30 min to inactivate complement, sterilized by Millipore filtration, and frozen.

The activity of the antisera was determined by the agglutination of bacterial cells. Cells were washed and resuspended in complete salts solution to a concentration of approximately  $5 \times 10^8$  cells/ml. 0.2 ml of this suspension was mixed with 0.2 ml of serial dilutions of serum and incubated at 30 C for 30 min. The degree of agglutination was determined by microscopic examination.

Antiserum was adsorbed with LPS by mixing the serum with an equal volume of complete salts containing purified LPS at a concentration of 100 ug/ml. The mixture was incubated for 2 hr at 30 C and then centrifuged at 27,000 x g for 15 min. The supernatant was carefully removed and centrifuged again.

Labelling of cells with ferritin-conjugated antibody.--The indirect method was used to label the LPS on the cell surface with ferritin-conjugated antibody. Cells were suspended to a concentration of approximately  $9 \times 10^7$  cells/ml in a dilute medium containing 0.1% trypticase in complete salts. A 30 ml volume of this suspension was mixed with 5 ml of rabbit anti-LPS antiserum and incubated for 30 min at 30 C. The cells were pelleted by centrifugation at 12,000 x g for 10 min, washed three times with 40 ml volumes of trypticase medium, and resuspended in 10 ml of the same medium. To this was added 0.5 ml of commercially prepared ferritin-conjugated IgG fraction of goat anti-rabbit immunoglobulin (IgA + IgG + IgM) (Cappel Laboratories, Inc., Downingtown, Pa.). The suspension was incubated for 30 min at 30 C, diluted to 40 ml with trypticase medium, and centrifuged at 3000 x g for 10 min. The pellet was washed once in 40 ml of complete salts, and resuspended in 20 ml of complete salts. The suspension was divided between two 15 ml glass centrifuge tubes and centrifuged at 3000 x g for 10 min. One pellet was fixed for thin sectioning and the other was resuspended in 10 ml of 0.05M  $MgCl_2$  and repelleted. It was then resuspended in several drops of the supernatant and transferred to a plastic microtube. This tube was placed in a large tube and centrifuged at low speed for several minutes. The microtube was cut off just above the pellet and the cells were transferred to specimen holders for freeze-

etching. This procedure was necessary because of the very small size of the pellet.

In some experiments, cells were fixed in glutaraldehyde before antibody labelling. Cells from 40 ml of culture were fixed for 1 hr in 20 ml of 4% glutaraldehyde in buffered salts (see section on thin sectioning). A 20 ml volume of 0.5% trypticase medium was added and the suspension was centrifuged at  $3000 \times g$  for 10 min. The cells were resuspended in 40 ml of dilute trypticase medium and incubated for 1 hr. This treatment was designed to eliminate any unreacted glutaraldehyde and prevent non-specific uptake of antibody. The cells were pelleted, resuspended in dilute trypticase medium, and labelled with antibody as previously described.

Except for the incubations with antisera, all manipulations were carried out at 0-4 C.

Thin sectioning.--Cells were fixed for thin sectioning in 4% glutaraldehyde in a buffered salts solution consisting of 0.22 M NaCl, 0.01M  $MgCl_2$ , 0.01M KCL, and 0.02M potassium phosphate buffer, pH 7.4. The concentration of Mg ion was reduced from the concentration in complete salts to prevent the formation of a precipitate with the phosphate buffer.

A 4 ml volume of the 4% glutaraldehyde fixative was added to 40 ml of an actively growing culture and the suspension was set at 4 C for 30 min. Cells were harvested by centrifugation resuspended in 10 ml of fresh fixative, and set for 2 hr in the cold. Cells were pelleted, washed three times in buffered salts, and pelleted again. The supernatant was carefully removed and 2 ml of 2%  $OsO_4$  in buffered salts was added without resuspending the pellet. The cells were fixed in the



cold for 2 hr, and then washed with repeated changes of buffered salts and finally with distilled water. The cells were repelleted as necessary.

Cells were dehydrated thru a series of 25, 50, 75, 95, and 100% ethanol and placed in acetone for embedding in plastic. The pellet was broken into small pieces and embedded in an Epon-Araldite mixture by the method of Mollenhauer (38).

Antibody labelled cells were fixed and embedded as described, omitting the initial glutaraldehyde fixation in the medium. Cells which were fixed with glutaraldehyde prior to antibody labelling were postfixed with  $\text{OsO}_4$  and embedded as described.

Isolated cell envelopes were fixed by resuspending in 4% glutaraldehyde in phosphate buffered 0.01M  $\text{MgCl}_2$ , postfixed in  $\text{OsO}_4$ , and embedded as described for whole cells.

Embedded material was sectioned with a diamond knife (DuPont de Nemours and Co.) in a Porter-Blum MT-2 ultramicrotome (Sorvall, Inc., Norwalk, Conn.). Sections were poststained with lead citrate (51) and uranyl acetate.

Positive and negative stains.--Isolated cell envelopes were positively stained by mixing a suspension with 0.5% aqueous uranyl acetate. A small drop of the mixture was applied to a formvar coated grid and immediately drawn off with filter paper. Purified LPS was suspended in 0.01M  $\text{MgCl}_2$  and stained with uranyl acetate as described. Purified peptidoglycan was suspended in distilled water and negatively stained with potassium phosphotungstate. Grids were examined in the electron microscope immediately.

Freeze-etching.--Glycerol-treated cells were prepared by mixing a

culture with an equal volume of 40% (v/v) glycerol in complete salts and incubating for 15 min at 4 C. Other specimens were frozen in the appropriate suspending medium without cryoprotective agents. Pellets of the various materials were resuspended in very small quantities of the supernatant and transferred to specimen discs with a drawnout Pasteur pipette. Gold specimen discs with a central hole were routinely used. For very small specimens, such as antibody labelled cells, gold discs without a well were used. Specimens were frozen by plunging them into liquid Freon 22 held at -150 C or a mixture of liquid and solid N<sub>2</sub> at a temperature of -209 C.

Specimens were freeze-etched according to the methods of Moor and Mühlethaler (40) using a Balzers BA 360M freeze-etch apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.). The specimen was transferred to the precooled (-150 C) stage and placed under a high vacuum. The specimen temperature was raised to -100 C and it was fractured with a cold microtome until a smooth surface was obtained. The surface was etched for 0 to 2 min and then replicated.

The replicas were cleaned by floating them on 50% (v/v) Chlorox overnight and rinsed on several changes of distilled water. Replicas of ferritin labelled cells required an additional cleaning on 40% (w/v) chromic acid. Replicas were picked up on uncoated 300 or 400 mesh grids.

Double-replica freeze-etching.--Complementary replicas were prepared by the methods of Mühlethaler et al. (42). A suspension of glycerol-treated cells was frozen in a sandwich of grids (28). Two 3 mm copper discs were flared using a special press. Two nickel London finder grids were similarly bent. One of the finder grids was placed on a copper disc and a small quantity of vaseline was applied to the edges. The

second finder grid was placed on top of this and carefully adjusted so that the corresponding grid squares were superimposed. The cell suspension was applied to the grids, being sure that it penetrated through the grid openings of both grids. The second copper disc was set on top and the sandwich was clamped with a pair of forceps. The excess liquid was removed with filter paper and the sandwich was plunged into liquid-solid  $N_2$ .

The sample was fractured in the Balzers apparatus using a special hinged holder (custom designed and manufactured in this laboratory according to the design of Mühlethaler et al. (42)). The holder replaces the usual specimen stage. After fracturing, the specimens were replicated immediately without etching. The finder grids with the attached replicas were carefully separated from the copper discs and allowed to dry. The grids were placed in chloroform to remove the vaseline and then removed and dried. Replicas were cleaned overnight in 40% chromic acid. Distilled water was added to dilute the acid and the grids were transferred to fresh water. They were then removed and dried. If the manipulations were done carefully the replicas remained attached to the finder grids. This facilitated finding the corresponding halves of cells in the two replicas.

Electron microscopy.--All materials were examined with a Hitachi HU11-C or HU11-E electron microscope (Hitachi, Ltd., Tokyo, Japan) operated at an accelerating voltage of 75 Kv. Photographs were taken on DuPont Cronar film.

Amino acid analysis.--Amino acids and amino sugars were determined on a JEOL model JLC-5AH automated amino acid analyzer (JEOL U.S.A., Inc., Cranford, N. J.) according to Spackman et al. (66). Samples were hydrolyzed in 4N HCl for 11 hr at 105 C in sealed ampules.

Reagents.--The following chemicals were used: glutaraldehyde, 8% under  $N_2$  gas (Polysciences, Warrington, Penn.);  $OsO_4$  (Engelhard Industries, Newark, N. J.); and Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). The following enzymes were obtained from Sigma: ribonuclease, pancreatic, Type I-A; deoxyribonuclease, pancreatic; lysozyme, eggwhite, Grade I; protease, fungal, Type V; trypsin, pancreatic, Type III. All other reagents were reagent grade.

## RESULTS

Thin sections of whole cells.--In longitudinal sections the vibrios appeared as slightly curved rods with a fine structure typical of gram-negative bacteria (Fig. 1). Two double-track layers, the cytoplasmic membrane and the outer membrane, were visible in the cell envelope (Figs. 1, 2, and 3). The outer membrane was wavy, but in places appeared to be closely associated with the cytoplasmic membrane (Fig. 2). The fragmented appearance of the outer membrane was probably due to its undulating contour, since it appeared as a double-track only when it was perpendicular to the plane of the section.

In some cells there was a dense layer inside the cytoplasmic membrane (Figs. 1 and 3). The nature of this layer is unknown. The area between the inner and outer membranes was often fuzzy, but no organized intermediate layer was visible. The dense layer seen in E. coli (16) is seldom seen in marine bacteria, possibly because the peptidoglycan is very thin (24).

Purified peptidoglycan.--The method used to prepare peptidoglycan was similar to that of Braun and Rehn (10) and involved the treatment of crude cell walls with hot SDS. Cells were heated to inactivate autolytic enzymes and were lysed with Triton X-100 to minimize the fragmentation of the peptidoglycan which occurs during lysis of cells by mechanical means.

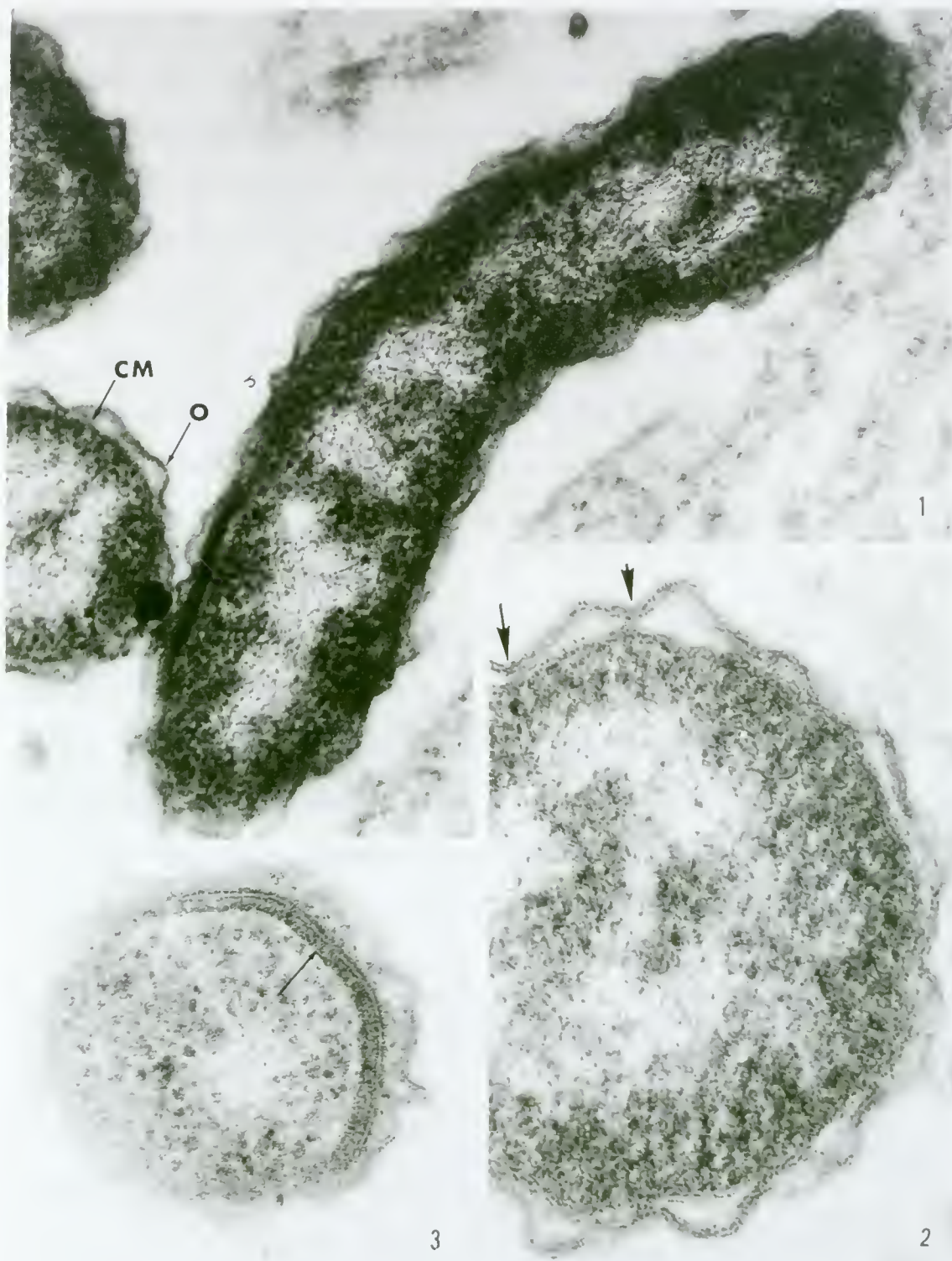
In order to determine if there was a lipoprotein covalently attached to the peptidoglycan, two samples were prepared. One sample was digested

Figs. 1, 2, and 3. Thin sections of cells fixed in glutaraldehyde and  $\text{OsO}_4$ . Note the double layered structure of the cell envelope.

Fig. 1. Longitudinal and cross sections of cells.  
(x 80,000).

Fig. 2. Cross section of a cell showing the double-track appearance of the cytoplasmic and outer membranes and close association of the two (arrows).  
(x 212,000).

Fig. 3. Cross section showing an extra layer (arrow) inside the cytoplasmic membrane. (x 167,000).



with trypsin, the other was not. The results of the amino acid analysis of these two samples were the same (Table 1). Both contained the usual amino acids and amino sugars found in peptidoglycan from gram-negative bacteria, that is, glucosamine, muramic acid, alanine, glutamic acid, and diaminopimelic acid, in a ratio of approximately 1:1:2:1:1. Other amino acids were present in very small amounts. Although the trypsin-treated sample was slightly cleaner, the results indicated that there was no lipoprotein attached to the peptidoglycan.

With the conditions used in the amino acid analysis neither muramic acid and serine nor diaminopimelic acid and methionine could be separated. Since the amounts of the other amino acids not normally found in peptidoglycan were low, it was unlikely that this affected the results significantly.

