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ULTRASTRUCTURE OF *VEILLONELLA* AND MORPHOLOGICAL CORRELATION OF AN OUTER MEMBRANE WITH PARTICLES ASSOCIATED WITH ENDOTOXIC ACTIVITY

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ABSTRACT

BLADEN, HOWARD A. (National Institute of Dental Research, Bethesda, Md.), AND STEPHAN E. MERGENHAGEN. Ultrastructure of *Veillonella* and morphological correlation of an outer membrane with particles associated with endotoxic activity. J. Bacteriol. **88**:1482-1492. 1964.—Normal, phenol-water extracted, and lysozyme-treated *Veillonella* cells were embedded in Vestopal W, sectioned, and examined by electron microscopy. Normal cells as well as the phenol-water extract (endotoxin) were examined by negative and positive contrast techniques. In thin sections of normal cells, three separate structural entities were observed surrounding the protoplasm, and were referred to as the outer membrane, the solid membrane, and the plasma membrane. The outer membrane was a membrane composed of two dense layers (30 A) separated by a less-dense layer (20 A), and followed a convoluted and continuous path around the cell. The solid membrane appeared as a taut, dense structure 100 to 500 A wide, and was separated from the outer membrane by up to several hundred Ångstroms. The plasma membrane was a unit-type membrane. After cells were treated with phenol-water, the outer membrane was absent, but the cells remained intact owing to the solid membrane. Observation of the phenol-water extract (endotoxin) revealed predominantly circular particles or discs which had approximately the same dimensions in height as the outer membrane had in width. Negatively stained whole cells showed similar structures on their surface. Lysozyme treatment of the cells did not affect the outer membrane; however, the solid membrane became diffuse and often disappeared, suggesting that the outer membrane and the solid membrane were separate structures.

The principal physical function of the cell wall or at least part of the cell wall is maintaining shape and rigidity of the organism (Work, 1961). Certain other properties, such as endotoxicity and O antigenic specificity, were ascribed to the cell wall of gram-negative bacteria. Chemically, cell walls of gram-negative bacteria were separated into an outer lipoprotein coat soluble in phenol separated by a less-dense layer from an inner mucopolypeptide layer termed the "R layer" (Weidel, Frank, and Martin, 1960), which appeared dense in the electron microscope. This latter layer contained lipopolysaccharide which was water-soluble after phenol extraction of cells, and was presumed to be the site of endotoxicity.

Mergenhagen, Hampp, and Scherp (1961) Mergenhagen, Zipkin, and Varah (1962), and Mergenhagen and Varah (1963) established that a phenol-water extract of *Veillonella* cells exhibited biological and immunological activities characteristic of endotoxins (O antigens), and that this water-soluble material was composed mainly of lipid and polysaccharide and was of high molecular weight. In an electron microscopic study of this endotoxin as well as of the ultrastructure of *Veillonella*, results were obtained which suggested a morphological correlation of the outer membrane of the cell with particles observed in phenol-water extracts (endotoxin). Further, when extracted with phenol, the outer unit-type membrane was removed and the cell retained its shape, owing to an inner solid membrane which was sensitive to lysozyme.

MATERIALS AND METHODS

Preparation of phenol-water extract (endotoxin) and residual cells after phenol treatment. A biologically active endotoxin-containing fraction was prepared from *Veillonella* (strain V2) by a slight modification of methods used previously (Mergenhagen et al., 1961, 1962). After phenol-water extraction of washed cells grown in 12 liters of a yeast extract-Trypticase-sodium lactate medium,

the aqueous phase was exhaustively dialyzed against distilled water, and the lipopolysaccharide-containing material was sedimented by centrifugation at 105,000 × *g* in a Spinco model L centrifuge. The sedimented material was pooled, redispersed in distilled water, and lyophilized. Residual cells after phenol-water extraction were collected, and were washed three times with distilled water before examination by electron microscopy.

Lysozyme treatment of Veillonella cells. Viable cells, grown in a yeast extract-Trypticase-sodium lactate medium for 24 hr, were washed once with phosphate buffer (pH 6.2; Difco), and were resuspended in 10 ml of this buffer containing 1 mg/ml of lysozyme (3 × crystalline; Nutritional Biochemicals Corp., Cleveland, Ohio). The reaction mixture was incubated in a water bath for 1 hr at 37 C. The cells were collected by centrifugation, washed once in distilled water, and immediately prepared for electron microscopy.

Electron microscopic procedures. Untreated *Veillonella* cells, as well as the phenol-water extracted and lysozyme-treated cells, were fixed and embedded in Vestopal W (Martin Jaeger Co., Geneva, Switzerland) according to the procedure described by Kellenberger, Ryter, and Séchaud (1958). Sections were cut with a LKB microtome and were stained with uranyl acetate for 1 to 2 hr.

Lyophilized phenol-water extracts (endotoxin), as well as whole *Veillonella* cells, were resuspended in distilled water, and were stained by placing a microdrop of the suspension on a Formvar-covered grid and withdrawing the surplus with a micropipette after 30 sec. Before the remaining suspension dried, staining solutions [2% phosphotungstic acid (PTA) adjusted to pH 5.3 with NaOH or 1% uranyl acetate adjusted to pH 5.0] were added to the grid, and the preparation was immediately blotted. Specimens were examined in a Siemens Elmiskop I electron microscope at plate magnifications of 40,000 to 120,000 diameters.

