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Cell Envelope Morphology of Rumen Bacteria

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The cell walls of three species of rumen bacteria (*Bacteroides ruminicola*, *Bacteroides succinogenes*, and *Megasphaera elsdenii*) were studied by a variety of morphological methods. Although all the cells studied were gram-negative and had typical cytoplasmic membranes and outer membranes, great variation was observed in the thickness of their peptidoglycan layers. *Megasphaera elsdenii* evidenced a phenomenally thick peptidoglycan layer whose participation in septum formation was very clearly seen. All species studied have cell wall "coats" external to the outer membrane. The coat of *Bacteroides ruminicola* is composed of large (approximately 20 nm) globules that resemble the protein coats of other organisms, whereas the coat of *Bacteroides succinogenes* is a thin and irregular carbohydrate coat structure. *Megasphaera elsdenii* displays a very thick fibrillar carbohydrate coat that varies in thickness with the age of the cells. Because of the universality of extracellular coats among rumen bacteria we conclude that the production of these structures is a protective adaptation to life in this particular, highly competitive, environment.

The surface of the gram-negative bacterial cell has been shown to be extremely complex. In some species, the outermost layer is a "mosaic" of lipopolysaccharide (LPS), protein, and phospholipid (14), whereas other species possess structured layers outside of the LPS-protein-phospholipid complex. These external "coats" are highly variable both in morphology and in chemical composition. The best-understood globular protein coat is that of *Spirillum serpens* (29), whose subunit chemistry has been described by Buckmire and Murray (7, 8). Morphologically similar coats have been found in a number of organisms (32, 36, 37, 44), many of which live in an aqueous habitat. Similar globular protein coats have also been found on the cells of certain gram-positive organisms (34).

Various extracellular carbohydrate "coats" have been described for a number of gram-negative bacteria. There is, for example, the LPS capsule of *Salmonella typhimurium* (40), the colanic acids of the *Enterobacteriaceae* (23), the capsule of *Aerobacter aerogenes* (46), the capsule of *Chondrococcus columnaris* (35), the outermost layer of marine pseudomonad B-16 (19, 20), and the polysaccharide adhesion material of certain marine organisms (17). Because these carbohydrate "coats" show little affinity for osmium or for conventional electron microscopic strains, they must either be reacted with specific polysaccharide stains (17, 24, 28, 35, 41) or studied by topographical methods (16).

Although it has been noted that the loss of a superficial cell coat has little effect on the survival of the cells in pure culture (30), we suggest that these external cell wall layers may play a very important role in cellular survival by conditioning the ionic and molecular environment of both cell wall-bound enzymes and of the cytoplasmic membrane (13, 14). We have, therefore, studied the cell surface of bacteria from an environment in which both shifts in pH and ion concentration, and the presence of potentially toxic molecules, challenge the cells of the bacterial population.

MATERIALS AND METHODS

Bacteroides ruminicola subsp. *ruminicola* (strain 23), *Bacteroides succinogenes* (S 85), and *Megasphaera elsdenii* (B159) were generously provided by M. P. Bryant, University of Illinois, Urbana, Ill. (4, 6, 39). The anaerobic techniques used to cultivate these three organisms were essentially those of Hungate (25), as modified by Bryant and Burkey (3). The cultures were grown in 300 ml of a prereduced rumen fluid medium (5) in a 500-ml round-bottom flask at 39 C on a rotary shaker (75 rpm).

For freeze-etching, cells were either frozen directly after harvesting by centrifugation or after three washes in "medium salts" solution, which contained all of the salts at the same concentration used in the medium. The centrifuged cells produced a thick,

viscous pellet. Pellet samples were frozen in Freon 22 and freeze-etched in a Balzers BA 360M apparatus by the method of DeVoe et al. (16). All preparations were etched for 1 min.

For shadowing, cells were harvested by centrifugation for 10 min at 15,000 × *g* and suspended in distilled water. The resultant suspension was dried down on Formvar-coated grids and shadowed with platinum at an angle of approximately 30° in a Balzers BA 360M apparatus.

Osmium tetroxide was purchased as a 4% solution under argon from Polysciences Inc., Rydal, Pa. Glutaraldehyde was purchased as a 70% solution under argon from Ladd Industries, Burlington, Vt. Both of these solutions were used immediately after opening to minimize oxidation. Ruthenium red was obtained from B.D.H. Chemicals, Toronto, Ont. Vestopal W and propylene oxide were purchased from Polysciences Inc., Rydal, Pa. Other reagents were the best grade obtainable locally.

To assess the effects of staining with ruthenium red and to provide a comparison with other morphological studies, cells were fixed by the following two methods. (Agar enrobing [31] was not used in any of these preparations to avoid the addition of extraneous polysaccharides.)

Cells were fixed and embedded for electron microscopy by the addition of one volume of fixative (1.2% glutaraldehyde in 0.067 M cacodylate buffer at pH 7.3) to nine volumes of the culture, and the "prefixation" was continued for 10 min at 22 C. This suspension was centrifuged in a Sorvall RC-2B refrigerated centrifuge for 10 min at 15,000 × *g*, and the pellets were suspended in fixative solution for 2 h at 22 C. The cells were further centrifuged and washed three times (10 min) in cacodylate buffer (0.067 M, pH 7.3) before fixation for 1 h in 1.33% osmium tetroxide in the same buffer. After three additional washes in cacodylate buffer, the cells were dehydrated through a graded series of acetone solutions (30, 50, 70, 90, 95, 100%), dehydrated further in propylene oxide, and embedded in Vestopal W.