It was possible that an autolytic enzyme was cleaving the lipoprotein, especially during the 30 to 45 minutes required to harvest the cells. To minimize this possibility a small sample of peptidoglycan was prepared by pouring an actively growing culture directly into hot SDS. The amino acid content of this sample was the same as before, and there was no evidence of a covalently linked lipoprotein (Table 1).

When negatively stained, the peptidoglycan appeared as cell shaped fragments which were distinctly fibrous (Fig. 4). The peptidoglycan was quite fragmented and did not resemble the finely granular "sacculi" obtained from other bacteria. The preparation shown was treated with trypsin, but the untreated material had the same appearance. No particles or granules were seen in either preparation.

Purified lipopolysaccharide.--The primary purpose of preparing LPS was to obtain a purified antigen to test the specificity of antisera,



Table 1. Amino acid analysis of purified peptidoglycan,<sup>a</sup>

Amino acids and amino sugars	Isolated from batch culture		Isolated by direct lysis in hot SDS	
	No trypsin treatment μmoles/ml	Trypsin treatment μmoles/ml	mole ratio <sup>e</sup>	mole ratio
Glucosamine <sup>b</sup>	0.402	0.474	0.96	0.440
Muramic acid <sup>c</sup>	0.457	0.477	1.08	0.452
Glutamic acid	0.467	0.517	1.11	0.487
Alanine	0.939	0.972	2.23	0.914
Diaminopimelic acid <sup>d</sup>	0.420	0.478	1.00	0.431
Glucosamine	0.399	0.480	0.95	0.422

<sup>a</sup> All other amino acids were present in trace amounts or absent.

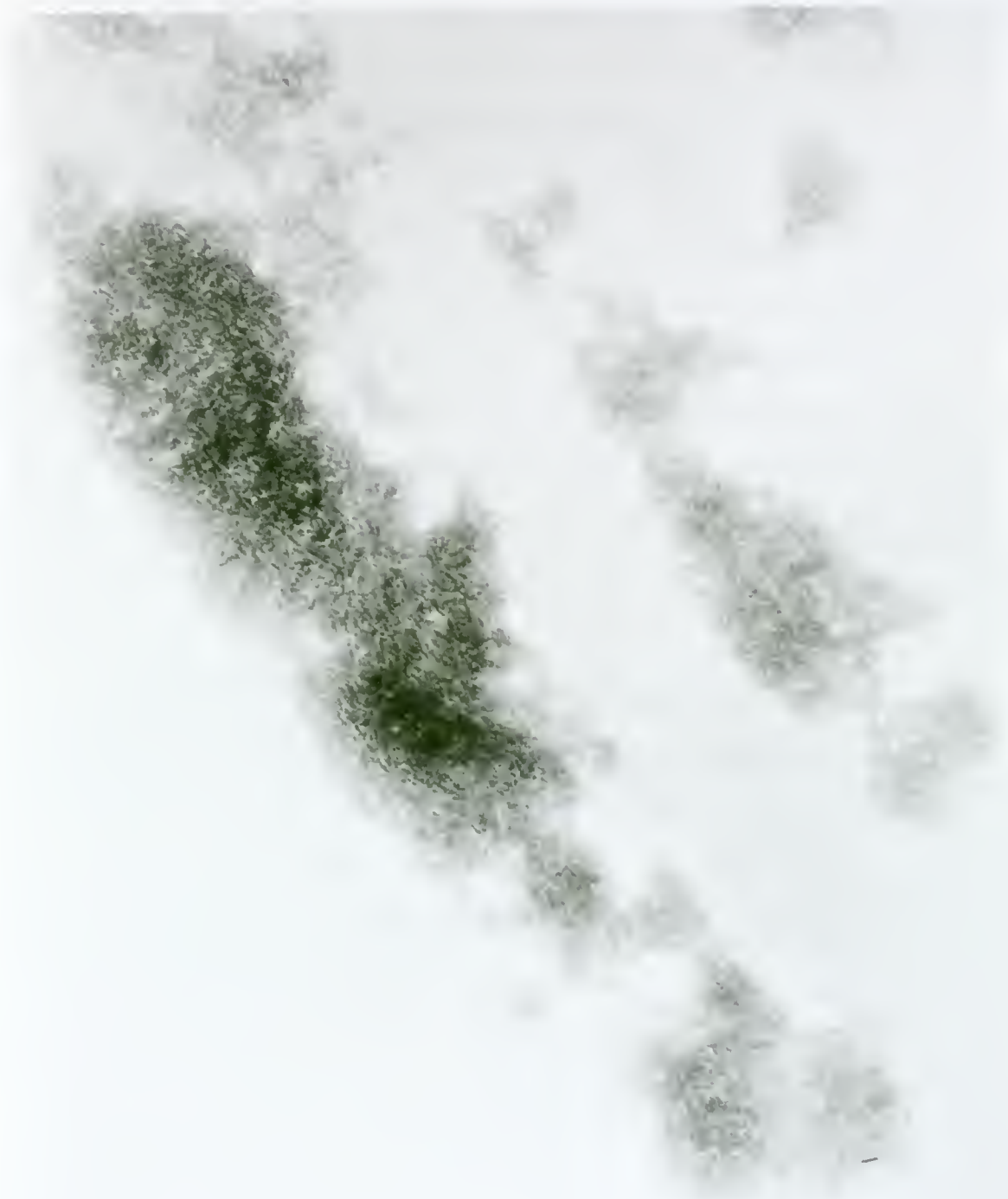
<sup>b</sup> Glucosamine elutes on both the long and short columns.

<sup>c</sup> Could not be separated from serine.

<sup>d</sup> Could not be separated from methionine.

<sup>e</sup> Diaminopimelic acid taken as 1.00.

Fig. 4. Purified trypsin-treated peptidoglycan negatively stained with potassium phosphotungstate. Note the fibrous nature of the layer. (x 224,000).



but the general nature of the LPS was also of interest. The standard method of preparing LPS from smooth strains of bacteria, phenol-water partition, was used to obtain crude LPS, and it was further purified by enzymatic treatments.

The results of preliminary work on the chemical analysis of the LPS indicated that it was reasonably pure and similar to that found in other bacteria. Keto-deoxyoctanoic acid and heptose were present (18, 75). Amino acid analysis demonstrated three major components, probably amino sugars, and small amounts of the various amino acids.

When uranyl acetate-stained preparations were viewed in the electron microscope the LPS had the appearance of ribbons of discs (Figs. 5 and 6). Similar results were reported by Shands et al. (62) for LPS from Salmonella. Only a few areas appeared to have a double-track structure.

Freeze-etching of cells without glycerol.--When cells were freeze-fractured in a salts solution without glycerol the cell envelope fractured in two planes, splitting the cytoplasmic membrane and the cell wall (Figs. 7-14). The fracture faces of the cytoplasmic membrane were similar to those seen in other bacteria. The convex face was a smooth surface densely covered with particles, 4 to 8 nm in diameter, which were sometimes arranged in a netlike array (Figs. 7-10). The concave face was more sparsely studded with particles (Figs. 7, 12, 13, and 14). In some preparations there were large circular areas devoid of particles on both the convex and concave fracture faces (Figs. 7, 9, and 12). There was no apparent correlation between the growth conditions of the cells and the presence or absence of these particle-free areas. Generally all or none of the replicas prepared from a single batch of

Fig. 5. Purified LPS positively stained with uranyl acetate showing ribbon structure and occasional areas (arrows) with double-track appearance. (x 168,000).

Fig. 6. Purified LPS positively stained with uranyl acetate showing ribbons and discs. (x 168,000).



cells demonstrated this feature, which indicates that it was a function of the condition of the cells and not an artifact of the freeze-etching process.

The cell envelope also fractured at another level, although with much less frequency and generally only in small areas. This fracture revealed two smooth faces and was probably splitting the outer layer. The convex face was often seen as patches of cell wall material on the cytoplasmic membrane (Figs. 7, 8 and 9). These patches appeared to originate from the outer cell wall layer (Figs. 7 and 9). The corresponding concave face was seen as holes in the cytoplasmic membrane (Figs. 7, 12, and 13). The location of these patches did not appear to be related to the particle-free areas on the cytoplasmic membrane.

The relationship between the smooth fracture face and the outer cell wall layer was more clearly shown in etched preparations (Fig. 11). Etching of preparations containing considerable amounts of salts often revealed a eutectic layer surrounding the cells (Fig. 8, and ref. 17). In some cases the eutectic layer was very thin, and although very fine surface detail may be obscured, the general structure of the surface was seen. This was demonstrated by the presence of flagella on the cell surface (Figs. 10 and 11). It was observed that the smooth convex fracture face is revealed by fracturing away a thin piece of the outer layer (Fig. 11).

Another concave fracture face was occasionally observed in cells fractured without glycerol. This surface was composed of globular subunits (Fig. 14). The corresponding convex face was not demonstrated in these preparations.

A more regular paracrystalline array of subunits was sometimes

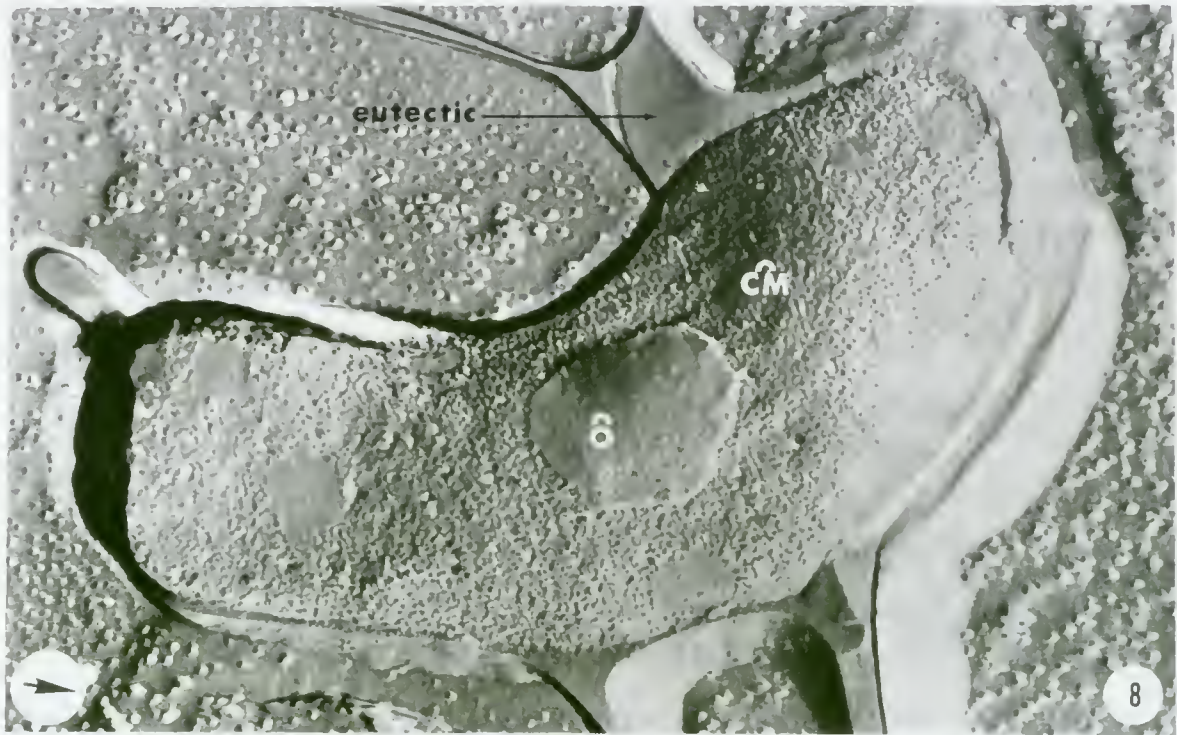
Fig. 7. Freeze-etched cells suspended in complete salts without glycerol. This low magnification view shows concave and convex fracture faces. (x 50,000).



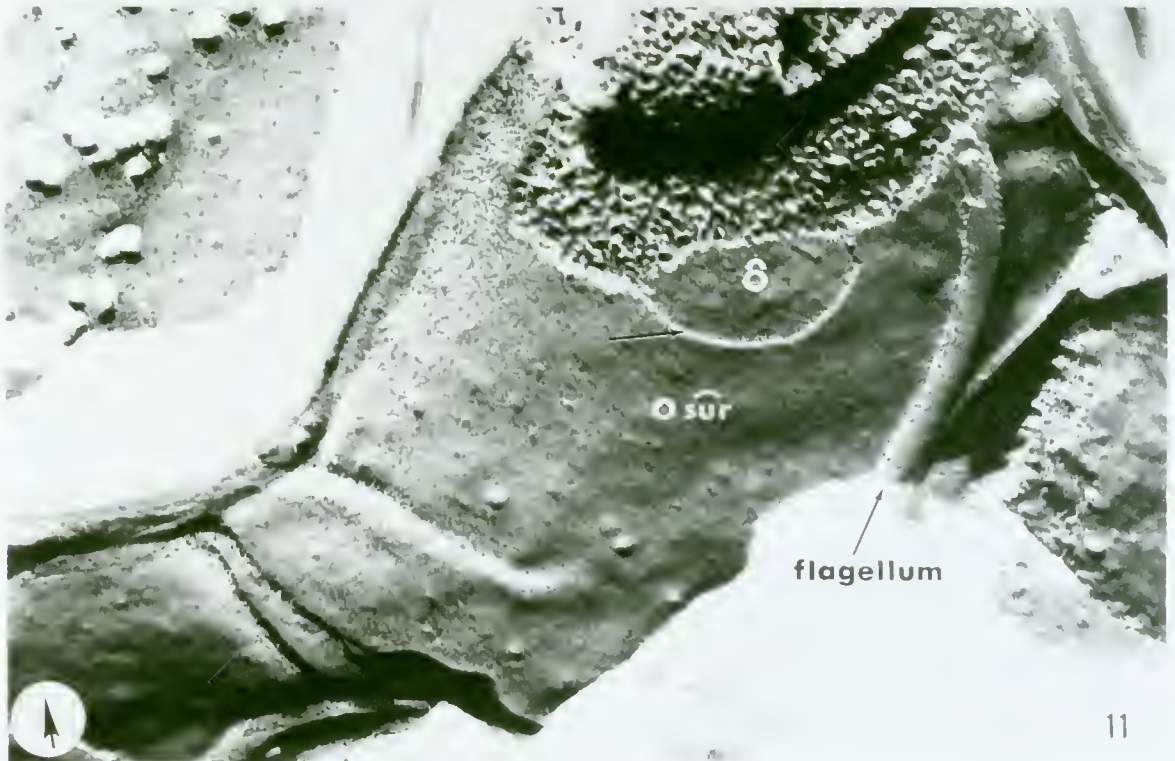
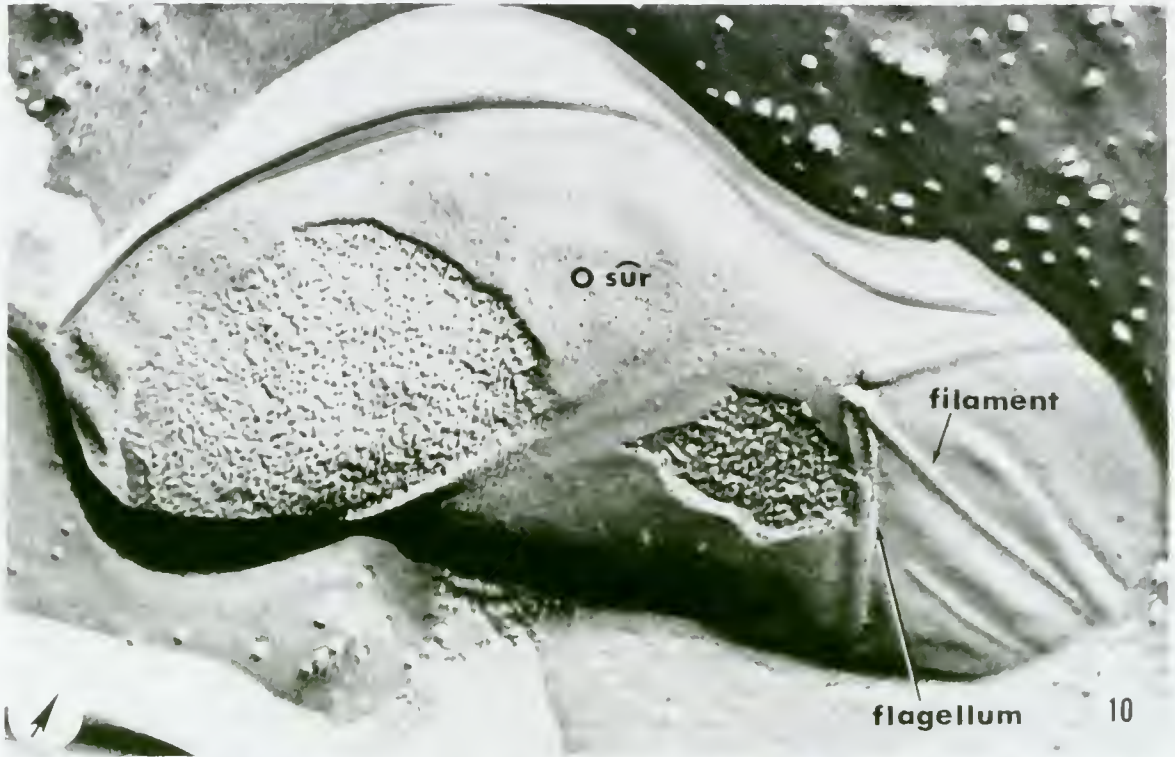