RESULTS AND DISCUSSION

General morphology of Veillonella (strain V2). The convoluted nature of the cell surface, as well as the diplococcal shape of the cell, were readily evident in negatively stained whole cells (Fig. 1). The exterior surface of the cell, when observed in this manner, appeared uneven, and was covered with numerous crooked, rod-shaped elements approximately 700 A wide. These elements were separated by dense lines which were undoubtedly areas of PTA deposition. Usually, one wide dense line which divided the cell into two was observed (Fig. 1). Occasionally, two dense lines representing the entire circular septum formation of the cell were seen when the cells were oblique to the beam (Fig. 5).

In thin sections, three separate structural entities surrounding the protoplasm of untreated cells were observed, and will be referred to as the outer membrane, the solid membrane, and the plasma membrane. The outer membrane appeared as two dense layers 30 A wide separated by a less-dense layer of 20 A (Fig. 2 and 3). These dimensions closely agree with those given by Kellenberger and Ryter (1958) for a structure which was located at the surface of *Escherichia coli*, and which they identified as the cell wall. Other gram-negative bacteria were shown to possess an outer membrane of similar dimensions, which was interpreted as the entire cell wall (Bladen and Waters, 1963) or part of the cell wall (Murray, 1963; Claus and Roth, 1964). The outer membrane did not appear rigid, but followed a continuous and extremely convoluted path around the periphery of the cell, although in a few cells it had fewer convolutions and was closer to the solid membrane. Frequently, circular structures of widely varying diameters were formed by the outer membrane at various places around the cell (Fig. 2). This outer membrane is rather similar to that described in *Spirillum serpens* as a unit-type membrane about 75 A overall, which followed a somewhat convoluted path around the periphery of the cell and was loosely disposed over a thin, taut, and dense layer (Murray, 1963).

The space between the outer membrane and the solid membrane, the next membrane inward, appeared to be of the same electron density as was the substrate surrounding the cell. Occasionally, parts of the outer membrane seemed to touch the solid membrane.

The solid membrane, situated between the outer membrane and the plasma membrane, appeared as a single dense layer which varied in thickness from 70 to 500 A (Fig. 2 and 4). The extremely thick (500 A) solid membrane was more prevalent in cells which lacked the outer membrane. Such cells were only rarely observed, perhaps suggesting that they were older. When 16-day-old cultures were examined, the outer-