When acid polysaccharides were to be specifically stained, one volume of a 0.15% aqueous solution of ruthenium red was added to one volume of culture, and the mixture was allowed to stand for 30 min at 22 C. One volume of fixative solution (1.2% glutaraldehyde in 0.067 M cacodylate at pH 7.3) containing 0.05% ruthenium red was added to nine volumes of this mixture, and the suspension was prefixed for 10 min at 22 C. The prefixed cells were centrifuged, suspended in fixative solution containing 0.05% ruthenium red, and then washed, fixed with osmium tetroxide, washed again, dehydrated, and embedded as above.

Sections were cut by using either an MT-2 or an LKB III ultramicrotome and mounted on clean, uncoated 400-mesh copper grids. Unstained sections of each of the preparations were examined, in addition to sections stained with uranyl acetate and lead citrate (38). All preparations were examined by using an AEI EM-801 electron microscope at an accelerating voltage of 60 kV, and images were recorded on Kodak no. 4489 electron microscope film.

RESULTS

Cells of *B. ruminicola* accumulate large amounts of electron-light carbohydrate material (Cheng, Hironaka, and Costerton, unpublished data), and the cytoplasm of some cells (Fig. 1 and 2) is nearly filled by this substance. The cytoplasmic membrane of these cells appears normal ("C" in Fig. 1 and 2), and the electron-dense peptidoglycan layer is thick enough (approximately 4 nm) to be seen clearly where it is well separated from the cytoplasmic membrane ("P" in Fig. 1). The outer membrane of the cell wall is unexceptional in cross-section ("O" in Fig. 1 and 2) and it is separated by an electron-light zone, which varies between 9 and 20 nm, from an electron-dense coat structure ("T" in Fig. 1 and 2). Careful study of closely packed cells indicates that the coat layer ("T") is the outermost cell wall component, for the coats of adjoining cells are closely apposed over long distances (Fig. 1 and 2). In very thin sections the coat layer is seen to exhibit a rough periodicity in cross-section (Fig. 2). Comparison of Fig. 1 and 2 shows that this coat layer is not stained by ruthenium red.

The regular globular nature of this structure is evident in shadowed (Fig. 3) and in freeze-etched (Fig. 4) preparations in which sublimation (etching) has revealed a globular surface pattern ("G" in Fig. 4) that is seen most clearly if the cells are washed to remove materials that may form a eutectic (16). Cleavage planes occur within the outer membrane ("O") and within the cytoplasmic membrane ("C").

In embedded and sectioned preparations of cells of *B. succinogenes* the cytoplasmic membrane and outer membrane are clearly resolved (Fig. 5), whereas the peptidoglycan layer is too thin to be resolved with any degree of certainty (21). A layer of material on the outer aspect of the outer membrane is stained by ruthenium red (Fig. 5). While this material covers much of the cell's surface it varies considerably in thickness and does not display any definite periodicity or regularity.

The outer surface of these cells lacked any distinguishable structural features when seen by shadowing or exposed by sublimation of freeze-etched cells (Fig. 6 and 7). Both the outer membrane and the cytoplasmic membrane constitute strong cleavage planes ("O" and "C" in Fig. 6 and 7) within the envelope of cells of this species, and the former exposes a smooth surface whereas the latter exposes a surface in which protein "studs" are unusually concentrated in a small number of well-defined areas ("A" in Fig. 6 and 7).