Fig. 8. Freeze-etched cell suspended in complete salts, showing the particle-studded convex fracture face of the cytoplasmic membrane and a patch of cell wall material. Etching has revealed a eutectic layer which sometimes surrounds the cells. (x 61,500).

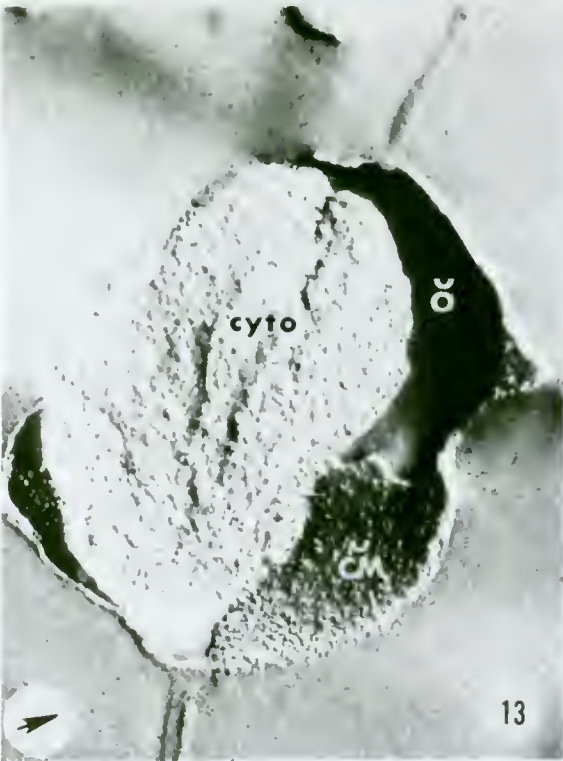
Fig. 9. Unetched convex fracture of cells without glycerol. Note the smooth fracture face of outer membrane and relation of this fracture to the outer surface (edge) of the cell. Large particle-free areas of the cytoplasmic membrane face are shown (arrow). (x 82,000).



- Fig. 10. Deep etched cell in complete salts, showing the smooth or slightly granular outer surface of the cell exposed by etching. Note the structure of the fractured sheathed flagellum and unidentified filaments. (x 83,200).
- Fig. 11. Etched cell in complete salts showing the smooth convex fracture face of the outer membrane and the true surface of the cell. Note the thickness of the fracture edge (arrow). (x 142,000).



- Fig. 12. Concave fracture of a cell in complete salts showing the particle-studded fracture face of the cytoplasmic membrane, and holes in the surface which reveal the smooth concave fracture face of the outer membrane. Particle-free areas (arrow) are also shown. (x 80,000).
- Fig. 13. Concave fracture of a cell clearly showing the smooth fracture face of the outer membrane. (x 68,000).
- Fig. 14. Concave fracture of a cell showing a surface with distinct subunit structure. (x 116,500).



seen (Fig. 15). Although it is not apparent in this figure, this array is located on the inner surface of the cytoplasmic membrane and possibly corresponds to the extra layer seen in thin sections (Fig. 3).

Freeze-etching of glycerol-treated cells.--The primary fracture plane in glycerol-treated cells was the cytoplasmic membrane, and the fracture faces were indistinguishable from those seen in cells without glycerol (Figs. 16 and 17). The outer layer also fractured revealing the two smooth faces previously described (Figs. 18 and 19). Frozen solutions of glycerol and water do not sublime significantly and therefore the outer surface of the cell could only be seen as an edge (Fig. 19).

A third fracture commonly occurred which revealed a rough convex surface and a concave surface composed of globular subunits (Figs. 20, 21, 22, and 23). This fracture plane has been demonstrated in other bacteria and generally was thought to split the outer membrane (26, 32, 71). Our results suggest that a more plausible interpretation is that the wall splits between the rigid layer and a globular protein layer which separates the rigid layer from the outer membrane.

The convex surface of the rigid layer was generally seen only at the ends of the fractured cell, with the envelope usually splitting at the level of the cytoplasmic membrane (Fig. 20). The surface is rough or granular with no clearly defined structure.

The concave globular layer appeared to be composed of globular units approximately 10 nm in diameter (Figs. 22 and 23). In some areas the subunits were in rows, but generally no ordered arrangement was apparent. The layer was usually only partially visible, extending from beneath the fractured cytoplasmic membrane. It was sometimes



Fig. 15. Freeze-etched cell in complete salts showing a paracrystalline array (arrow) inside the cytoplasmic membrane. a. (x 60,000)  
b. (x 167,000).

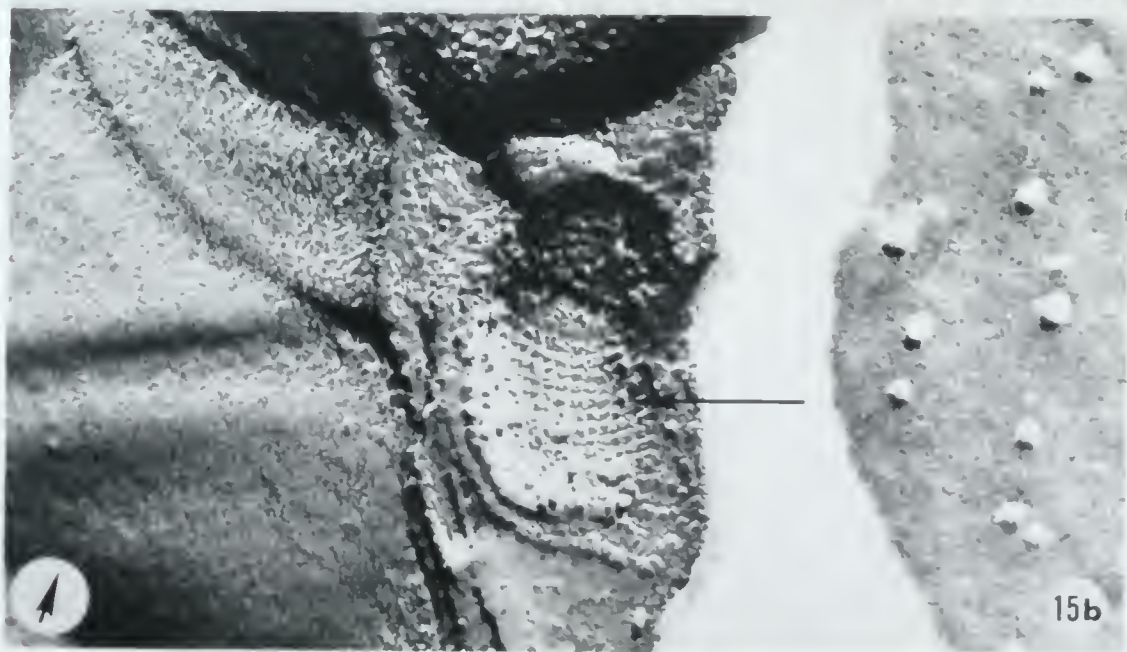


Fig. 16. Concave fracture of the cytoplasmic membrane in a glycerol-treated cell. Cells were suspended in complete salts containing 20% glycerol. The edge of the cell wall is visible (arrow). (x 106,000).

Fig. 17. Convex fracture of the cytoplasmic membrane in a glycerol-treated cell. Note the netlike arrangement of the membrane particles. (x 107,000).

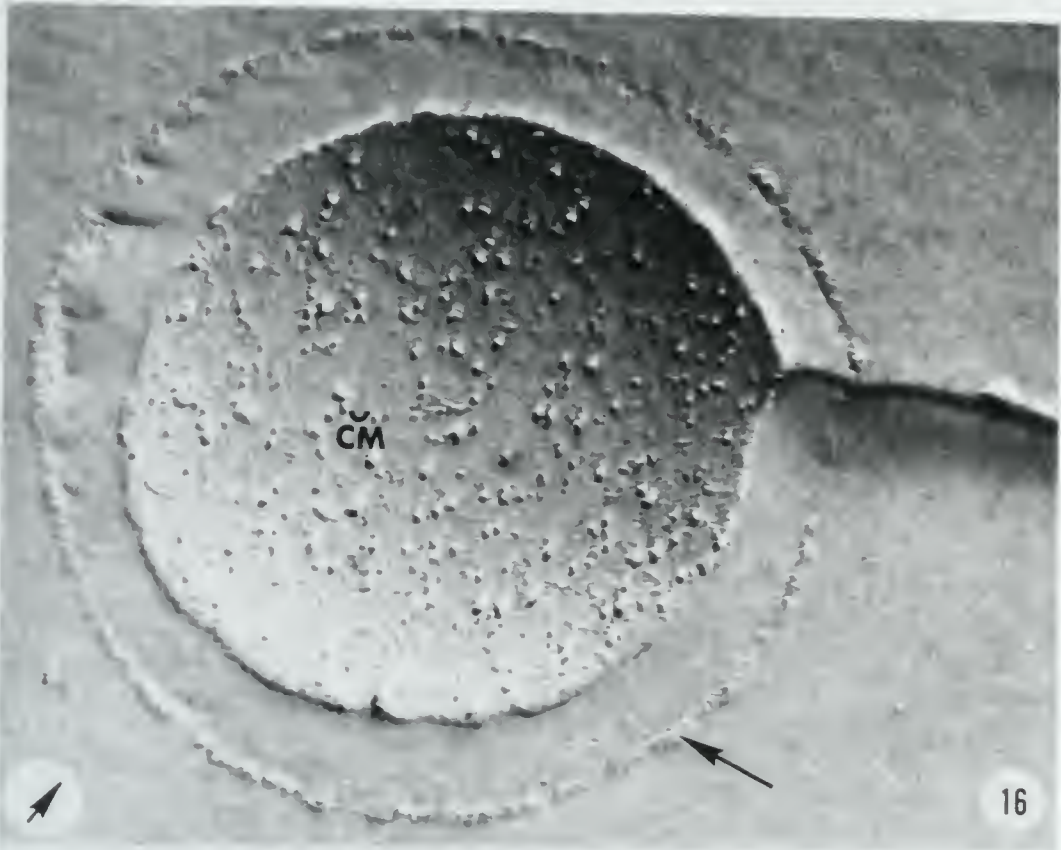
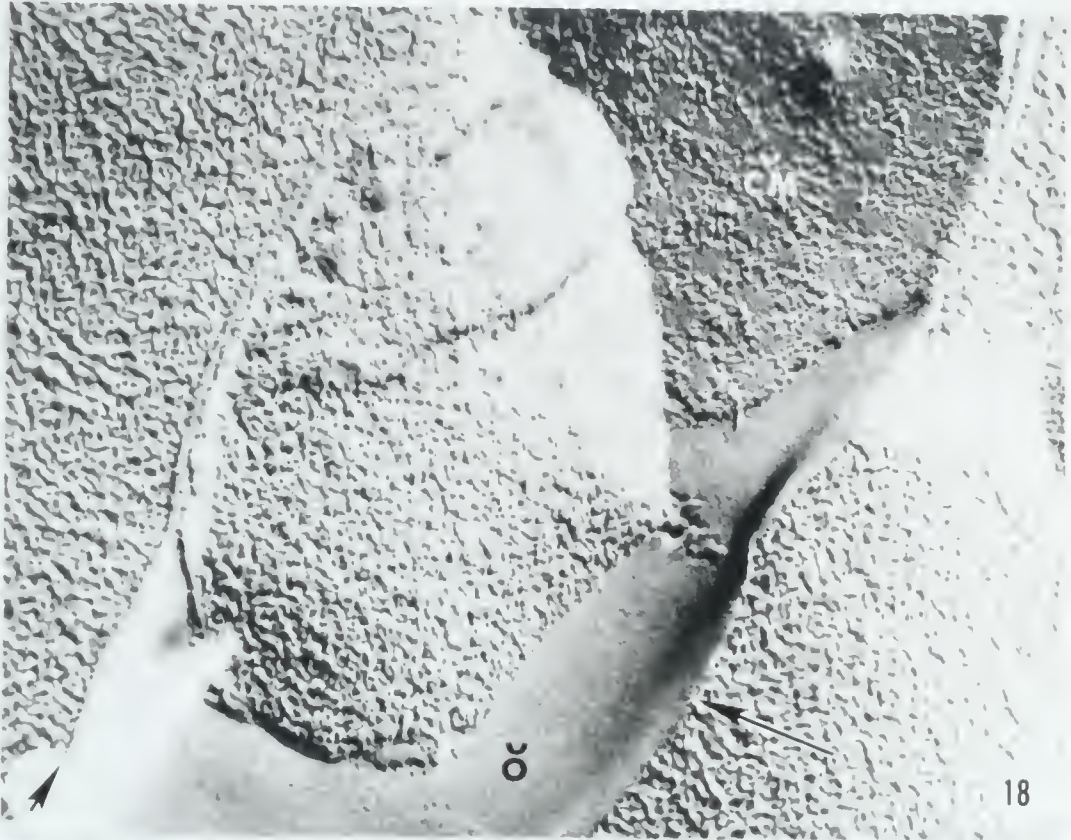


Fig. 18. Unetched fractured glycerol-treated cell showing the concave fracture face of the outer membrane. Note the width of the shadow cast by the layer (arrow) indicating that it is quite thin. (x 76,000).

