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Ultrastructure and Adhesion Properties of *Ruminococcus albus*

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Morphological studies have shown that cells of the anaerobic rumen bacterium *Ruminococcus albus* have electron-translucent granules of reserve carbohydrate in their cytoplasm, and that they have a polysaccharide "coat" layer external to their gram-negative cell wall. This coat layer, which stains specifically with ruthenium red, forms a compact mat of fibers adjacent to the cell, and fibrous elements also project as much as 0.6 μm from the cells. These radial fibers are clearly visualized by freeze-etching, and can be seen to extend throughout the extensive intercellular space in centrifuged pellets of these bacteria. Cells of *R. albus* adhere to cellulose fibers added to the culture medium, and the coat material is seen to mediate this adhesion in addition to its function in the general protection of these cells.

Isolates from the predominantly gram-negative bacterial populations of marine (11, 29) and freshwater (15) environments, soils (2, 3), infected tissue (25), and the bovine rumen (8, 9) often have extracellular coats external to the outer membrane. These coats are either composed of fibrous carbohydrate (2, 8, 11, 15, 25) or of highly ordered globular protein subunits (2, 8, 29); the former vary in thickness between 10 nm (29) and 1.2 μm (unpublished data) whereas the latter may be from 7 nm thick (8) in a single layer to ca. 20 nm thick and composed of five distinct layers (T. J. Beveridge, personal communication).

We have suggested (9) that these extracellular coat layers protect the cell against predatory bacteria (F. L. A. Buckmire, Bacteriol. Proc., p. 43, 1971), antibodies (22), antibiotics (9), and harmful molecules and ions by decreasing the already limited penetrability of the cell envelope and by ionic exchange (the Donnan effect). Other workers (11, 15, 18) have shown that the carbohydrate coats of some bacteria mediate the irreversible adhesion of bacteria to surfaces within their environment. In the most extreme case, the bacteria were shown to be entirely embedded in a continuous adherent layer of carbohydrate slime (18). Shilo (26) has shown that myxobacteria must adhere to blue-green algae in order to digest them, and the enzymes concerned are therefore considered to be bound to the myxobacterial cell surface, which is an extracellular coat (21).

Previous studies of isolated rumen bacteria (5, 8) and of natural rumen populations (6) have shown that extracellular coat layers are universally present in these organisms. We have extended these studies by a detailed examination of the cell envelope of *Ruminococcus albus*, which is an important cellulolytic rumen bacterium (14), and we have examined the spatial relationship between cells of this organism and the cellulose fibers whose digestion they accomplish.

MATERIALS AND METHODS

Organism and culture conditions. *R. albus* was generously provided by M. P. Bryant, University of Illinois, Urbana, Ill.

The anaerobic technique used throughout this investigation for culturing these rumen bacteria was essentially that of Hungate (12) as modified by Bryant and Burkey (4). In some cases, cells were cultured in artificial medium in the absence of rumen fluid (24) to ensure that carbohydrate coat material observed on this organism was truly of bacterial origin. Where cellulose decomposition was to be described, shredded Whatman #1 filter paper (0.5%) was added as the chief carbon source in the medium.

Natural rumen population. A sample of the natural bacterial population was collected from the rumen of fistulated cows 4 h after an all-concentrate (6) feeding. After filtering through four layers of cheesecloth, the rumen contents were centrifuged at 48,000 × *g* for 30 min, and the bacterial portion of the pellet (a slimy layer on the surface) was fixed, stained with ruthenium red, and embedded for electron microscopy.