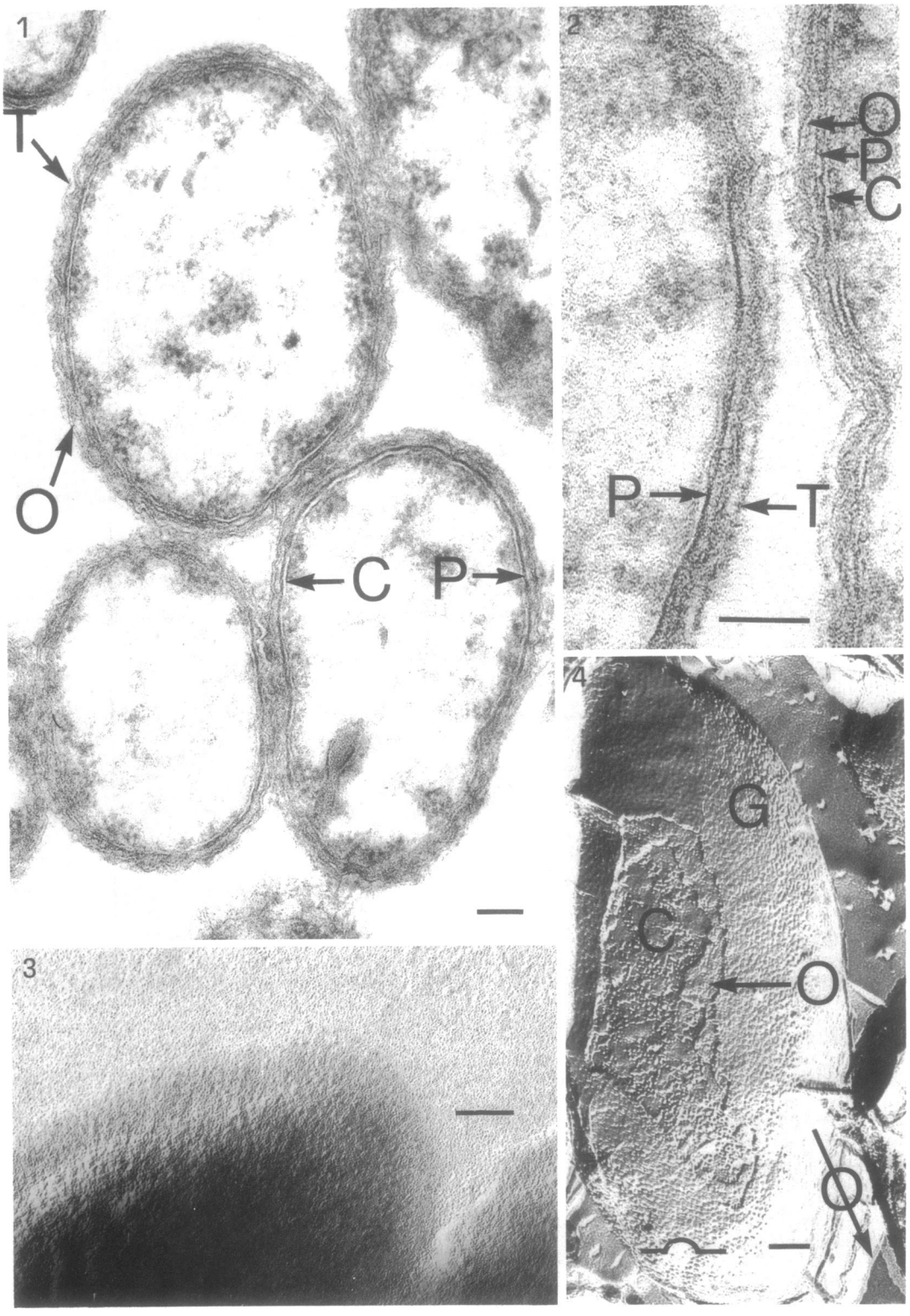


FIG. 1. *Electron micrograph of sectioned cells of Bacteroides ruminicola. Note the resolution of the component layers of the cell envelope, the close apposition of the coat layers of adjoining cells, and the presence of large amounts of electron-light cytoplasmic material. The bar in this and subsequent electron micrographs indicates 0.1 μm. Symbols used in this and the subsequent figures: A, concentration of granules in cytoplasmic membrane; C, cytoplasmic membrane; G, globular subunits; H, physiologically "young" cell; M, masses of intercellular fibers; O, outer membrane of cell wall; P, peptidoglycan layer; R, tangential section through coat layer; S, physiologically "old" cell; T, coat layer; ⌒, convex cleavage; and Ø, direction of shadow.*

Sectioned preparations of the cells of *Megasphaera elsdenii* show large cells with extensive cytoplasmic glycogen deposits (12). The cell envelope consists of a cytoplasmic membrane of normal proportions ("C"), an unusually thick (approximately 30 nm) peptidoglycan layer ("P"), a highly folded outer membrane ("O"), and a fibrillar coat layer ("T") which stains heavily with ruthenium red (Fig. 8–13). Some cells are surrounded by a thick (15 to 35 nm) homogeneous layer of electron-dense material, which has strands protruding from its outer edge (cell "H" in Fig. 8-13), whereas others have only protruding strands of electron-dense material at their surfaces (cells marked "S" in Fig. 8–12). These strands of electron-dense material extend up to 120 nm from the cells and often appear to connect adjacent cells (Fig. 8–12). Sections of cells stained with ruthenium red alone, which had not been subsequently stained with uranyl acetate or lead citrate, show that the cell coat material has a strong specific affinity for this stain (Fig. 13).

The outer membrane of these cells is highly folded (Fig. 8–12), whereas the very thick peptidoglycan layer follows an even contour, except where it forms a wedge-shaped thickening when septum formation is being initiated (1 in Fig. 8 and 2 in Fig. 11). The developing septum extends inward (3 in Fig. 9) until a complete septum is formed (4 in Fig. 12) to partition the daughter cells. The cytoplasmic membrane is very closely apposed to the inner surface of the peptidoglycan layer (Fig. 8–11) and follows the same smooth contour. The relative positions of these cell envelope structures is very evident at the high magnifications of Fig. 10 and 11.

Shadowed preparations of cells of *M. elsdenii* show the presence of a fibrillar component at the cell surface (Fig. 14), and freeze-etched preparations indicate that the cells are covered by these fibrous strands (Fig. 15–18). The strands occur on the cell surface, where they are exposed by sublimation, and they appear to form tangled eutectic-covered masses ("M" in Fig. 15) between the cells, which may correspond to the intercellular connections seen in sectioned material (Fig. 8–11). The cells used in this freeze-etched preparation had been washed free of medium components by three 10-min washes in "medium salts" solution to minimize eutectic formation (16). The cell envelope of cells of this species contains two cleavage planes, and that of the outer membrane is stronger than that seen in most gram-negative bacteria. The surface exposed by this plane is heavily and very evenly studded with very small (<5 nm) particles ("O" in Fig. 15–18). This finely studded pattern is also seen at outer aspects of the outer membrane in areas where the eutectic is sufficiently thin to allow its resolution (Fig. 15–18). The surface exposed by the cleavage plane within the cytoplasmic membrane of these cells is relatively heavily studded (2, 16, 22) with spherical and rod-shaped particles (Fig. 15–18) of the size (5 to 12 nm) usually seen at this level of the cell envelope of gram-negative bacteria.

The loci of septum formation are especially prominent in cells of this species. A well-defined furrow can be seen at the cell surface and in the outer membrane cleavage plane (Fig. 15, 17, and 18) at the point where the septum is being initiated. The cytoplasmic membrane is also indented by septum formation, to an extent that depends on the depth to which the septum has penetrated into the protoplast. The outer membrane and the cytoplasmic membrane are furrowed to a similar extent in the early stages of septum formation (1 in Fig. 18), but then the latter becomes more indented (2 in Fig. 17), and the difference is accounted for by the clear thickening of the zone between the cleavage planes (* in Fig. 17), which results from the thickened peptidoglycan layer. At a later stage the cytoplasmic membrane is indented to a much greater extent than the outer membrane ("3" in Fig. 18), and the wedge-shaped thickening of the peptidoglycan layer is clearly seen in the space between the cleavage planes (* in Fig. 18). Many cells are seen in which two septa are developing at the same time (Fig. 11 and 18).