Fig. 19. Convex fracture of a glycerol-treated cell showing the smooth fracture face of the outer membrane. In preparations with glycerol the outer surface of the cell can only be seen as an edge (arrow). (x 92,000).



Figs. 20 and 21. Freeze-etched glycerol-treated cells showing the rough convex fracture face of the rigid layer.

Fig. 20. (x 100,000).

Fig. 21. (x 157,500).

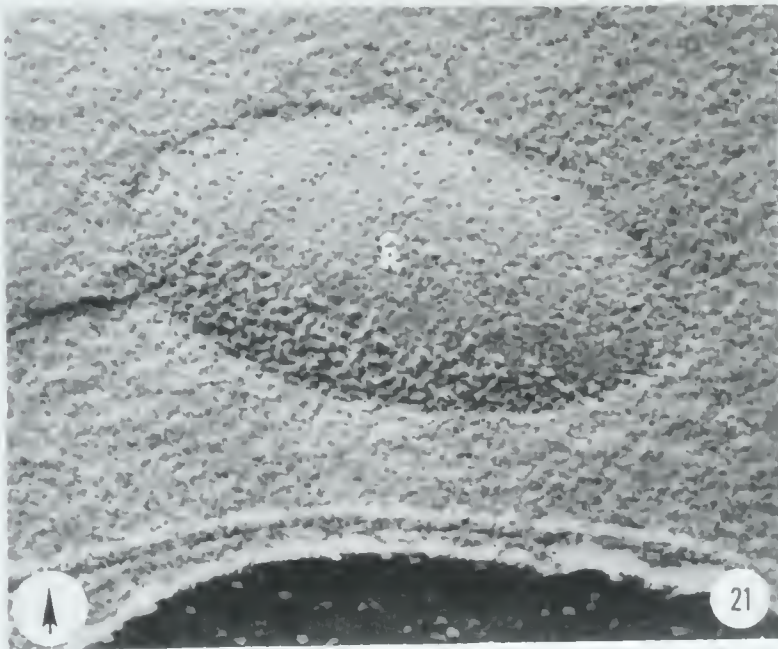
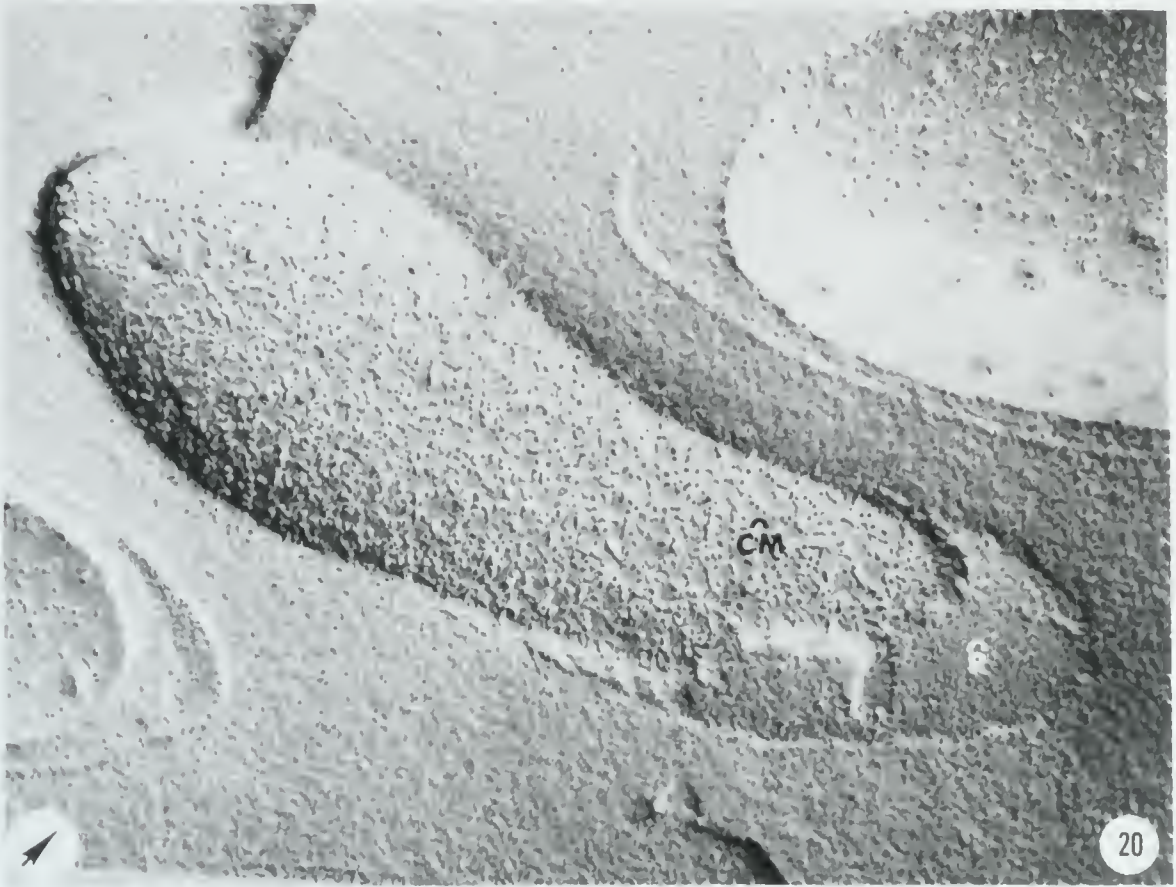
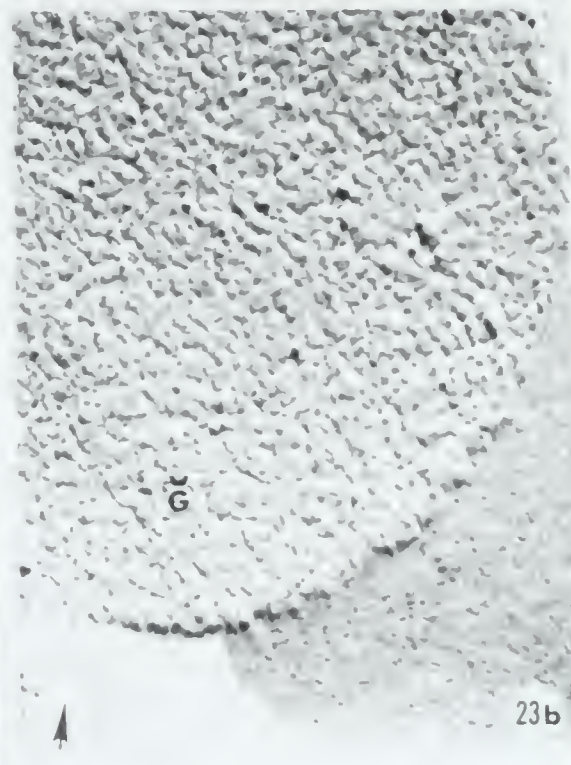
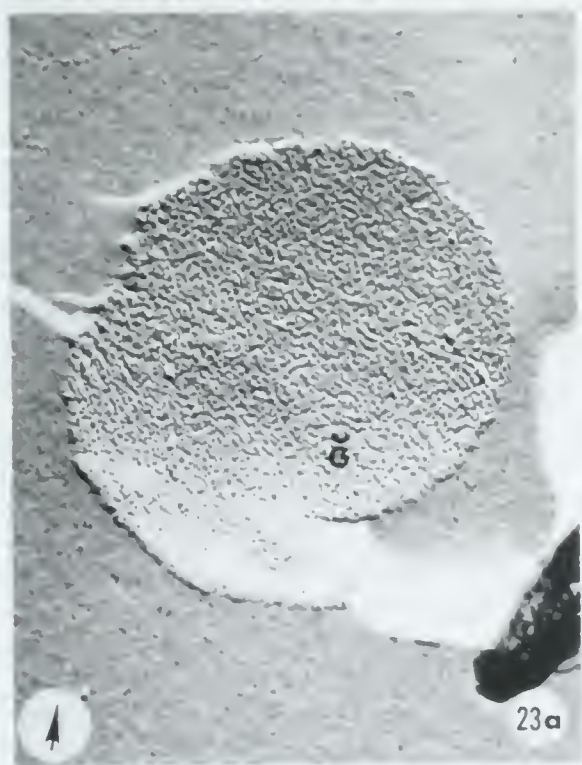
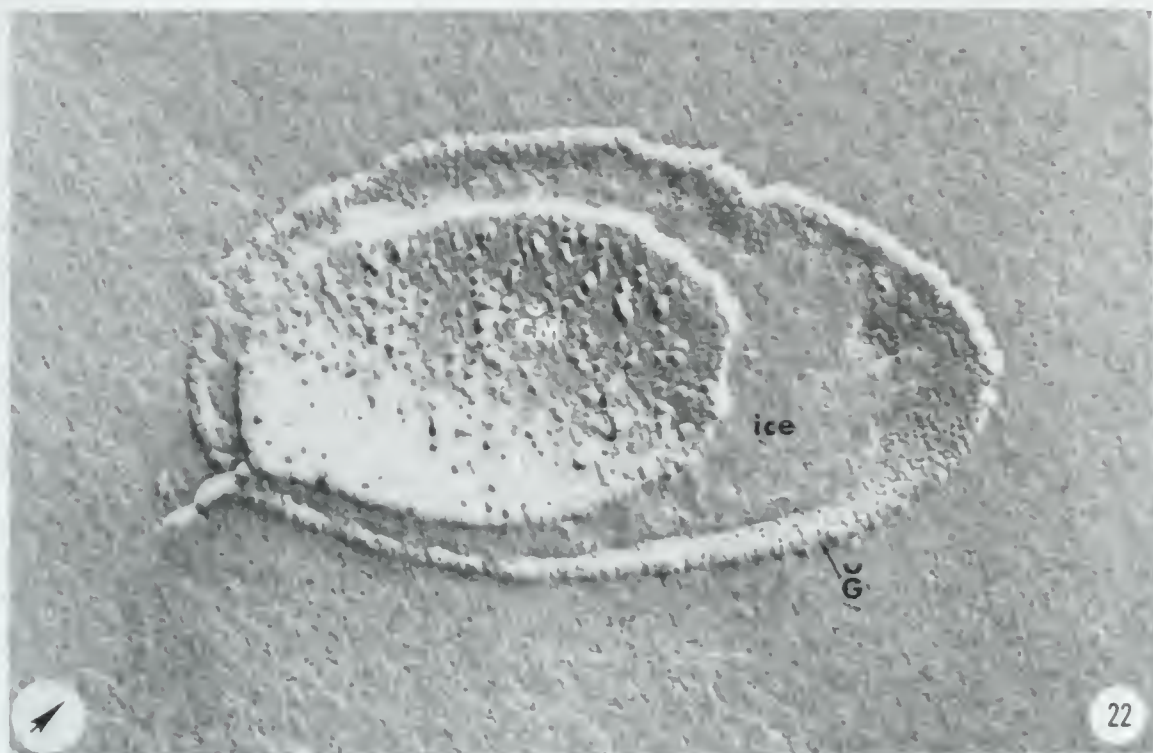




Fig. 22. Concave fracture of a glycerol-treated cell. Note the material, apparently ice, on the surface of the globular layer. (x 122,000).

Fig. 23. Concave fracture of the globular layer in a glycerol-treated cell. a. (x 66,000), b. (x 167,000).



obscured by an intermediate layer which appeared to be ice (Fig. 22). This suggests that the fracture faces were separated by fluid prior to freezing.

In areas where the globular layer was incomplete it was apparent that the layer was in fact composed of individual subunits, and that the subunits were backed by a smooth surface (Fig. 24). This smooth surface was probably the true inner surface of the outer membrane as opposed to the smooth fracture face of the outer membrane.

The cell envelope was also seen in profile in cross fractured cells (Figs. 25 and 26). The inner and outer membranes were routinely observed, and in some cases an intermediate layer was visible. The membranes occasionally had a double-track appearance, but there is no good explanation for this.

Using double-replica technique, complementary replicas of glycerol-treated cells were prepared. The matching fracture faces of individual cells were located in both replicas. In the figures shown, one of the negatives was inverted before printing so that the images would appear superimposable rather than as mirror images. The results of the double-replica work clearly demonstrated that the three pairs of fracture faces previously described were complementary, and each pair was produced by a single fracture (Figs. 27, 28, and 29). It was also apparent that while a rather thin layer was fractured away to expose the smooth convex face, a thick piece of wall was removed in exposing the rough convex face. This suggests that the rough face is at a lower level in the cell envelope.

Structure of isolated cell envelope fractions.--The study of isolated cell envelopes and various fractions of envelopes by freeze-

- Fig. 24. Concave fracture of a glycerol-treated cell. The globular layer is incomplete and the individual subunits (arrow) and the smooth backing layer are visible. The smooth surface is thought to be the true inner surface of the outer membrane. a. (x 60,000), b. (x 157,000).
- Fig. 25. Cross fractured cell envelopes of three adjacent cells showing the edges of the cytoplasmic membrane, rigid layer, and outer membrane. The membranes have a double-track appearance. (x 132,000).
- Fig. 26. Edge fractured cell showing the fracture surface of the membrane and the cross fractured cell wall. (x 132,000).