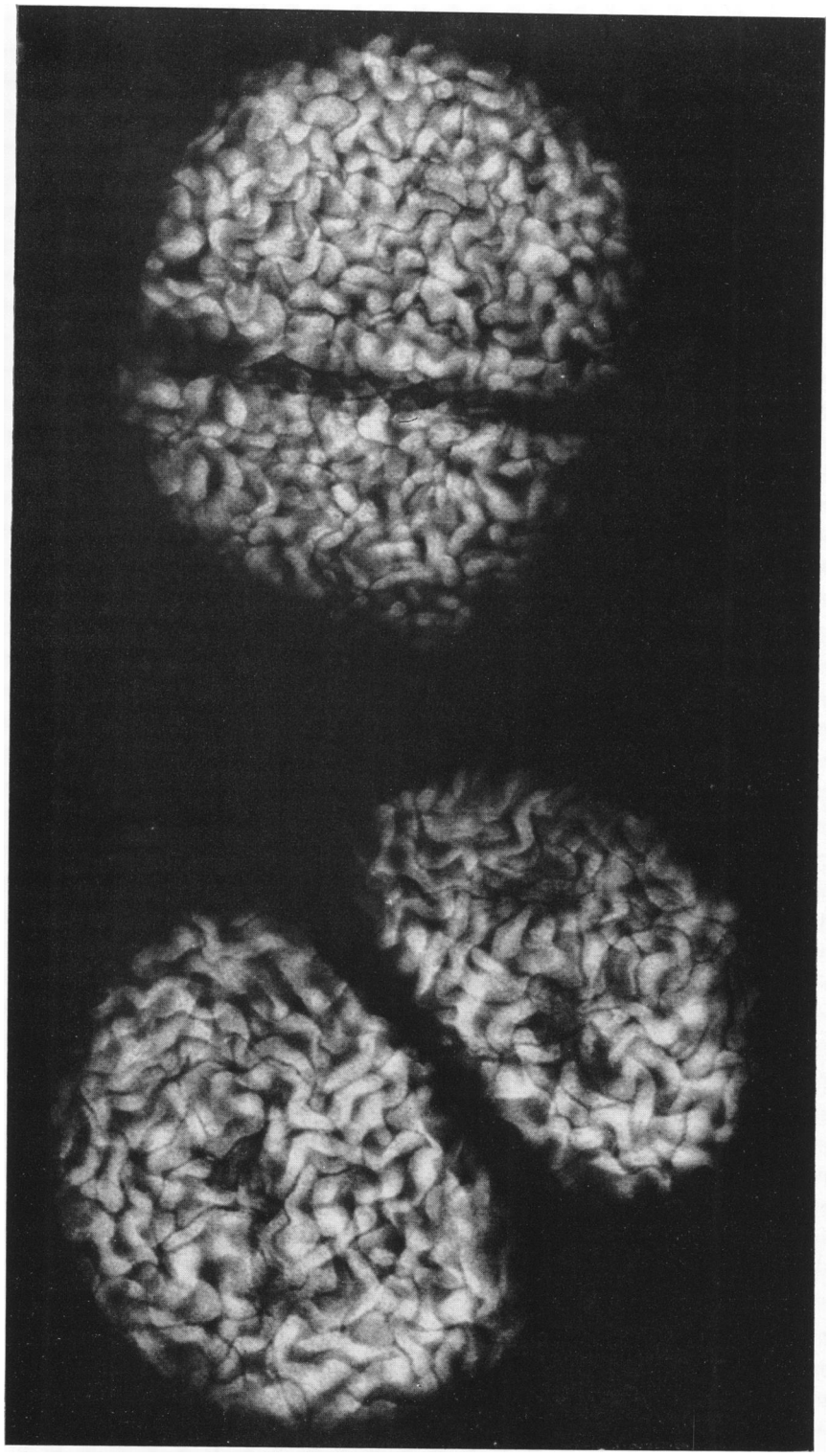


FIG. 1. *Extremely convoluted and uneven nature of the cell surface is evident when Veillonella cells are negatively stained with phosphotungstic acid. The diplococcal shape can easily be seen, and one half of the cell appears larger than the other. 104,000* ×.

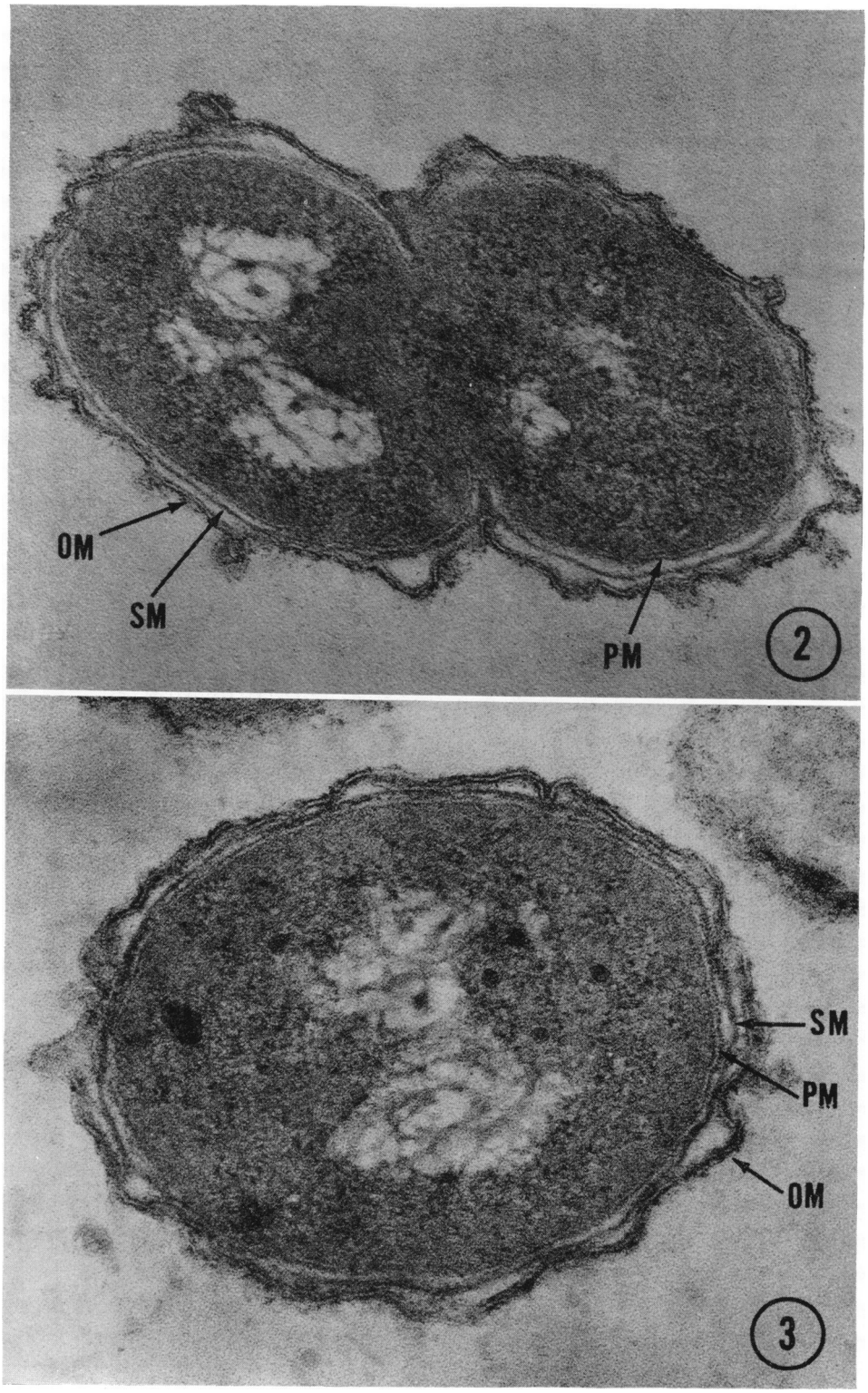
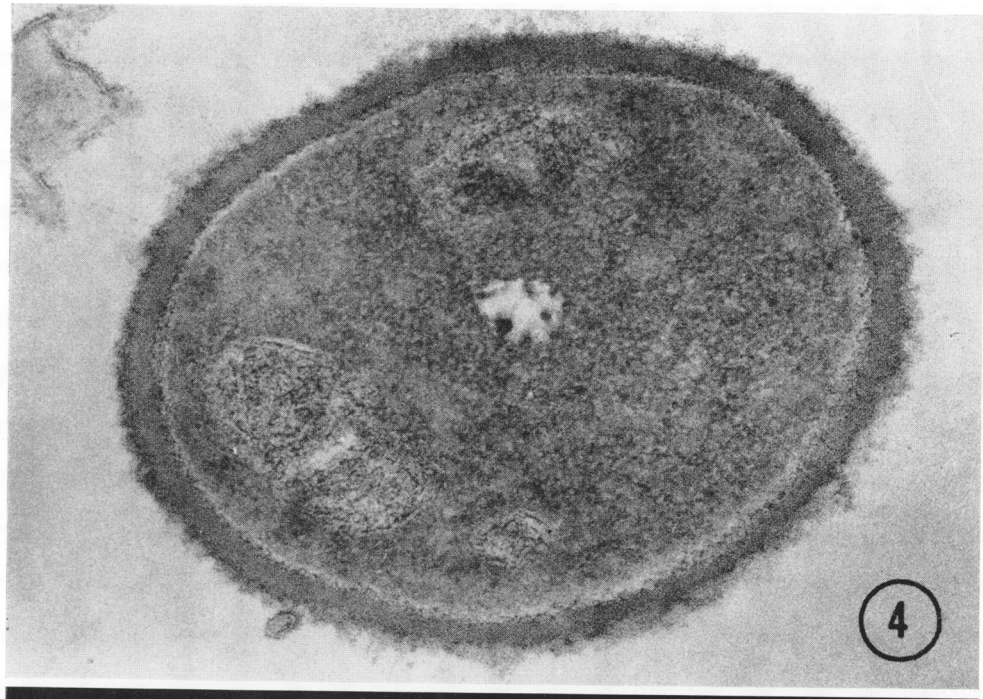