DISCUSSION

The cells of the three pure cultures used in this study (*B. ruminicola*, *B. succinogenes*, and *M. elsdenii*) all accumulate an electron-light material in discrete deposits in their cytoplasm. This material has been identified as glycogen in

FIG. 2. *Electron micrograph of a section of cells of Bacteroides ruminicola that were stained with ruthenium red. Note the resolution of the constituent layers of the cell envelope and the regular periodicity of the globular components of the coat layer (T).*

FIG. 3. *Shadowed preparation of cells of Bacteroides ruminicola showing the array of globular subunits at the surface of the cells.*

FIG. 4. *Freeze-etched preparation of washed cells of Bacteroides ruminicola showing the globular subunits (G) at the cell surface that is exposed by etching, the cleavage plane within the outer membrane (O), and the particle-studded cleavage plane of the cytoplasmic membrane (C).*

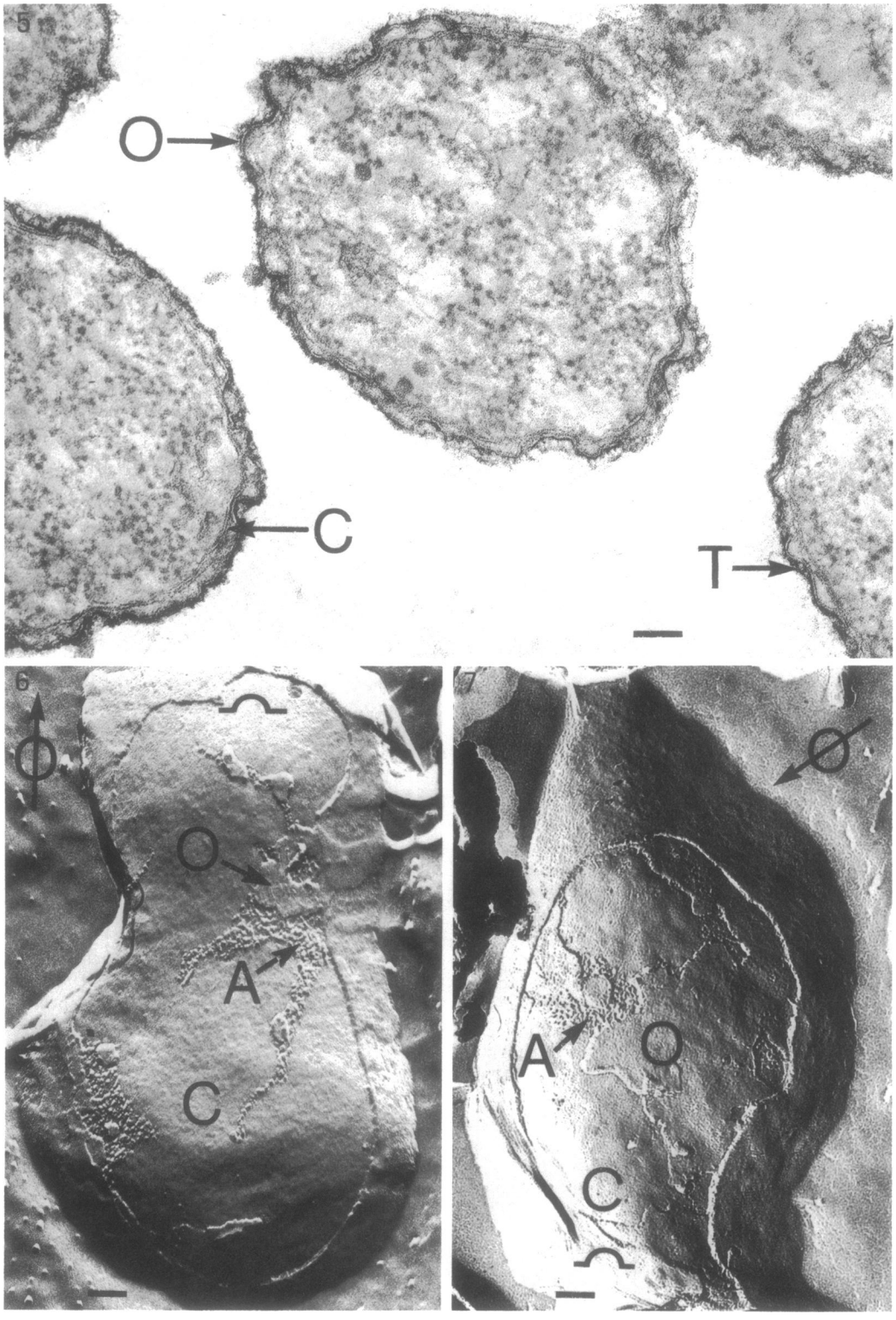


FIG. 5. *Electron micrograph of a section of cells of Bacteroides succinogenes that were stained with ruthenium red. Note the resolution of the cytoplasmic membrane (C) and the outer membrane (O) and the presence of a thin and irregular layer of electron-dense material external to the latter.*

FIG. 6 and 7. *Freeze-etched preparations of washed cells of Bacteroides succinogenes showing the smooth cell surface that is exposed by sublimation, the smooth cleavage plane of the outer membrane (O), and the sparsely particle-studded cleavage plane of the cytoplasmic membrane (C). Note that the particles in this latter cleavage plane are concentrated in certain relatively small areas (A).*