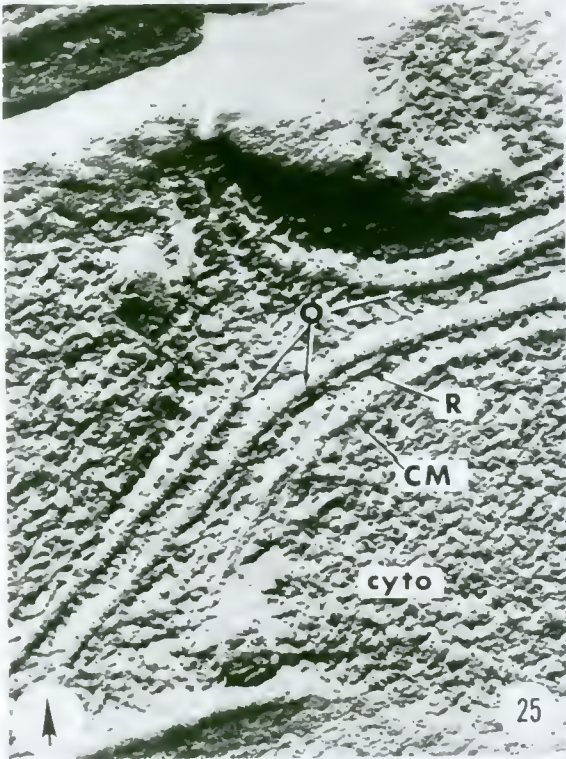
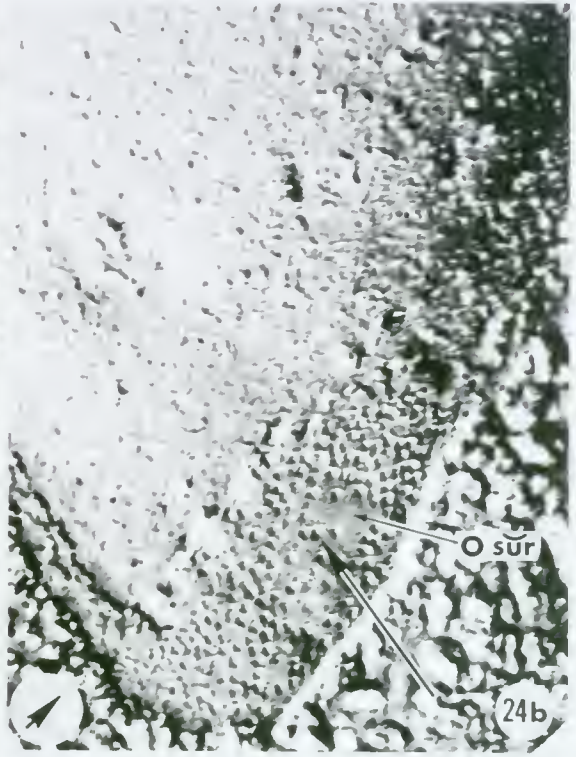
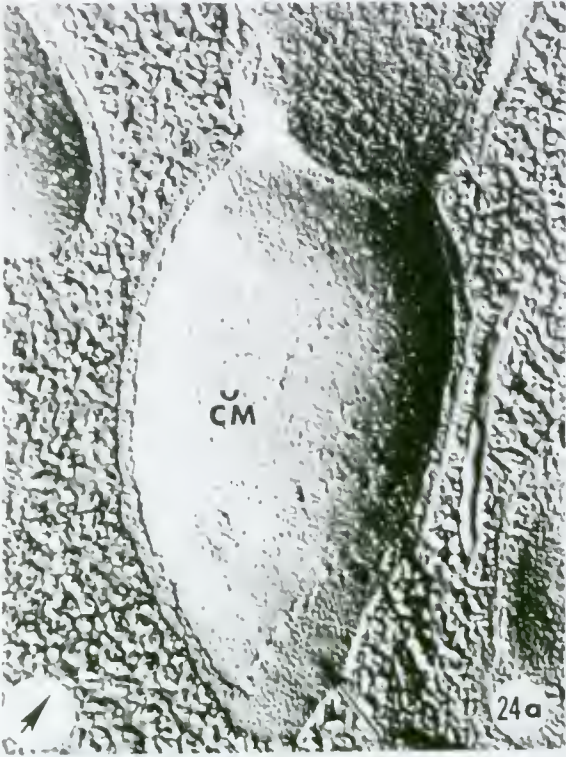


Fig. 27. Complementary surfaces of a glycerol-treated cell observed by double-replica technique. This pair of photographs clearly shows that the membrane faces are produced by fracturing. PHB, poly- $\beta$ -hydroxybutyrate granule. (x 80,000).

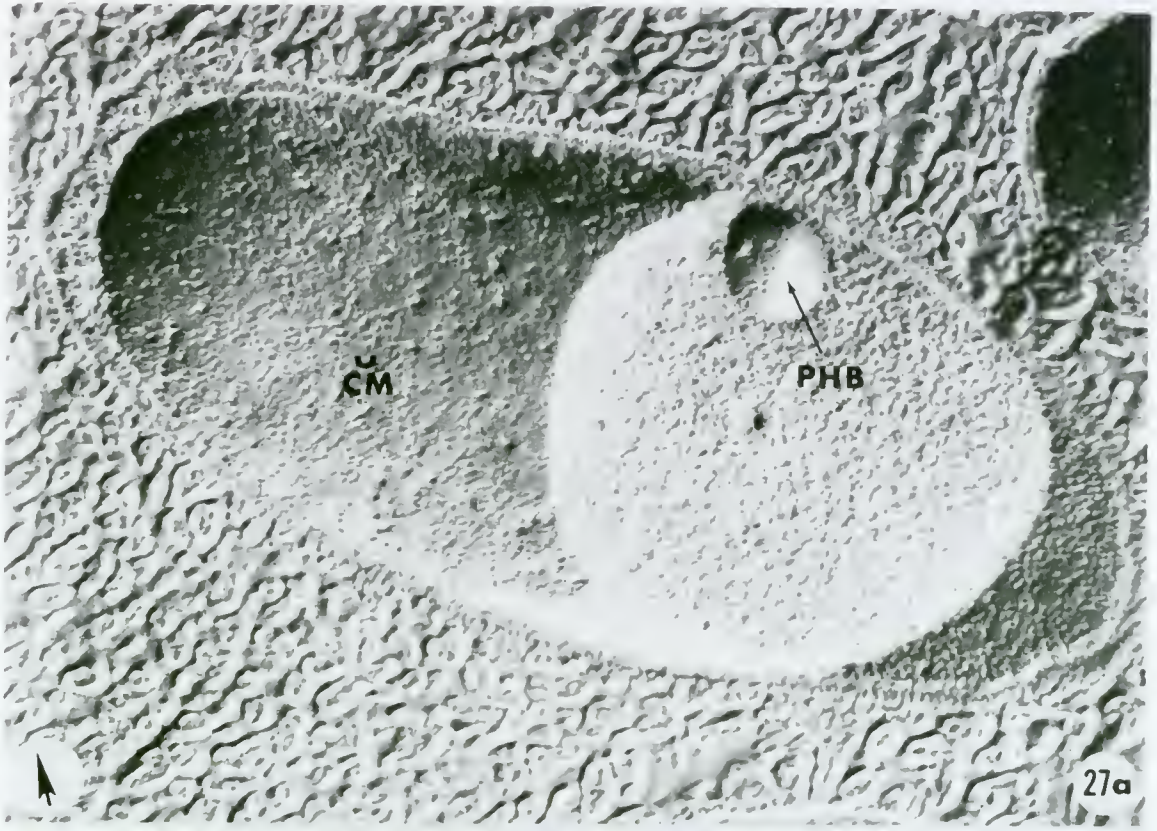


Fig. 28. Complementary fracture faces of a glycerol-treated cell showing the relationship between the concave globular layer and the convex rough faces. (x 75,000).





Fig. 29. Complementary fracture faces of a glycerol-treated cell showing the relationship between the smooth concave and convex fracture faces. (x 100,000).



etching offers several theoretical advantages over using whole cells. Since the fractions are of less complex composition, it should be easier to correlate a particular fracture face with the cell wall component which is being studied. In whole cells only those surfaces which are natural fracture sites can be seen. Using isolated cell envelopes it should be possible to observe other surfaces, such as the inner surface of the cytoplasmic membrane, by merely etching away the ice. In the case of marine bacteria there is an additional advantage. Cell envelopes can be frozen in distilled water or dilute salt solutions thereby reducing the tendency to form eutectic layers which obscure etch surfaces.

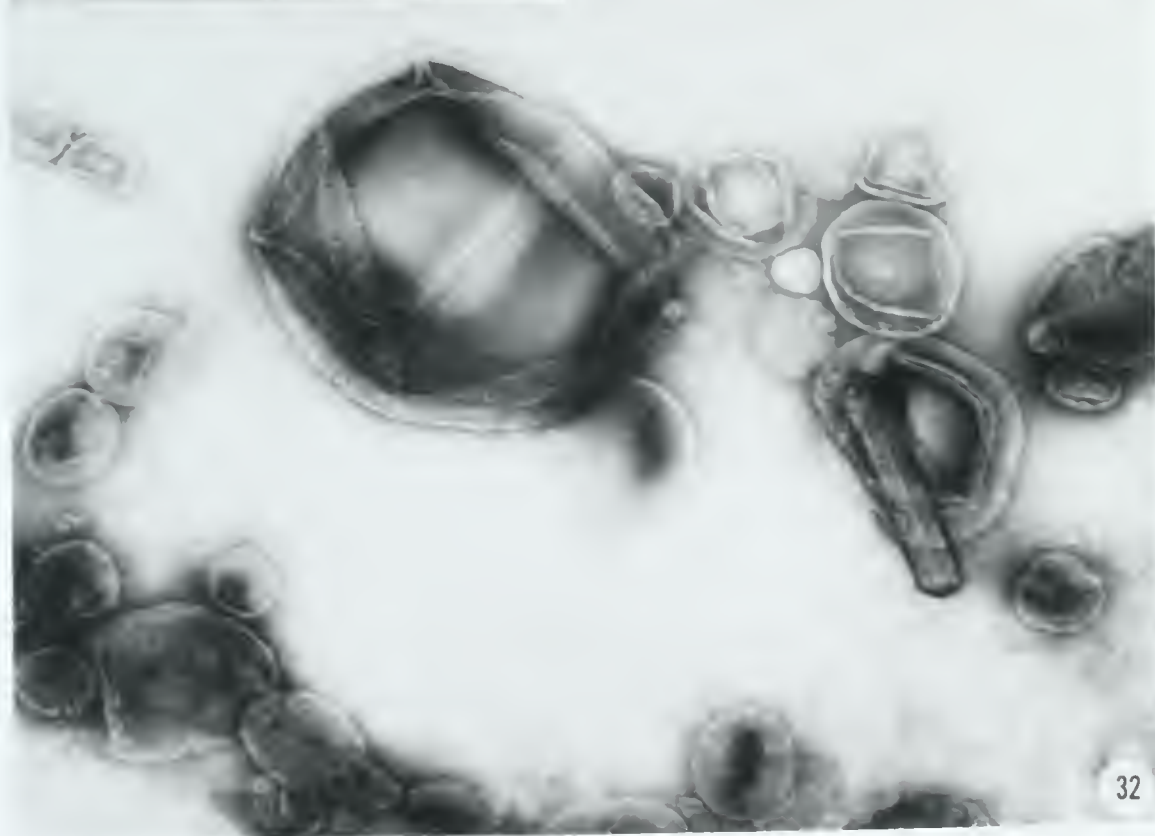
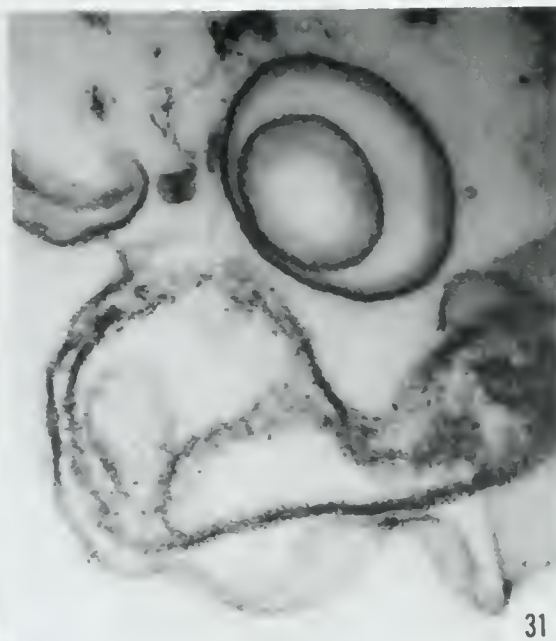
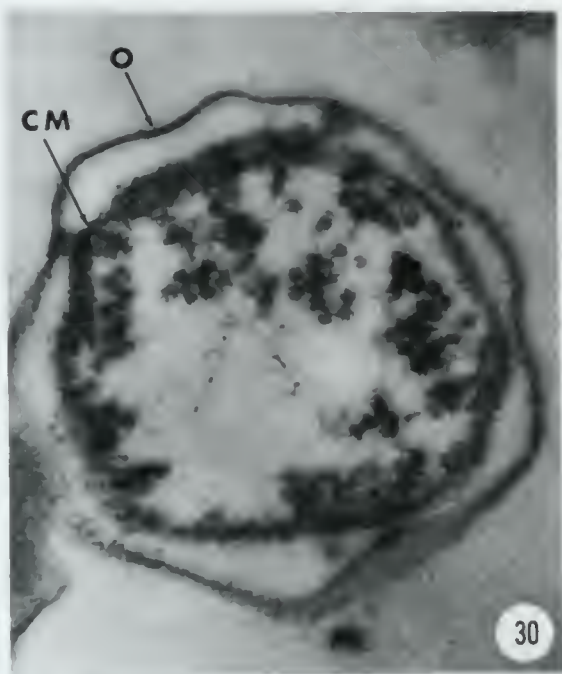
Complete cell envelopes were prepared by lysing the bacteria in a French pressure cell. Examination of thin sections revealed that a variety of different structures were produced in this lysis process (Figs. 30 and 31). The majority of the envelopes were double-membrane structures which were either open C-shaped fragments or closed vesicles. Single membrane vesicles were also seen and it was not possible to determine if these were formed from the cytoplasmic membrane or the outer membrane. As in whole cells, the rigid layer was not visible.

The various structures were also seen in uranyl acetate stained envelopes (Fig. 32). Similar results were obtained with potassium phosphotungstate and ammonium molybdate negative stains. No subunit structure was observed in any of these preparations.

Freeze-etching of these complete envelopes yielded little additional information. The cytoplasmic membrane fracture faces and the globular layer were observed, but a variety of other surfaces could not be identified (Figs. 33 to 36). Many of the envelope frag-

Figs. 30 and 31. Thin sections of isolated cell envelopes prepared by lysing bacteria in a French pressure cell. (x 127,000).

Fig. 32. Isolated cell envelopes positively stained with uranyl acetate, showing double membrane fragments of various sizes. (x 151,000).