FIG. 2 and 3. *Three separate structures surround the cell: the outer membrane (OM), solid membrane (SM), and the plasma membrane (PM). The outer membrane, a unit membrane, appears extremely convoluted, whereas the solid membrane tightly follows the periphery of the protoplasmic constituents. Several circular structures are also evident in the outer membrane. 108,000 ×; 120,000 ×.*



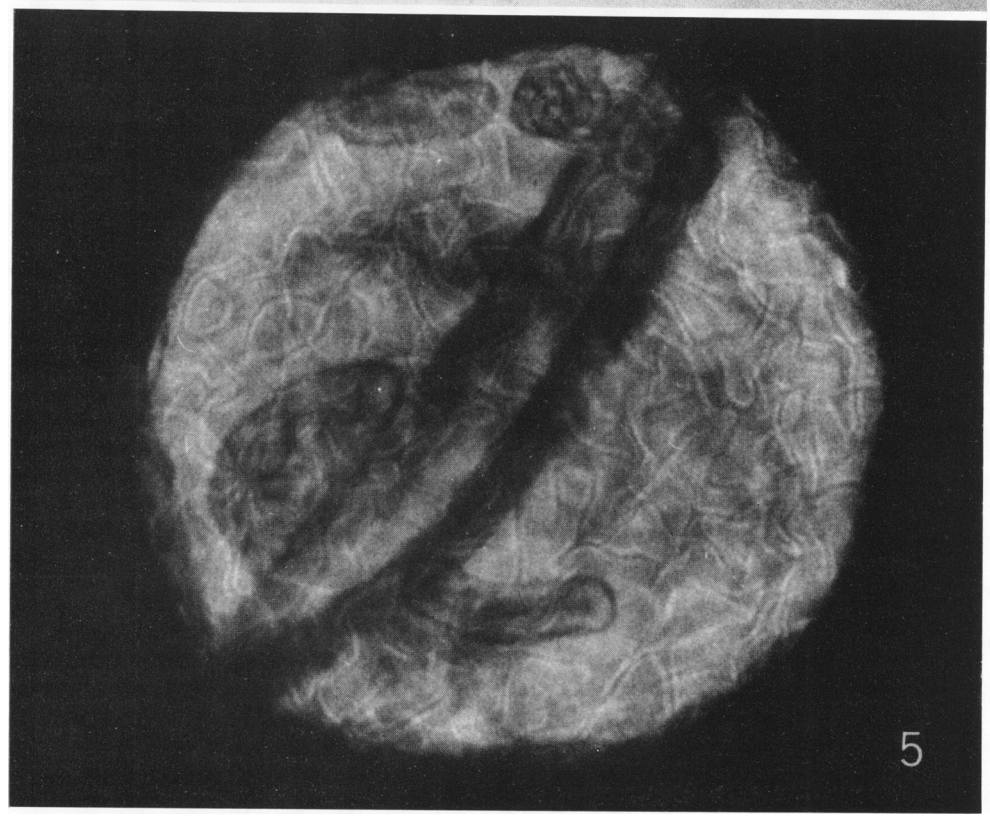


FIG. 4. *Cell which is devoid of the outer membrane. The solid membrane is extremely thick. 100,000 ×.*

FIG. 5. *Negatively stained whole cell demonstrating the visualization of intracytoplasmic membranous elements through a transparent cell surface. Both sides of cell septum formation are also seen. 137,000 ×.*