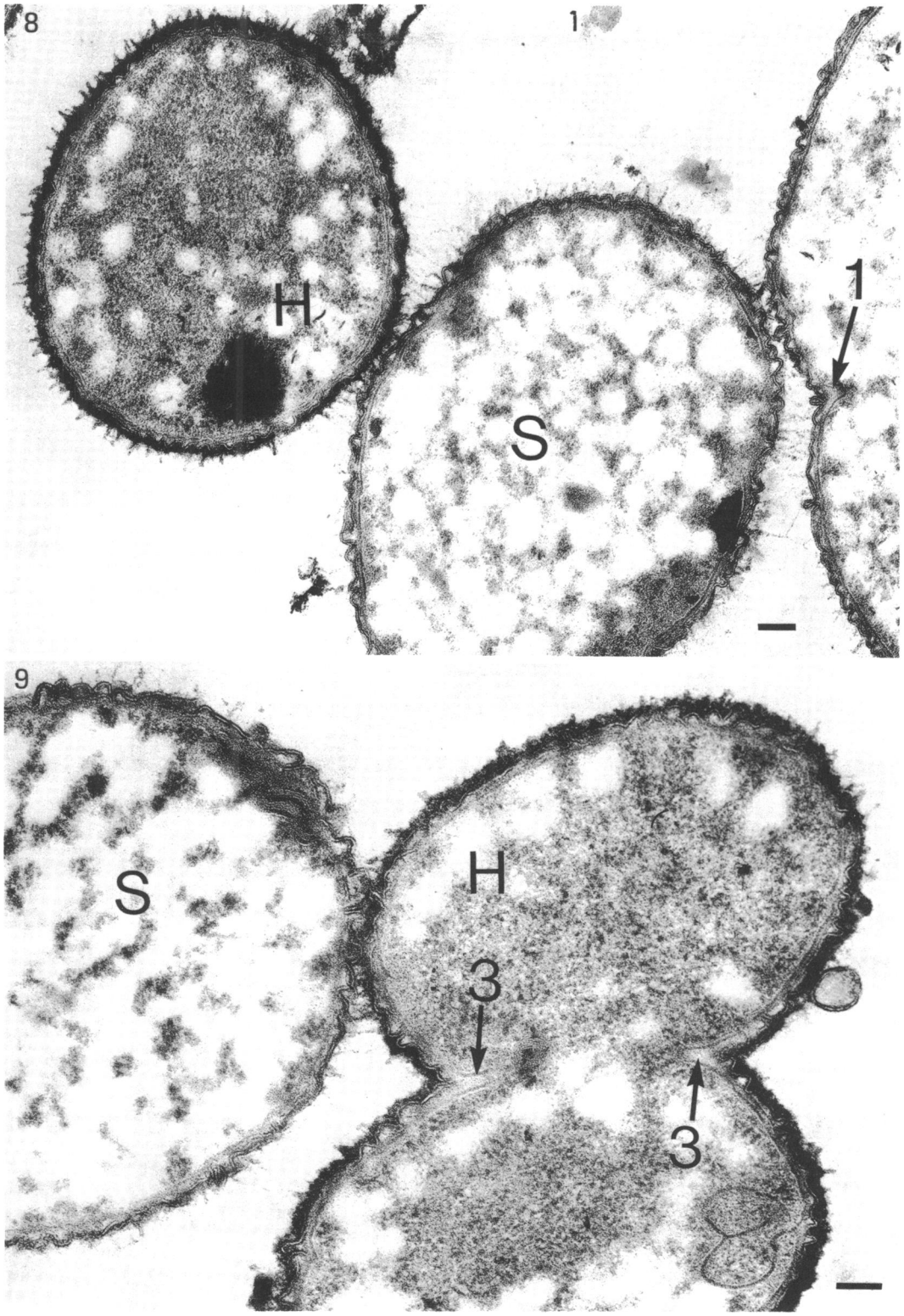


FIG. 8 and 9. *Electron micrographs of cells of Megasphaera elsdenii stained with ruthenium red. Note the partially complete septum no. 3 in Fig. 9, the striking differences between the coat layers of young (H) and old (S) cells, and the apparent intercellular adhesions formed by the coats of adjoining cells.*