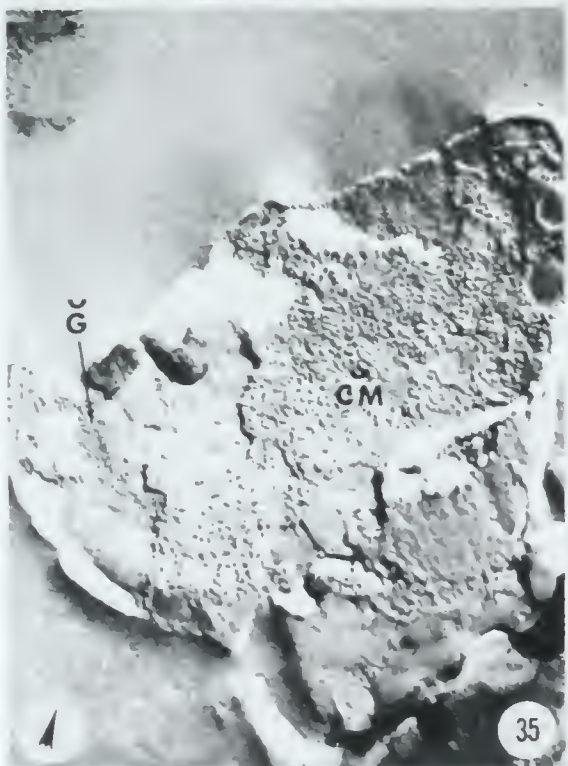
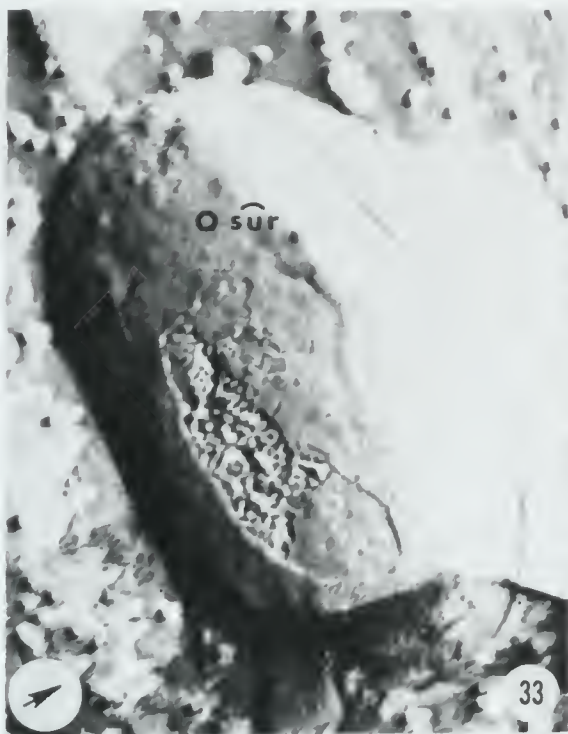
Figs. 33, 34, 35, and 36. Freeze-etched isolated cell envelopes. Envelopes were prepared by lysing bacteria in a French pressure cell and suspended in 0.01M  $MgCl_2$ .

Fig. 33. This fragment shows a typical cytoplasmic membrane fracture face and the outer surface exposed by etching. (x 116,000).

Fig. 34. An unfractured fragment exposed by etching. (x 58,500).

Fig. 35. A concave fracture exposing the globular layer. (x 58,500).

Fig. 36. This fragment shows the globular layer apparently unfractured and exposed by etching alone. (x 157,500).





ments were unfractured and revealed by etching (Fig. 35). The envelopes were suspended in 0.01M  $MgCl_2$  rather than in complete salts to minimize the formation of eutectic layers. The true outer surface of the envelopes appeared smooth or finely granular. The granularity may represent the true structure of the surface or it may be an artifact of very low angle shadowing since it is only seen in areas where the surface is sloping away from the direction of shadow.

The true inner concave surface of the cytoplasmic membrane should be revealed by etching but was not recognizable. Surprisingly, the concave globular layer was exposed by etching (Fig. 36). This indicates that this layer separated from the rough surface during the preparation of the envelopes, and therefore could not be an internal fracture surface of a membrane.

Cell walls were prepared by lysing cells with Triton X-100. Schnaitman (59) has shown that in the presence of magnesium this detergent solubilizes the cytoplasmic membrane leaving only slightly altered cell walls. The freeze-etched appearance of these cell walls was very complex and difficult to interpret. During preparation the walls apparently packed together and flattened out, and possibly turned inside out, making it difficult to recognize concave and convex surfaces.

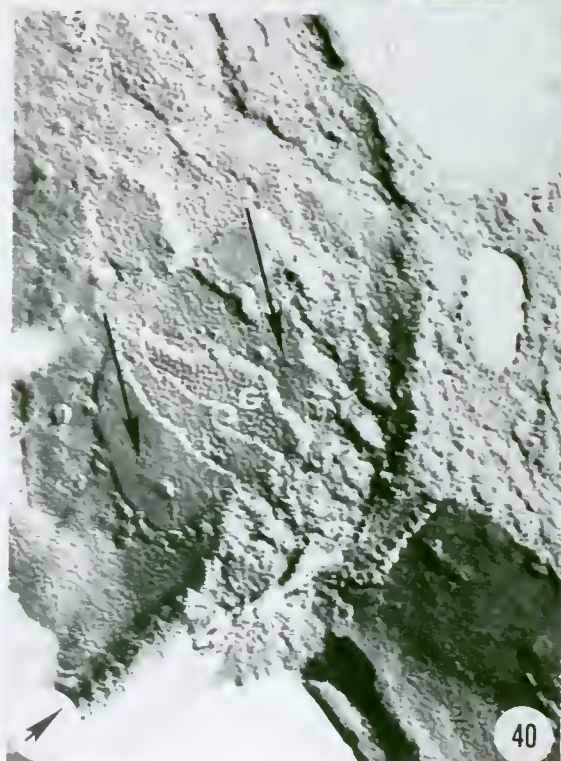
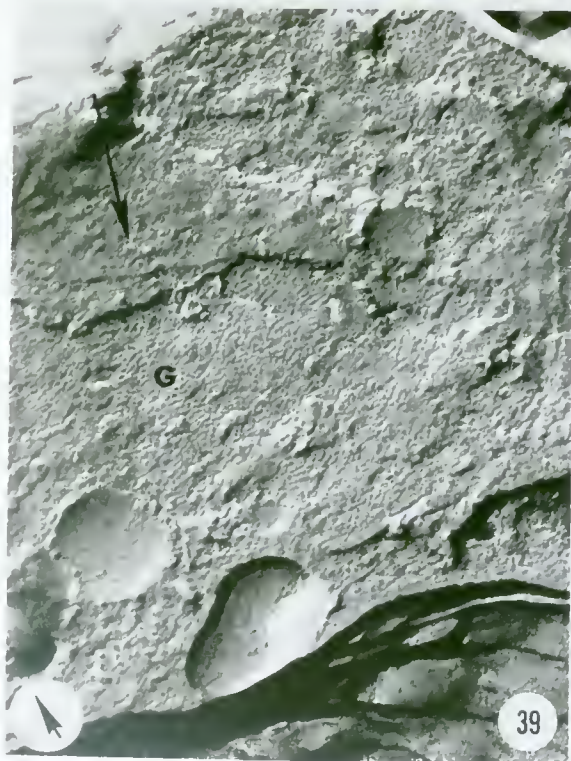
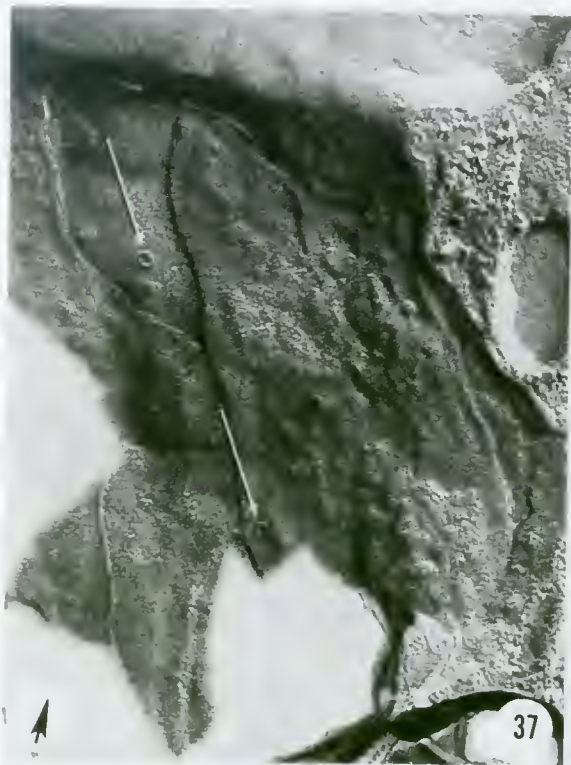
A variety of surfaces were observed (Figs. 37 to 40). As expected, no typical cytoplasmic membrane fracture faces were seen. Many of the surfaces were composed of large circular structures which may have been flattened vesicles (Fig. 38). Since the cytoplasmic membrane is absent, the inner surface of the rigid layer should be exposed. This surface was seen superimposed on the globular layer (Figs. 39 and 40).

Figs. 37, 38, 39, and 40. Freeze-etched cell walls. The walls were prepared by lysing cells with Triton X-100 and suspended in 0.01M MgCl<sub>2</sub>. (x 90,000).

Fig. 37. This fragment has a smooth surface and shows circular structures (arrows) of unknown nature.

Fig. 38. Cell walls showing large circular structures which are probably flattened vesicles.

Figs. 39 and 40. Fractured cell walls showing the globular layer and adjacent layers (arrows). It is not apparent whether these are concave or convex surfaces.



When these walls were partially digested with lysozyme they had a different appearance. Concave surfaces were observed which had a distinct fibrous appearance (Figs. 41, 42, and 43). These surfaces were exposed by etching. When this surface was fractured away the globular layer was revealed (Figs. 42 and 43). This fibrous layer was not seen in any other type of preparation and probably represents the partially digested peptidoglycan.

Forsberg et al. developed a procedure which allows the removal of the outer membrane from a marine pseudomonad (22, 23). Application of this washing procedure to the marine vibrio caused the release of outer membrane material, but did not markedly affect the viability of the culture. The solubilized material was collected by ultracentrifugation, and the pellet obtained was a clear gelatinous material unlike the white, easily resuspended cell wall material produced in the other procedures.

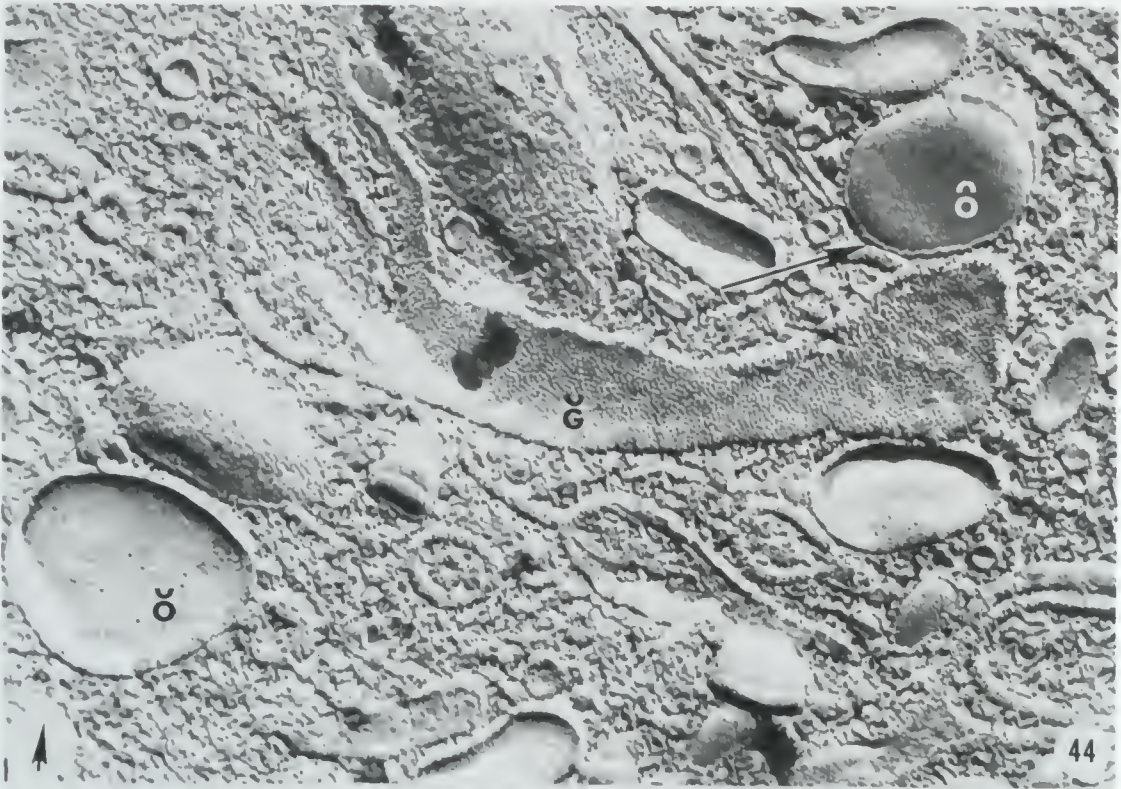
Freeze-etching of this material revealed variously shaped vesicles which were seen in cross section and as concave and convex fracture faces (Figs. 44 and 45). Slight etching exposed an edge around the convex faces demonstrating that these faces are produced by fracturing. Both of the fracture faces were smooth and appeared identical to the smooth faces seen in whole cells. A few patches of globular layer were also observed in these preparations (Fig. 44). These layers were generally larger than the membrane vesicles and appeared flatter and more cell shaped, suggesting that they were part of a larger fragment of cell wall or had some innate structure which prevented them from forming vesicles.

- Figs. 41, 42, and 43. Freeze-etched cell walls prepared by lysing cells with Triton X-100 and partially digesting with lysozyme.
- Fig. 41. Note the fibrous nature of the concave surface (arrows) and the patch of ice which indicates that this is an etch surface. (x 66,000).
- Fig. 42. This fracture shows the relationship of the globular layer and the concave fibrous layer. (x 66,000).
- Fig. 43. In this area the fibrous material is emerging from the ice background (arrow) indicating that this is an etch surface. (x 100,000).



Fig. 44. Freeze-etched isolated outer membrane material suspended in 0.01M  $MgCl_2$ . Cross fractured vesicles and the smooth concave and convex fracture faces are shown. Etching has revealed the outer surface of a vesicle (arrow). A large fragment of the globular layer is also shown. (x 86,000).

Fig. 45. Fractured outer membrane vesicles showing the fracture face and unfractured surface (arrow). (x 135,000).





Localization of LPS with ferritin conjugated antibody.--Three different antigen preparations were used to produce antisera. The activities of the antisera were determined by agglutination of whole cells. The highest titer was produced by injecting whole cells, but it was found that adsorbing this antiserum with purified LPS would reduce the agglutination activity by only 50%. Repeatedly adsorbing with LPS had no further effect. Only the antiserum produced by injecting partially purified cell walls was specific for LPS. This antiserum was of lower titer than the others, but its activity could be completely adsorbed with LPS and it was used for all labelling experiments.