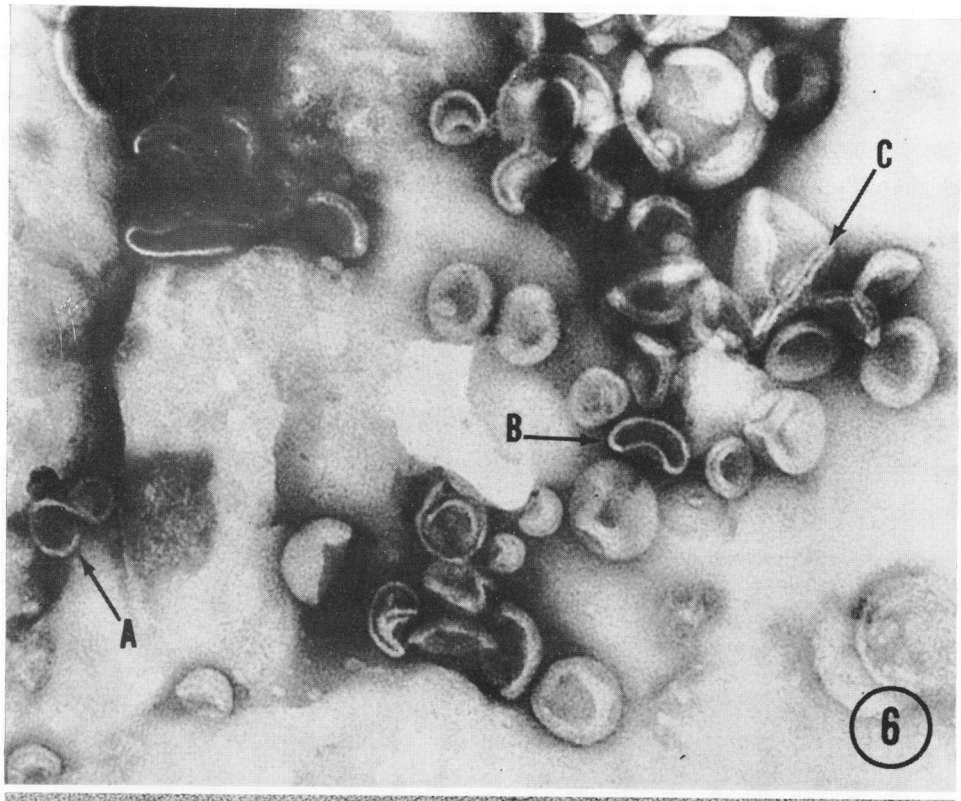
most structure of most cells appeared to be a thick solid membrane similar to that seen in Fig. 4. The solid membrane appeared to be a rigid structure which followed the periphery of the protoplasm rather than the convolution of the outer membrane. The solid membrane was usually separated from the plasma membrane by distances up to several hundred Ångstroms (Fig. 2 and 3). The area between the solid membrane and plasma membrane, and that between the solid membrane and outer membrane, appeared similar in density. A similar dense, taut structure was observed in *S. serpens* by Murray (1963), who regarded it as the innermost layer of the cell wall, and in *E. coli* by Murray (*personal communication*), and Bladen (*unpublished data*). The recent discovery of a solid dense layer in the area between the outer unit membrane, frequently described as whole or part of the cell wall, and the inner unit membrane, which is the plasma membrane, is undoubtedly due to better methodology.

The plasma membrane was most frequently seen as a unit-type membrane consisting of two dense layers (25 A) separated by a less-dense layer of 30 A (Fig. 2 and 3). Occasionally, cells which did not have this three-layered structure were observed; rather, the plasma membrane appeared to consist of an outer dense layer (25 A) separated from the cytoplasmic constituents by a space of 25 to 30 A. No structural connections between the plasma membrane and solid membrane were noted. The former occasionally appeared to be continuous with an intracytoplasmic membranous element, and it was observed that an individual cell had either several intracytoplasmic membranous elements or none. These elements were not found in any specific type of arrangement, but were seen as membranes throughout the cytoplasm of the cell. Structures similar in morphology to the intracytoplasmic membranous elements were also observed in some negatively stained whole cells (Fig. 5). This phenomenon of viewing intracytoplasmic membranous elements through an electron transparent bacterial cell wall was recently reported by Bladen, Nylen, and Fitzgerald (1964).

Morphology of particles present in the phenol-water extract of Veillonella cells. When negatively stained with PTA, the phenol-water extract (endotoxin) from *Veillonella* cells contained particles in a variety of shapes, predominantly circular, which ranged in diameter from 250 to 1,400 A (Fig. 6). Frequently, the particles had dense centers with less-dense borders (35 A). The dense centers did not have any specific structural arrangement, and no regular size was evident. Many particles appeared as twisted flat discs (Fig. 6, A) or had indented centers (Fig. 6, B). Occasionally, the twisted shapes gave one the opportunity to view what was probably the side surface of the particle (Fig. 6, C), which appeared as two electron-lucent layers 30 A wide separated by a dense area of the same width.

When positively stained with uranyl acetate, particles present in the phenol-water extract appeared somewhat different than when negatively stained with PTA (Fig. 7). The edges of particles in direct contact with adjacent ones were straight. Usually, the particle was surrounded by two dense layers approximately 15 to 20 A wide separated by a less-dense layer 40 A wide. Occasionally, the area contained within the inner circle was slightly more dense than that between the outer two dense layers. A third small circle was sometimes observed within this inner dense area. It was interesting to note the correspondence between the size of the particle border observed in the phenol-water extract and the dimensions of the outer membranes as seen in thin sections. Further, there was some resemblance in the general shape of the particles and the outer surface of the whole cell when both were negatively stained with PTA.