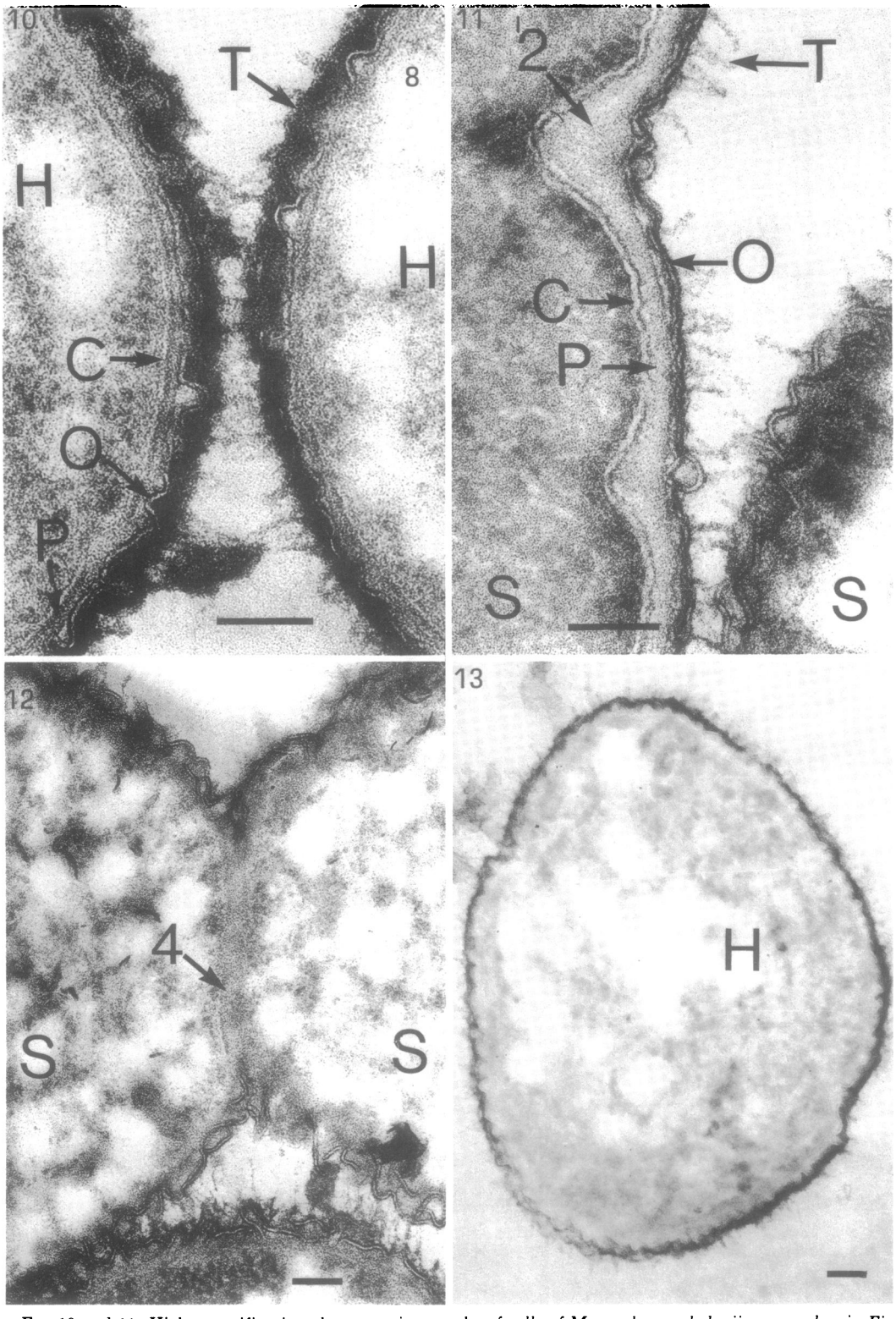


FIG. 10 and 11. *High-magnification electron micrographs of cells of Megasphaera elsdenii prepared as in Fig. 8 and 9. Note the clear resolution of the cytoplasmic membrane (C), the peptidoglycan layer (P), which is exceptionally thick in the cell in Fig. 11, the sites of septum initiation no. 2 in the cell in Fig. 11, the clearly resolved outer membrane (O), and the different coat layers (T) of the young and old cell types.*

FIG. 12. *Electron micrograph of cells of Megasphaera elsdenii prepared as in Fig. 8 and 9, showing a completed septum dividing two daughter cells.*

FIG. 13. *Electron micrograph of a cell of Megasphaera elsdenii prepared as in Fig. 8 and 9, except that neither uranium nor lead staining of section was performed. The membranes of the cell envelope are not clearly resolved, but the coat layer is intensely stained by ruthenium red.*

the case of *M. elsdenii* (12), and its deposition has been shown to increase with increasing physiological age of the cells of this species. Some of the cells of the mixed bacterial population of the rumen also contain these cytoplasmic inclusions, and the number of such cells has been shown to increase with the use of fine-particle-size concentrate feed (12). Cheng et al. have suggested that lysis of these bacteria may contribute to the high carbohydrate levels and high viscosity of the rumen fluid (11) seen in cows that "bloat" because of this feeding regime.

The ctyoplasmic membranes of cells of the three pure cultures studied show no unusual features when viewed in profile in sectioned material. Freeze-etching has shown that the distribution of protein studs in the cytoplasmic membrane of *B. ruminicola* is similar to that seen in most bacteria (2, 16, 22, 36, 43), whereas the protein studs in *B. succinogenes* are sparse and show extreme concentration in certain loci, and those of *M. elsdenii* are closely packed and virtually continuous.

The peptidoglycan-lipoprotein complex lies immediately outside the cytoplasmic membrane of gram-negative cells (14), and this complex is rendered electron-dense by conventional staining for electron microscopy (31). This inelastic cell wall layer is partially responsible for the shape of the bacterial cell (19, 20, 21), but its thickness varies considerably among different organisms. In the marine pseudomonad B16 it comprises only 1.2% of the cell wall and is so thin that it is only visible when separated from other cell wall components (21), whereas in *Escherichia coli* this layer comprises 2.0% of the cell mass (45) and is seen as an electron-dense layer 2 to 3 nm thick (31) in sectioned preparations. The peptidoglycan layer of the rumen bacteria studied here varies from the very thin structure of *B. succinogenes* to the approximately 4-nm layer of *B. ruminicola* and the exceptionally thick (approximately 30 nm) layer of *M. elsdenii*.

Murray et al. (31, 42) have shown that gram-negative bacteria divide by septum formation and that the centripetal growth of the peptidoglycan layer produces this structure which divides the daughter cells. Because of their phenomenally thick peptidoglycan layer, cells of *M. elsdenii* are excellent material for the examination of the process of septum formation, and thick septa can be clearly seen in all stages of cell division. In this respect those cells resemble the trichome-forming *Simonsiella* (31) and *Alysiella* (27), and *M. elsdenii* also resembles these organisms in that the elastic (15) outer layers of the cell wall take no significant part in the centripetal growth of the septum. Freeze-etching has shown this developing septum directly, and its location is reflected by a "furrowing" at the cell surface in *M. elsdenii*.