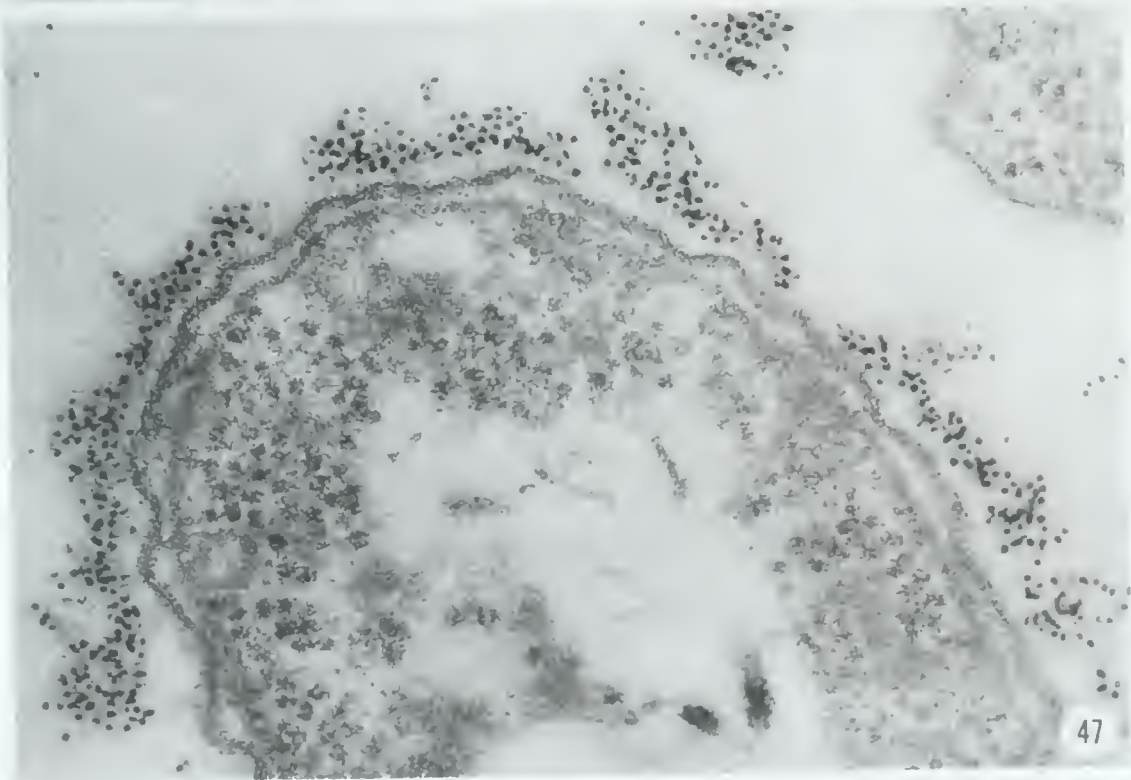
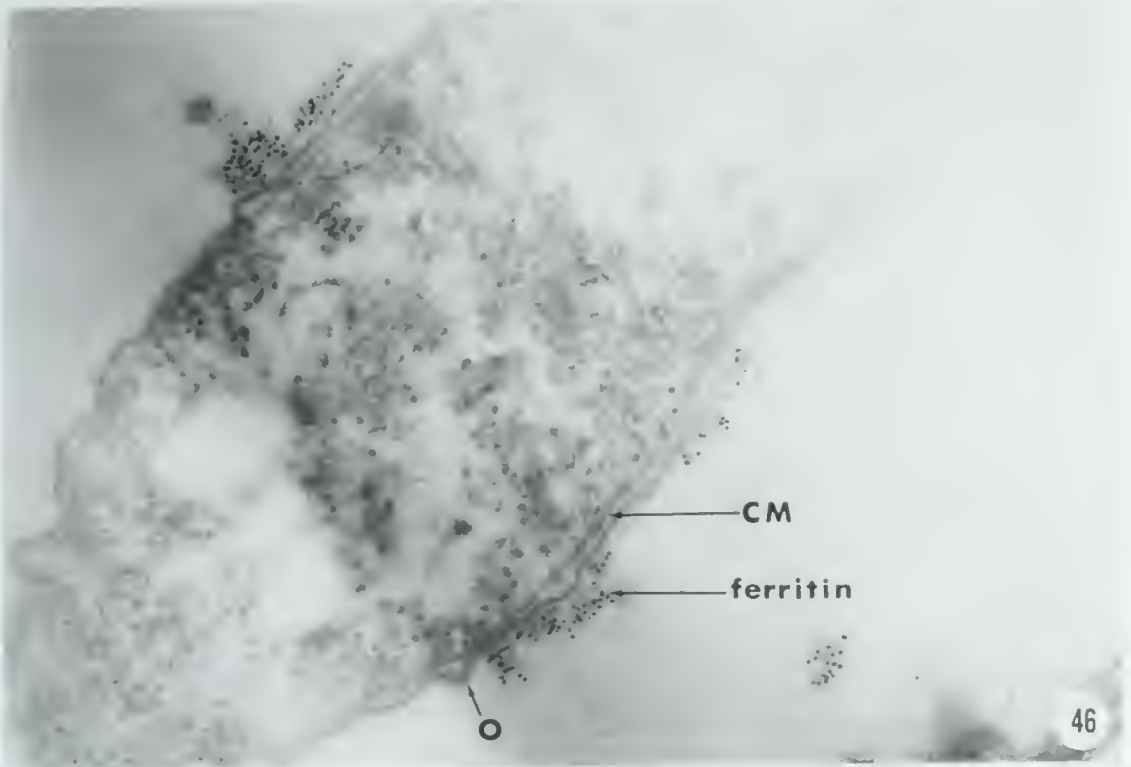
Cells were labelled by the indirect method using anti-LPS antiserum followed by ferritin-conjugated anti-rabbit immunoglobulin antiserum. The specificity of the labelling was determined by treating cells with anti-LPS antiserum or LPS adsorbed anti-LPS antiserum followed by ferritin-conjugated antiserum. Cells were also treated with ferritin-conjugated antiserum alone. The cells were examined in the electron microscope without staining and the degree of labelling was determined. The results are shown in Table 2. Neither unfixed nor glutaraldehyde fixed cells were labelled by ferritin-conjugated antiserum alone, and they were only slightly labelled by LPS adsorbed antiserum. Using unadsorbed antiserum the fixed cells were more heavily labelled than the unfixed cells. This may have been caused by loss of labelled LPS from the cell surface during the washing procedure.

Thin sections of labelled cells revealed that the ferritin was localized in a band external to the outer double-track layer (Figs. 46 and 47). In cells which were not fixed before labelling, the ferritin

Table 2. Determination of the specificity of antibody labelling.

Cells	Treatment	Result
Unfixed cells	Anti-LPS then ferritin-conjugated antiserum	Labelled
Unfixed cells	LPS adsorbed anti-LPS then ferritin-conjugated antiserum	Unlabelled
Unfixed cells	Ferritin-conjugated antiserum only	Unlabelled
Glutaraldehyde fixed cells	Anti-LPS then ferritin-conjugated antiserum	Labelled
Glutaraldehyde fixed cells	LPS adsorbed anti-LPS then ferritin-conjugated antiserum	Unlabelled
Glutaraldehyde fixed cells	Ferritin-conjugated antiserum only	Unlabelled

- Figs. 46 and 47. Thin sectioned cells labelled with ferritin. The cells were treated with rabbit anti-LPS antiserum, washed, and then labelled with ferritin-conjugated goat anti-rabbit antiserum.
- Fig. 46. This cell was fixed following the antibody treatments. (x 80,000).
- Fig. 47. This cell was fixed in glutaraldehyde before antibody treatments. Note the distance between the ferritin molecules and the outer double-track. (x 167,000).



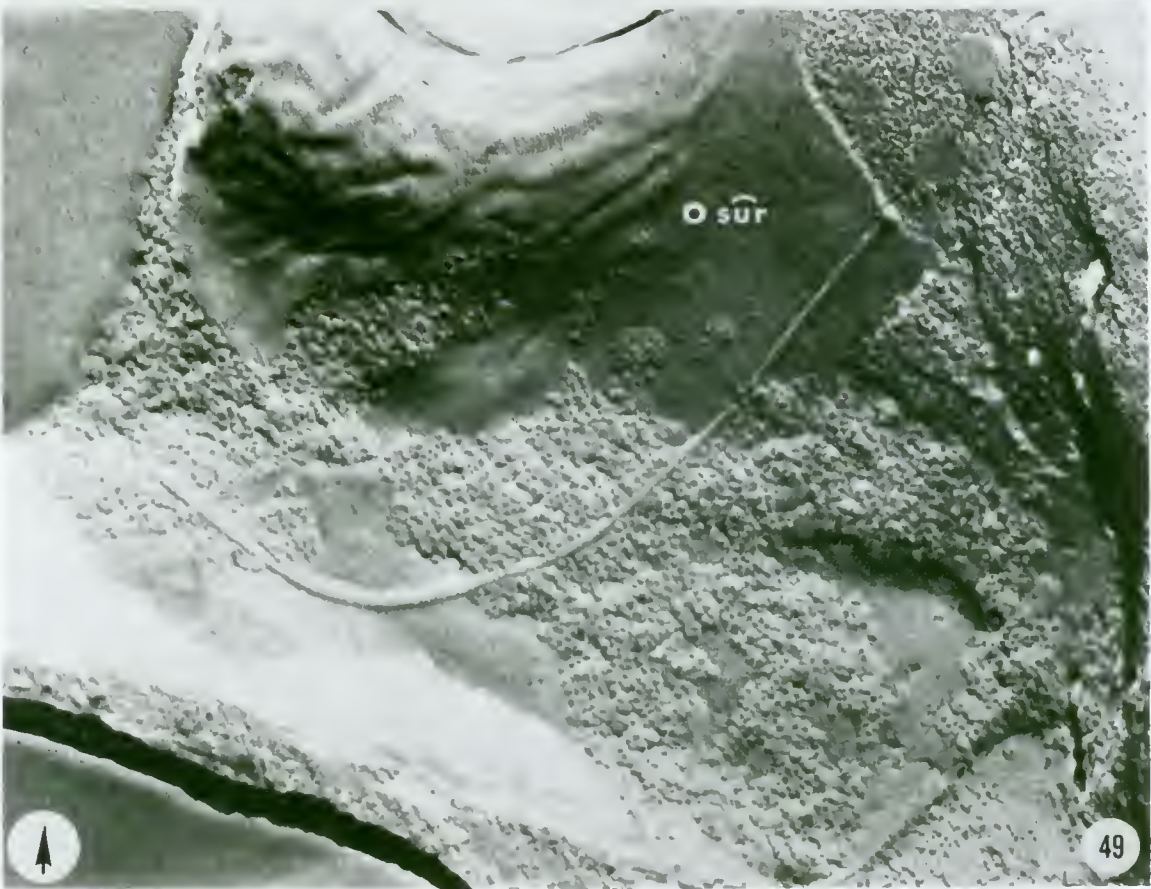
was limited to small areas. The labelling was heavier and more uniform in fixed cells. In all cases the ferritin was a considerable distance from the outer double-track. This is similar to the results of Shands (61), and was probably due to the fact that the polysaccharide side chains extend out from the cell surface and are not stained by the heavy metal stain. The use of indirect labelling puts two antibody molecules between the antigen and the ferritin molecules which could account for a 20 to 50 nm space.

For freeze-etching, ferritin labelled cells were suspended in 0.05M  $MgCl_2$ . It was found that this solution would maintain the integrity of the cells and still allow the deep etching needed to expose the cell surface. The presence of ferritin confirmed that the outer surface of the cells was revealed by etching. It was difficult to compare the appearance of the ferritin in thin sections and in freeze-etching. All of the particles in freeze-etched cells were lying close to the surface of the cell. It is possible that during the freezing process the ferritin molecules are excluded from the growing ice crystals thus packing them down onto the cell surface.

With unfixed cells the ferritin molecules were clustered in patches on the cell surface (Figs. 48 to 51). The envelope fractured normally revealing the convex cytoplasmic membrane and outer membrane fracture faces (Figs. 50 and 51).

Glutaraldehyde fixed cells were heavily labelled (Fig. 52). The ferritin was evenly distributed on the cell surface and many individual ferritin molecules were seen. In unfixed cells the LPS was freely mobile in the membrane and was probably drawn together by the divalent

Figs. 48 and 49. Freeze-etched unfixed cells labelled with ferritin and suspended in 0.01M MgCl<sub>2</sub>. The LPS on the cell surface was labelled indirectly with ferritin-conjugated antibody. The cell surface was exposed by deep etching. The ferritin molecules are clustered in patches. (x 92,000).



Figs. 50 and 51. Convex fractured unfixed cell labelled with ferritin. Note the relationship of the smooth fracture face to the ferritin labelled surface. (x 92,000).



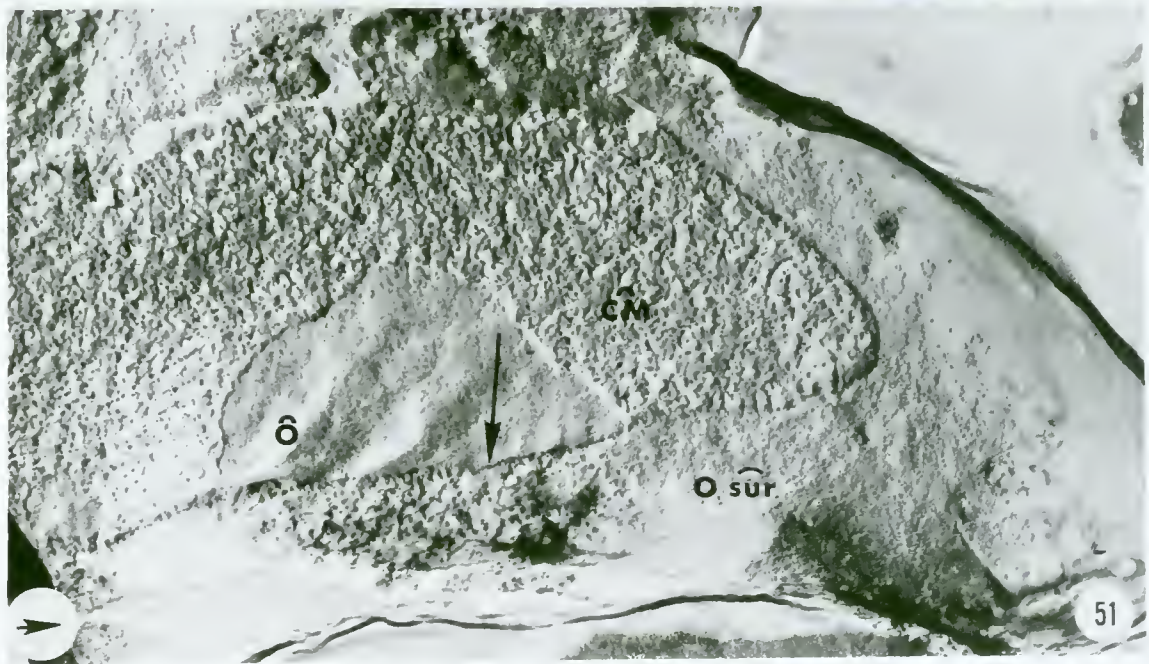
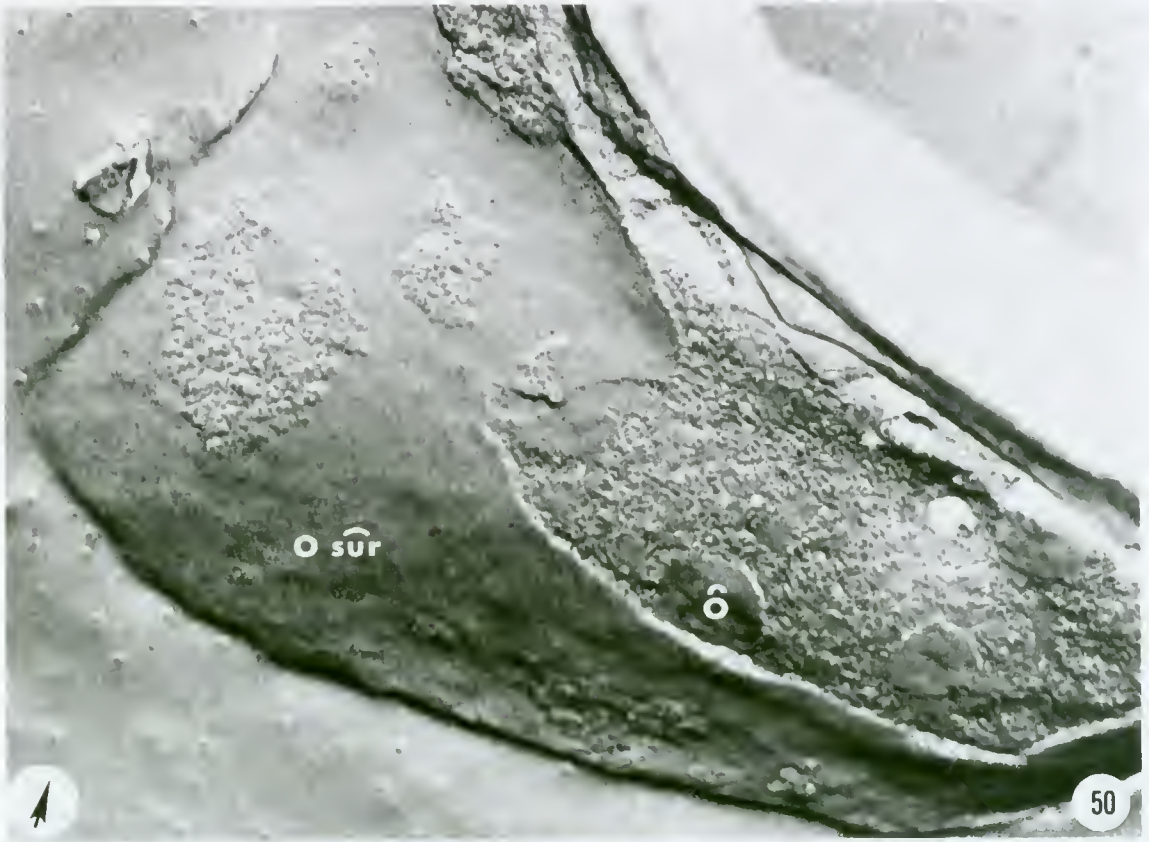