Morphology of Veillonella extracted with phenol-water. After treatment of cells with phenol-water, which extracted both protein (phenol layer) as well as lipopolysaccharide (water layer), the general shape of the cells was comparable to that of untreated cells. However, one major difference was noticed immediately. This was the absence of the outer membrane (Fig. 8) which was so evident in normal cells (Fig. 2 and 3). After phenol-water extraction, the outermost structure observed was a single dense layer which did not resemble the three-layered membrane previously observed, and which probably represented the solid membrane. The presence of a three-layered plasma membrane was rather difficult to discern, as was occasionally the solid membrane due to the presence of a very fine amorphous-appearing material present in all cells extracted with phenol-water. Except for the presence of this fine amorphous material, the protoplasmic constituents and



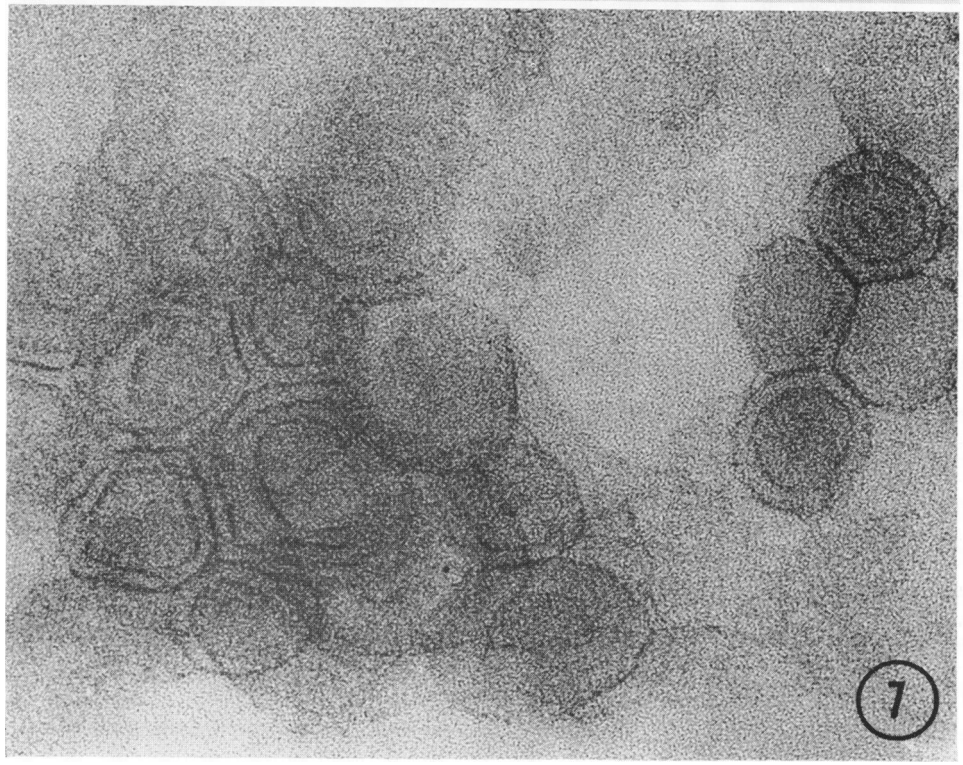
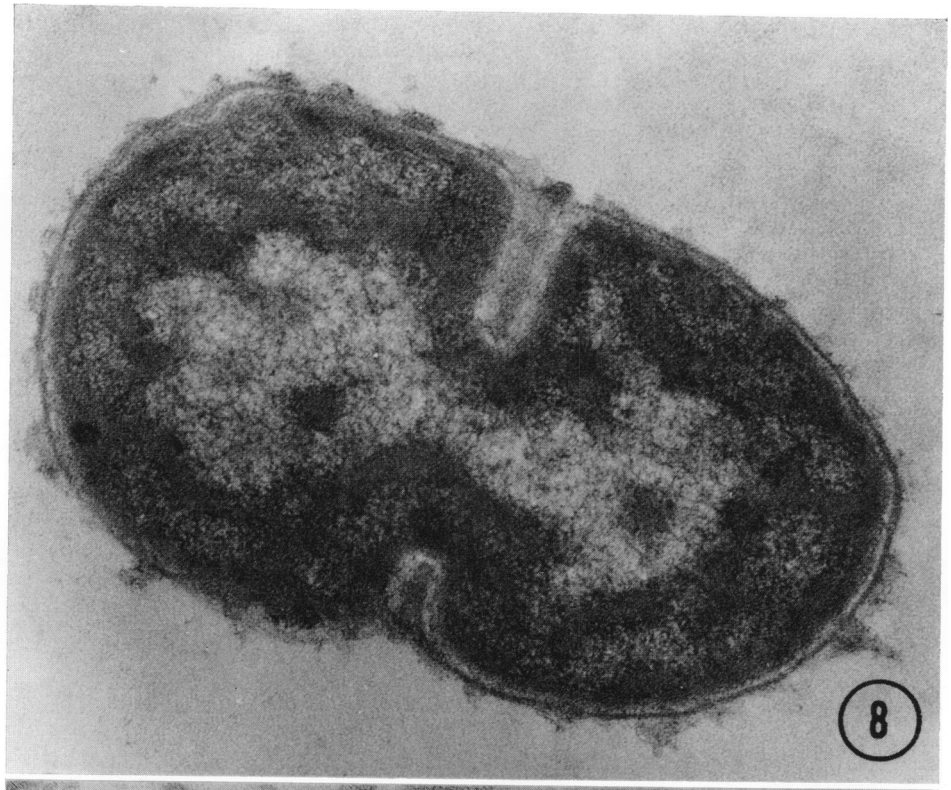


FIG. 6. *Electron micrograph of negatively stained particles seen in the phenol-water extract of Veillonella cells. Many of the particles appear to be flat discs, and some are twisted (A) or have indented centers (B). Some are twisted in such a manner as to show the side view of the particle (C). 174,000 ×.*