The outer membrane of the rumen bacteria studied here in sectioned material is seen to be folded to different extents in different species, but freeze-etching shows a flat profile for this layer of the cell wall, and we must conclude that

FIG. 14. *Shadowed preparation of cells of Megasphaera elsdenii showing the fibrous nature of the external coat material.*

this folding is an artifact of fixation and/or of shrinkage upon embedding (2). This layer is a complex of phospholipid, protein, and LPS (15), and it cleaves in its hydrophobic median zone upon freeze-etching (16, 18). The cleavage plane in the outer membrane of the cell wall of both *B. ruminicola* and *B. succinogenes* is similar to that of most gram-negative bacteria, but that of *M. elsdenii* is covered by very small (<5 nm), globular particles that resemble the spherical subunits of the outer membrane of the cell wall of *Pseudomonas aeruginosa* seen by Gilleland et al. (22). This globular pattern is also seen at the outer aspect of the outer membrane, which is revealed by sublimation (etching), when eutectic formation has been prevented. This suggests that the outer membrane of the cell walls of cells of this species has a hydrophobic median zone sufficiently well defined to constitute a cleavage plane (16, 18), but that discrete globular elements are also present in the median zone and that these structures are sufficiently large to be seen at the outer aspect of this layer. The chemical nature of these globular

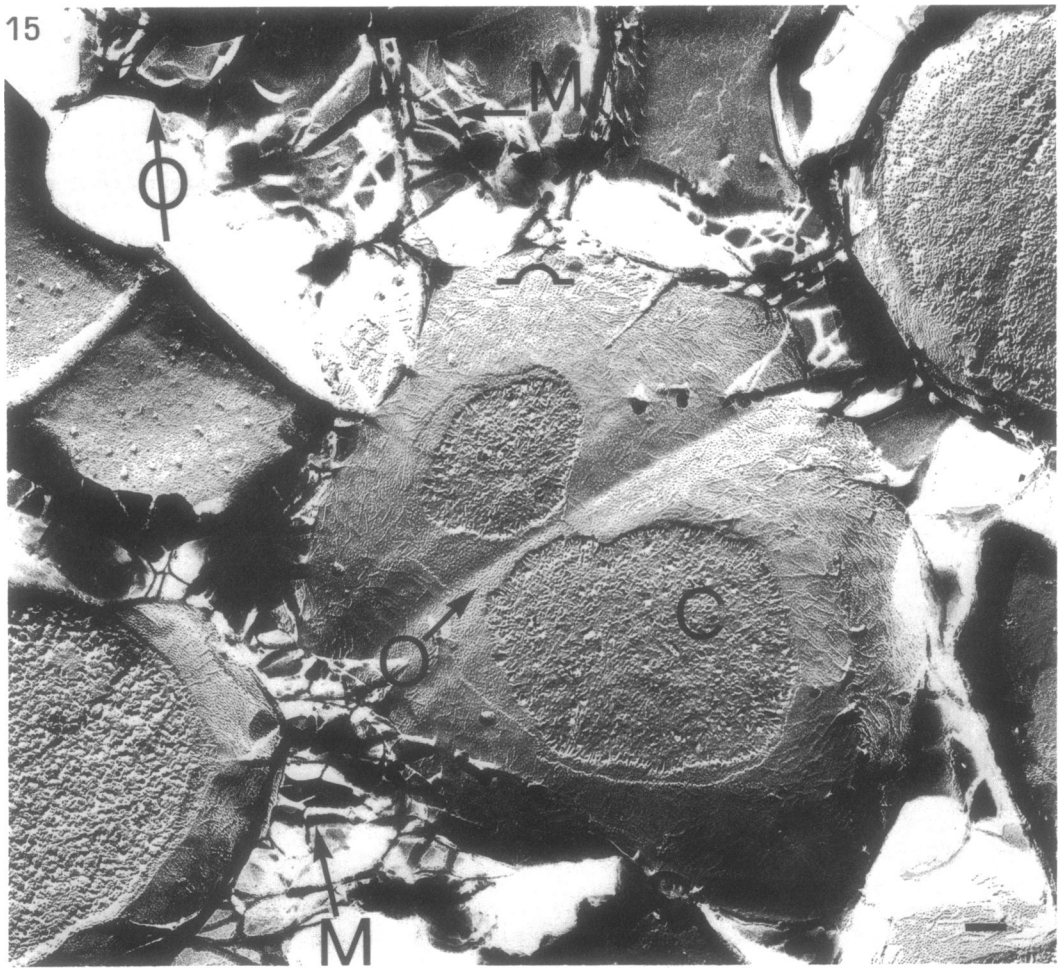
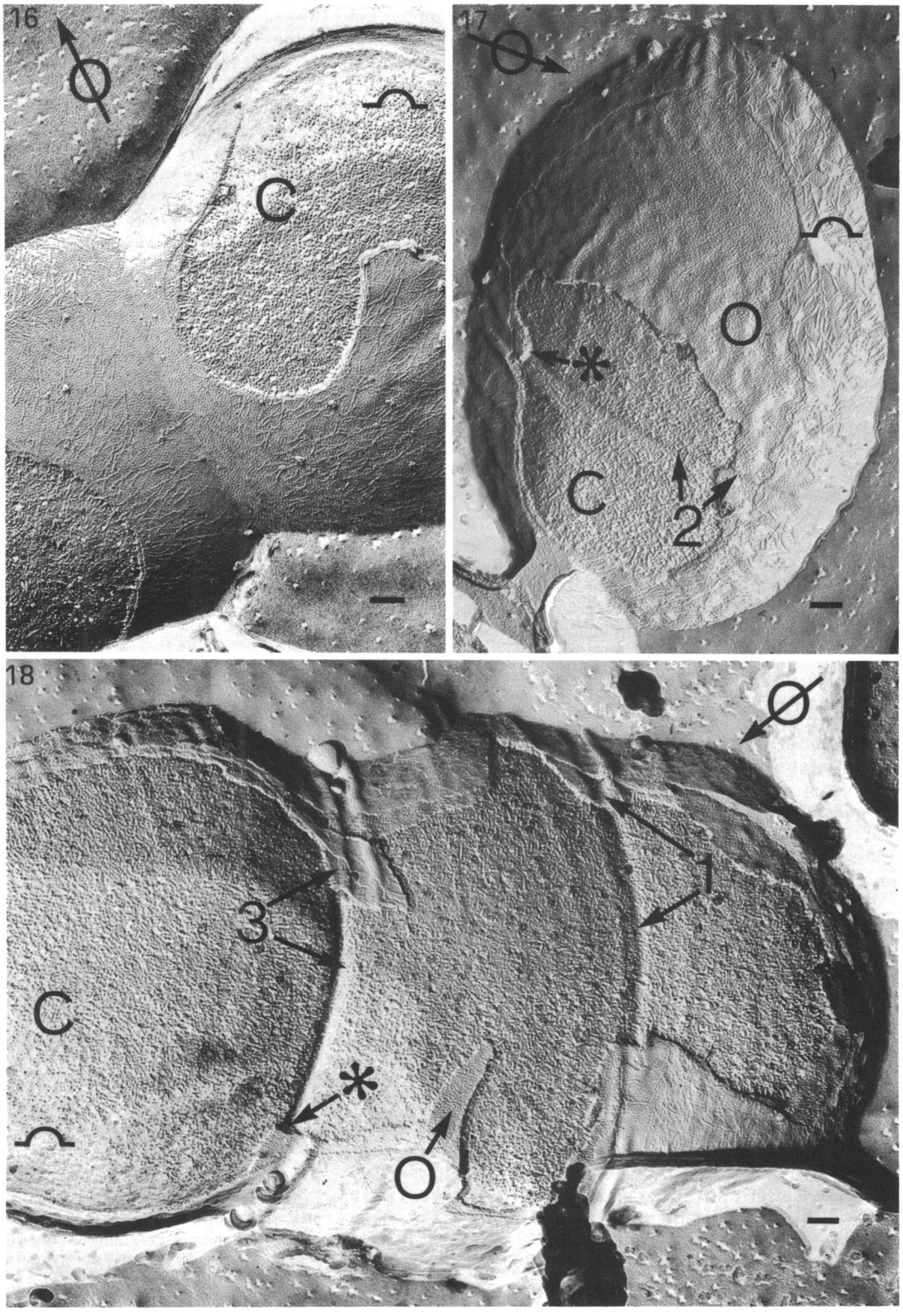