Fig. 52. Ferritin labelled glutaraldehyde fixed cell. The ferritin molecules are more evenly distributed than in unfixed cells. Note the relationship of the smooth convex fracture face and the ferritin covered surface exposed by etching. The fracture edge is quite thin (arrow). (x 157,000).



antibody. Glutaraldehyde fixation stabilized the membrane and prevented the LPS from moving. The relationship between the smooth fracture face and the ferritin labelled outer surface was clearly shown by these experiments (Fig. 52).

## DISCUSSION

It was shown in this study that the cell envelope of the marine vibrio MW40 freeze-fractures in three planes. The relationships between the various fracture faces were proven by double-replica technique. It has been firmly established in previous studies (65, 71) that one fracture splits the cytoplasmic membrane, and this interpretation is consistent with the results of this study. Lickfeld et al. (32) recently offered an alternate explanation, but it appears to be untenable.

Localizing the other two fracture planes is more difficult. As described in the Results section, these fractures are interpreted as follows. One fracture splits the outer membrane revealing smooth convex and concave faces. The second fracture splits the cell wall at an intermediate level exposing a rough convex face and a concave face which is composed of globular subunits superimposed on a smooth surface.

The smooth fracture faces were seen primarily when the cells were freeze-etched without glycerol, but were demonstrated in glycerol-treated cells. The rough convex face was only seen in glycerol-treated cells, but the globular concave face was observed with and without glycerol. Although the two fracture planes have not been clearly demonstrated in the same cell, they have been observed in different cells in the same replica. It can thus be concluded that while the

presence or absence of glycerol tends to determine the location of the fracture, it does not alter the appearance of the fracture faces as others have implied (26, 71). The action of glycerol as it affects the fracturing process is not known.

If it is accepted that there are two distinct fracture planes in the cell wall, it must be decided where the fractures occur. It is logical to assume that if the outer membrane is a lipid bilayer, it will fracture along its hydrophobic center as has been demonstrated for other membranes. In other studies only the globular-rough fracture plane was observed and it was postulated that this represents the splitting of the outer membrane (26, 32, 71). In view of our results this seems unlikely.

A survey of membrane fracture faces in a variety of natural and artificial membranes reveals that although they may be studded with particles, the portion of the face which associated with the lipid is generally smooth. The smooth cell wall fracture faces would therefore fit this description while the rough convex face would be atypical.

The globular layer could represent an unusual type of membrane face, but the dimensions of the layer seem to preclude this. The subunits are 10 nm in diameter, and our results show that they are nearly spherical. The outer double-track measures about 7.5 nm and could not accommodate a layer of 10 nm globules. While the double-track does not represent the entire thickness of the outer membrane, it does reflect the size of the lipid portion between which the globular layer is supposed to be located. A similar problem exists in explaining the particles seen in most membranes, and it has been proposed that these particles are intercalated into the lipid layer. The globular layer,

however, is a continuous surface, and such an explanation would require that the entire outer half of the outer membrane be composed of the globular units. This seems to be unlikely, and the demonstration of a smooth surface in areas where the subunits are missing tends to disprove it.

The relative location of the two fracture planes can be inferred from the thickness of the fracture edges, although it is difficult to actually measure these edges. Only a thin layer is fractured away to expose the smooth convex face, and this is consistent with the belief that this fracture removes the outer half of the outer membrane. The fracture edge down to the rough convex face is thicker, indicating that it represents a layer in the middle of the cell envelope.

It was observed in this study that the globular layer is sometimes obscured by a covering of ice. A similar result was obtained in complementary replicas of E. coli, in which it was observed that the suspending medium could intrude between the globular and rough faces (71). This could not occur if these faces were the hydrophobic portion of a membrane. The demonstration that in isolated cell envelopes the globular layer can be exposed by etching alone clearly proves that this face is not a membrane fracture face.

The observation of smooth fracture faces in isolated outer membrane material also supports our interpretation. A similar study was reported by Forge et al. (21) but the results and conclusions are very vague and can not be directly compared to our results.

Little direct evidence has been offered in support of the alternate interpretation. Gilleland et al. (26) stated that in cells showing both cross-fractured envelope and surface fractures, the rough convex face

could be seen to originate from the inner track of the outer double-track. Lickfeld et al. (33) demonstrated that the cross-fractured outer membrane does have a double-track appearance in some areas, although there is no plausible explanation for this. They also showed that the cross-fractured cell wall is composed of two layers, that is, the outer layer and the rigid layer (similar to Fig. 26). It appears probable that the double-track layer observed by Gilleland et al. is actually two layers, and the rough face corresponds to the inner of these layers. This layer is probably the rigid layer.

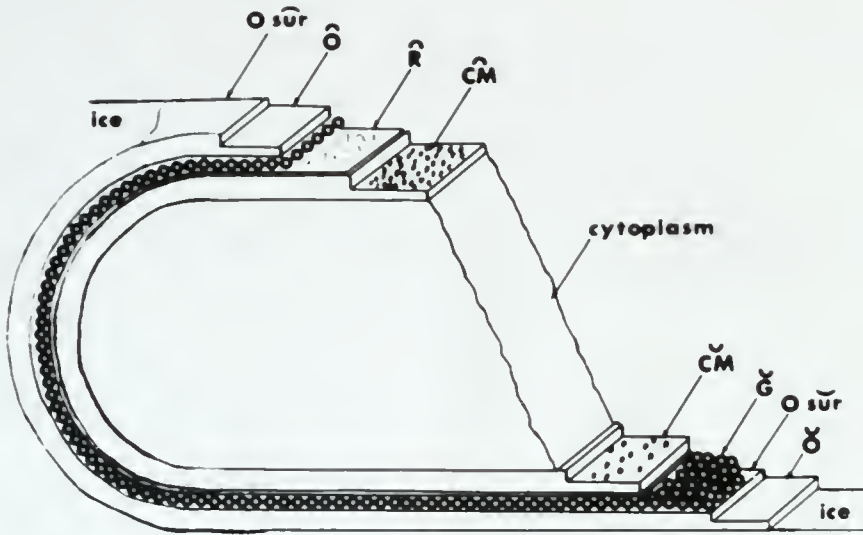
Van Gool and Nanninga (71) observed that the globular-rough fracture was probably located in the outer membrane since the rough face was separated from the cytoplasmic membrane by an intermediate layer which they believed was the rigid layer. Careful examination of their results indicates that this layer is actually the unfractured surface of the cytoplasmic membrane.

In the same study it was stated that no cell wall fracture plane was present in lysozyme produced spheroplasts. It seems unlikely that digestion of the peptidoglycan layer would eliminate the globular-rough fracture plane if it was located in the outer membrane. DeVoe et al. (17) reported a smooth fracture face in spheroplasts of a marine pseudomonad freeze-etched without glycerol. There was no cell wall fracture plane in cells which lacked the outer membrane.

It therefore appears that the majority of the results of this and other studies are compatible with our interpretation. From the freeze-etching work a model of the cell envelope of the marine vibrio can be proposed (Fig. 53). This illustrates the location of the fracture planes and relationship of the various faces.



Fig. 53. Diagrammatic representation of the cell envelope of the marine vibrio MW40 as determined by freeze-etching. The locations of the fracture planes are illustrated.



The cytoplasmic membrane fractures exposing particle-studded faces. The true surfaces of the membrane are only seen as edges. The particles are believed to represent proteins which are a part of the membrane (7). In bacteria there has been no direct evidence to substantiate this conclusion, and no one had demonstrated any relationship between the nature of the particles and the physiological state of the cells. Large particle-free areas were sometimes present on both fracture faces. Similar areas have been reported in other studies (44, 65). These patches were larger and more numerous in cells which were subjected to the minimum amount of handling before freezing. This indicates that these areas are normally present in actively growing cells and during storage the membrane rearranges.

The second fracture separated the cell wall exposing the rough convex face of the rigid layer and a concave face composed of globular subunits. In areas where the globular layer is incomplete a smooth surface is revealed which is probably the true inner surface of the outer membrane. This fracture may occur in a hydrophobic plane formed by the lipoprotein attached to the peptidoglycan and the protein layer superimposed on the inside of the outer membrane. Such a hydrophobic association was postulated by Schnaitman (60).

The organism used in this study does not have a covalently linked lipoprotein on the peptidoglycan, at least during exponential growth. It is not known whether the lipoprotein is entirely absent or present but not covalently linked. In either case, this may account for the fact that this fracture plane is not as prevalent as in E. coli or Pseudomonas. This fracture was not seen in a marine pseudomonad (17) which also lacks a lipoprotein (24).

dePetris (16) and Thornley and Glauert (69) demonstrated a globular layer between the rigid layer and the outer membrane, and found that this material was eliminated by digesting the cells with proteolytic enzymes. Fischman and Weinbaum (20) studied negatively stained E. coli cell walls and observed a regular hexagonal array of globular units. This array was removed by proteolytic enzymes. The subunits were 13 to 14 nm in diameter which is somewhat larger than the subunits seen in freeze-etching.

Schnaitman (58) examined the protein content of the cell wall of E. coli and found a major protein with a molecular weight of approximately 44,000. The size and amount of this protein would make it a likely prospect for the subunits of the globular layer. These proteins are quite hydrophobic and could be postulated to bind the rigid layer to the outer membrane. The proteins released from P. aeruginosa by EDTA extraction have been directly associated with the units seen in freeze-etching (26). These units are probably comparable to the globular subunits seen in this study.

In the marine pseudomonad (23) the entire outer membrane is removed by treatments which eliminate the magnesium ions from the cell envelope. In this procedure a protein is also released. These results are consistent with our interpretation of the work with P. aeruginosa and may indicate that one role of divalent cations is in binding the globular layer to the rigid layer. E. coli is not markedly affected by chelating agents, and this binding may be of less importance in this organism since it has a more continuous protein layer and a much larger number of lipoprotein molecules bound to its peptidoglycan (9).

The third fracture splits the outer membrane revealing smooth

fracture faces. It is thought that this membrane is very low in enzymatic activity and this may be reflected by the absence of particles on the fracture faces. The fracturing of the outer membrane also indicates that it is probably a lipid bilayer with a hydrophobic center as has been assumed.

The patches of cell wall material seen in some preparations were also reported by Bayer and Remsen (4). These patches may correspond to the areas of adhesion between the cell wall and the cytoplasmic membrane observed in E. coli (3).

The true outer surface of the outer membrane is exposed by etching. This surface is smooth or finely granular. In other studies a subunit structure was reported on the cell surface (4, 19, 26).

In summary, it appears that the technique of freeze-etching is a valuable tool in studying the structure of the cell envelope. Although further work is needed to clearly prove the location of the fracture planes and new techniques must be developed to correlate the chemical nature of the envelope components with the structures seen in freeze-etching, this study has demonstrated several important points. The location of the fracture plane in the cytoplasmic membrane and the nature of the faces was confirmed. The existence of a globular layer in the cell wall was confirmed and this layer was localized in the middle of the cell wall, probably forming a fracture plane with the rigid layer. It was shown that the outer membrane fractures and the fracture faces are free of particles. Although the work was not completely successful, it was found that the technique could be applied to isolated cell envelope components. And finally it was demonstrated that ferritin labelled antiserum could be used to localize

a component on the cell using freeze-etching. This technique may prove very useful in localizing other envelope components which are present in a less continuous layer on the cell surface.

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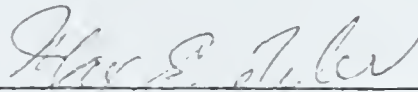
## BIOGRAPHICAL SKETCH

Jack Thornton Crawford was born August 7, 1945, in Hamilton, Ohio. He graduated from Hamilton Taft High School in June, 1963. The following fall he entered Bowling Green State University and graduated from the Ohio State University in June, 1967, with a Bachelor of Science degree in microbiology. In September, 1967, he entered the University of Florida and held a graduate teaching assistantship from September, 1967, until August, 1972, and a graduate teaching assistantship from September, 1972, to June, 1973.

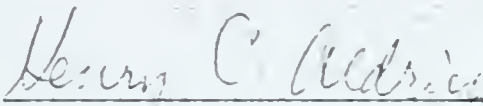
He is a member of the American Society for Microbiology and the Southeastern Branch of the American Society for Microbiology. At present, he is a candidate for the Ph.D. degree in microbiology.

Mr. Crawford is married to the former Kay Woodten and they have two sons - Brent and Gregory.

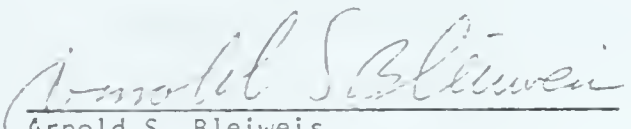
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Professor of Microbiology


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Henry C. Aldrich  
Associate Professor of Botany

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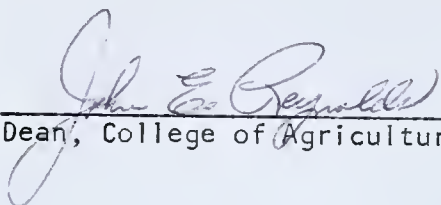
  
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Associate Professor of Microbiology

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December, 1973

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