FIG. 7. *Positively stained particles seen in the phenol-water extract of Veillonella cells. The edges of these particles appear flat when in contact with adjacent ones. Two dense layers form the borders of the particles. Several particle centers seem more dense than the surrounding rim. 375,000 ×.*



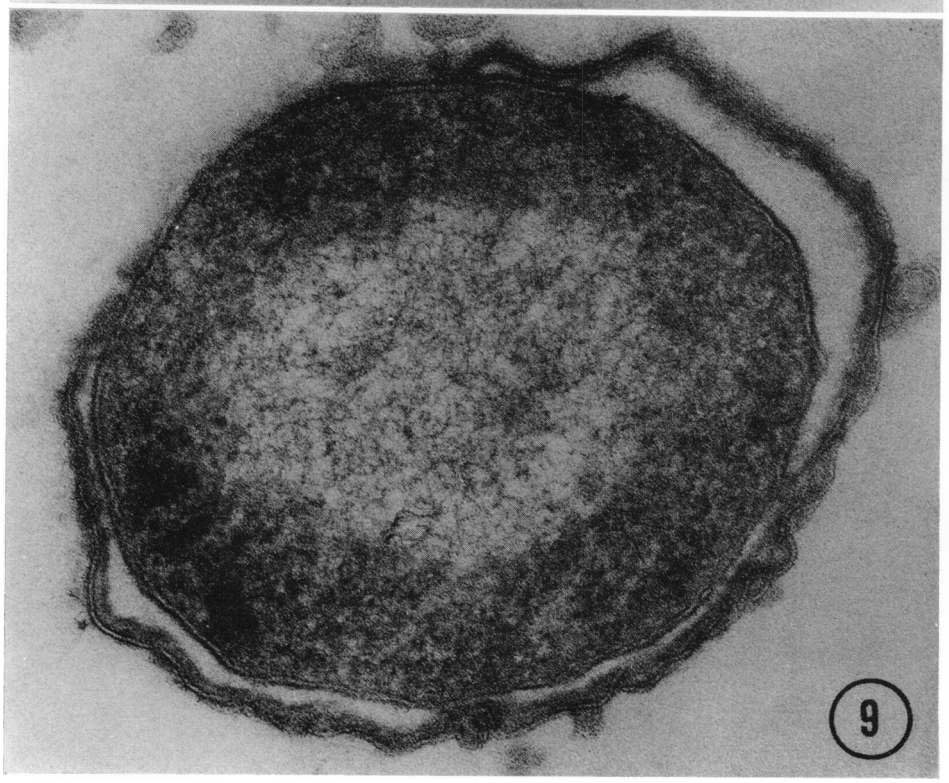


FIG. 8. *Electron micrograph of a Veillonella cell after phenol-water extraction. When compared with Fig. 2 and 3, it is evident that the outer membrane is absent. The solid membrane is now the outermost layer, and appears to be responsible for maintaining cell integrity. Except for a fine amorphous material, the cytoplasmic and nuclear material are normal. 124,000* ×.

FIG. 9. *After treatment with lysozyme, the cells become distended and frequently are round. The outermost membrane is the three-layered outer membrane. The solid membrane appears diffuse and is not taut. 120,000* ×.