FIG. 15–18. *Freeze-etched preparations of cells of Megasphaera elsdenii showing the fibrous nature of coat material at the cell surface, which is exposed by sublimation. In Fig. 15 tangled intercellular masses (M) of these fibers can be seen. The surface exposed by the cleavage plane of the outer membrane (O) is covered by very small (<5 nm), tightly packed, spherical particles. The cytoplasmic membrane cleavage plane (C) is heavily studded with larger particles. The site of septum formation is revealed by a furrow at the cell surface and, in the early stages of septum formation (no. 1), the cleavage planes of the outer membrane and the cytoplasmic membrane are indented to the same extent. In later stages of septum formation (no. 2 and 3), the cleavage plane of the cytoplasmic membrane is much more deeply indented than that of the outer membrane, and the developing septum is seen as a thick wedge of material (*) between these two cleavage planes.*



FIGS. 16–18

elements is currently under investigation in our laboratories.

The cells of each of the three species studied here in pure culture, and most of the mixed bacteria in samples of rumen contents, have additional cell wall layers outside of the outer membrane. *B. ruminicola* was seen to have a highly ordered globular surface layer morphologically identical with the protein coats of other gram-negative (7, 8, 32, 36) and gram-positive (34) bacteria. Both topographical preparations and sectioned material show that this layer is indeed the outermost structure of the cell envelope, and its failure to react with ruthenium red shows that it is not composed of carbohydrate (28). This observation may be of some taxonomic importance because the cells of *Bacteroides succinogenes* have a carbohydrate coat.

Ruthenium red has been shown to be a specific stain for mucopolysaccharides and polysaccharides (28), for hyaluronic acid (24), and for glycoproteins and proteoglycans (33), and it has been used to stain the surface structures of mycobacteria (35), of various aquatic bacteria (17), and of *Diplococcus pneumoniae* and *Klebsiella pneumoniae* (41). Among the rumen bacteria studied here, cells of *B. succinogenes* had thin, irregular, and unstructured accretions of ruthenium red-positive material at their surface, whereas cells of *M. elsdenii* were surrounded by thick fibrillar masses of this material. A previous study (12) has given us the means of assessing the physiological age of a given cell of this species by reference to cytoplasmic glycogen deposits, and it is clear that younger cells have the thickest carbohydrate coat, and that the coat consists only of protruding strands at the surface of older cells.

Recent work in several areas has made it clear that the physiology and morphology of cells in pure batch culture do not always accurately reflect that of cells of the same strain in mixed populations in a natural environment that has more of the characteristics of a continuous culture. Bae et al. (1) have examined bacterial cells directly from soil and have found that most of these cells are surrounded by thick capsules, and Fletcher and Floodgate (17) and Jones et al. (26) have shown that many aquatic bacteria are surrounded by an acidic polysaccharide capsule, which mediates their adhesion to solid surfaces. Kaiser and Starzyk (27) have shown that cells of *Alysiella* adhere to the oral epithelium by means of a capsular structure. Shands has shown (40) that pathogenic cells of *Salmonella typhimurium* are surrounded by a very thick capsule within the tissue of an infected mouse, and these current observations indicate that most bovine rumen bacteria have both additional surface layers and extensive capsules.

We have suggested (13, 14) that the cell walls of gram-negative bacteria protect these organisms from ionic effects by acting as an ion-exchange material and from toxic molecules by the provision of a selective molecular sieve, and that the cell wall-bound enzymes (9) of these cells are protected from low pH denaturation in the culture by the protective effect of the cell wall (10). Because all of the rumen bacteria examined in this study have thick and complex cell walls, this may represent an adaptation to their variable and challenging environment.

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