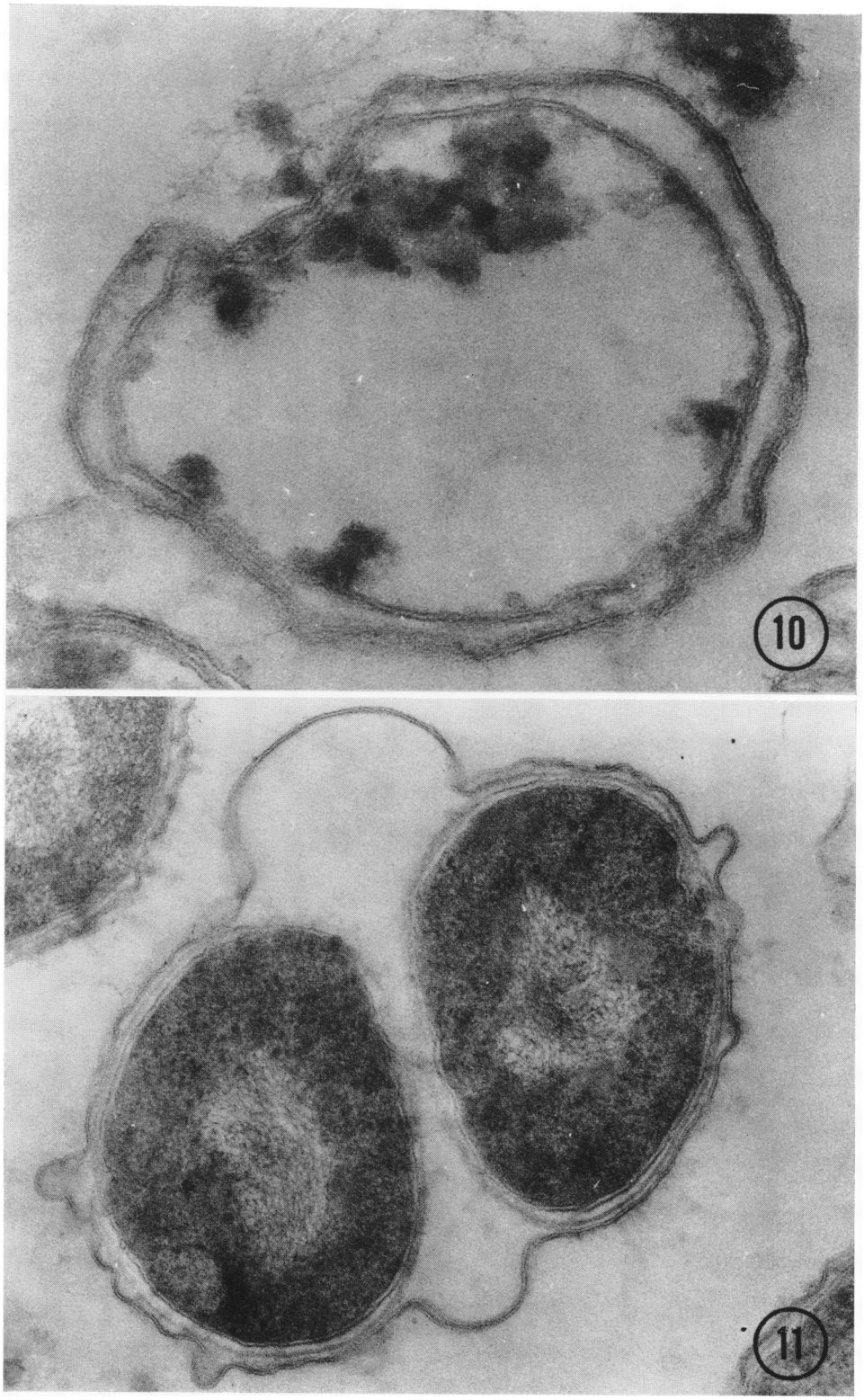


FIG. 10. *In cells which are greatly distended and grotesque after treatment with lysozyme, the solid membrane is very diffuse and is frequently absent, whereas the outer membrane and the plasma membrane appear normal. 120,000 ×.*

FIG. 11. *Cell which has been acted upon by lysozyme but which still appears relatively normal in morphology. The solid membrane does not appear as diffuse as those seen in Fig. 9 and 10. 82,000 ×.*

nuclear material appeared normal. The general preservation of the cells after phenol-water treatment was an unexpected result, because *E. coli* cells treated in a similar manner exhibited gross morphological changes. These results are of interest because it was presumed that, in endotoxin extracted from whole cells by the phenol-water method, protoplasmic contamination is likely to be extensive (Fukusi et al., 1964). This did not appear to be true with *Veillonella*, because the solid membrane was intact around the cell, maintaining its shape, and the protoplasmic constituents seemed reasonably normal; therefore, any gross protoplasmic contamination of the endotoxic extract is unlikely. Further, the apparent lack of effect of phenol on the solid membrane would suggest that it is not predominantly protein in nature.

Fragments of a multilayered membrane were evident around the periphery of the phenol-water treated cells, but in no case were these fragments continuous around the cell (Fig. 8). Several strands of these fragments appeared to be composed of five alternating dense and less-dense layers. Occasionally, the central dense layer was seen as two adjacent dense layers, suggesting that they were actually remaining parts of the outer membrane which adhered together along one side.

Morphology of lysozyme-treated cells. The presence of a three-layered convoluted outer membrane which was similar to bacterial structures previously identified as the cell wall, as well as the existence of a rigid solid membrane which, after removal of the outer membrane, appeared wholly responsible for cell integrity, make a distinction of the actual cell wall difficult. One characteristic of the cell wall is its susceptibility to lysozyme. Treatment of *Veillonella* cells with lysozyme revealed that the general shape of some cells became distended and round (Fig. 9). The outer three-layered membrane was evident around the periphery of most cells, and was considered analogous to the outer membrane observed in normal cells. The solid membrane was frequently missing but, when present, appeared diffuse or broken and, in many cases, adhered to the inner layer of the outer membrane rather than to the cytoplasmic membrane. The plasma membrane remained intact.

In most lysozyme-treated cells, the solid membrane was either completely lacking or very diffuse and broken, and the cells were greatly distorted (Fig. 10). When the solid membrane was absent, the protoplasmic constituents were usually aggregated in many small clumps, with most of the area contained within the plasma membrane empty (Fig. 10). It was interesting to note that in the occasional cell which had normally distributed protoplasmic constituents, as well as some sort of normal shape, the solid membrane was still largely present (Fig. 11).

Salton (1952) showed that the substrate for lysozyme was the cell wall itself, and reported that wall preparations from susceptible species were completely solubilized by lysozyme treatment. If this is true, one could interpret the action of lysozyme on the solid membrane, and not the outer membrane, as a demonstration that the former is actually the cell wall responsible for cell integrity and composed of mucopolypeptides, reported to be the site of lysozyme action. The outer membrane, then, could be just a loose covering structure. An alternative interpretation is that the solid membrane is actually the inner layer of a five-layered cell wall, the outer membrane being the outer layer. This interpretation would not preclude the mucopolypeptide composition or site of lysozyme action. However, the widely varying distances between these structures, as well as the possible nonrigidity of the outer membrane with no obvious connections to the solid dense layer, would suggest that the outer membrane is a separate entity from the solid dense line.

With regard to the foregoing observations, the following conclusions seem justified. The outer three-layered membrane is wholly or partly responsible for endotoxic activity and O antigenic specificity. The outer membrane does not function in maintaining cell integrity. The cell wall is actually a single, dense, taut membrane around the periphery of the cell. The outer three-layered membrane and the inner solid membrane are separable structures. Protoplasmic constituents are not removed during phenol-water extraction, because the solid membrane remains intact. Present concepts concerning the relationship of chemical information to the ultrastructure of various bacterial cell walls may require re-evaluation.

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