Isolation of coat material. Late logarithmic-phase (14 h) cells of *R. albus* (O.D. 1.40 at 660 nm, Gilford spectrophotometer) were harvested, washed twice with glass-distilled water, and freeze-dried. Ten grams of the dried cells were suspended in 500 ml of glass-distilled water in a 1-liter flask. This mixture was heated for 4 h in a boiling water bath. The

suspension was centrifuged at 25,000 × *g* for 30 min; the supernatant fluid was retained, and the bacterial cell pellet was collected for ruthenium red embedding to see whether the outer carbohydrate coat was removed. Two volumes of cold absolute alcohol were added slowly to the supernatant fluid at 5 C to precipitate the polysaccharide-containing outer layer. This mixture was stored in the refrigerator (4 C) overnight and centrifuged at 48,000 × *g* for 10 min. The supernatant fluid was discarded, and the white precipitate was suspended in slightly alkaline water and reprecipitated with two volumes of absolute alcohol. The white precipitate was suspended in water and dialyzed against glass-distilled water for 4 days at 4 C. The solution remaining in the dialysis tube was freeze-dried and was examined by negative staining for electron microscopy.

Ruthenium red embedding procedure. When the outer carbohydrate coat was to be demonstrated, a 20-ml sample of each culture (from various stages of growth) was centrifuged (15,000 × *g*, 10 min), and the pellet was suspended in a 0.15% aqueous solution of ruthenium red (17) (B. D. H. Chemicals, Toronto, Ont.) and held at room temperature for 30 min. The sample was then centrifuged (18,000 × *g*, 10 min), and the pellet was suspended and fixed for 1 h in 1.2% glutaraldehyde (purchased as a 70% solution under argon from Ladd Industries, Burlington, Vt.) in a 0.067 M cacodylate buffer (pH 7.3) with 0.05% ruthenium red. The sample was then washed three times for 10 min each in ruthenium red and cacodylate buffer and then postfixed 3 h at room temperature (22 C) in 1.33% osmium tetroxide (purchased as a 4% solution under argon from Polysciences Inc., Rydall, Penn.) in ruthenium red and cacodylate buffer. After osmium fixation, the sample was washed three times for 10 min in ruthenium red and cacodylate buffer and then subjected to an acetone dehydration series of 30 min in each of 30, 50, 70, 90, and 100% acetone by volume. Freshly distilled acetone was diluted to 30, 50, and 70% with ruthenium red and cacodylate buffer, but the 90% acetone was made by dilution of 100% acetone with distilled water. The sample was then washed twice for 20 min in 100% propylene oxide (Polysciences Inc., Rydall, Penn.) and embedded in Vestopal (Polysciences, Rydall, Penn.).

Morphological control preparations for embedding were prefixed by addition of 1/10 volume of 5% glutaraldehyde in 0.2 M phosphate buffer (pH 6.2) to a sample from the culture medium. After 20 min, the sample was centrifuged (15,000 × *g*, 10 min), and the pellet was suspended and fixed for 2 h at 20 C in 5% glutaraldehyde in 0.2 M phosphate buffer (pH 6.2). After fixation, the sample was enrobed (20) in 4% agar and washed five times (15 min) in 0.067 M cacodylate buffer (pH 6.5) before 2-h postfixation in 1.33% osmium tetroxide in 0.067 M cacodylate buffer (pH 6.5). After five washes (15 min each) in the cacodylate buffer (0.067 M, pH 6.5), the sample was subjected to an acetone dehydration series as described above. The agar cores were then washed twice in 100% propylene oxide (20 min each) and embedded in Vestopal.

Ultramicrotomy. Ultrathin sections of material embedded in plastic were cut with a Reichert model OM U2 ultramicrotome.

Staining sections. To ensure that increased contrast in the outer carbohydrate coat was due to ruthenium red staining and not to secondary staining of sections with uranyl acetate (used as a 1% aqueous solution at pH 5) and lead citrate (23), unstained sections were examined in the electron microscope after mounting on clean 400 mesh copper grids, while other sections were stained with uranyl acetate and lead citrate.

Freeze-etching. Samples for freeze-etching were collected from the culture medium and centrifuged to a thick pellet from which droplets were mounted on gold disks and frozen in Freon 22 (19). By using a Balzers BA 360 M apparatus, samples were freeze-cleaved and then sublimated for 1 min by the method of DeVoe et al. (10) before platinum shadowing and carbon coating to form the freeze-etch replica.

Negative staining. Negative staining of whole cells and isolated coat material was done by mixing one volume of 2% zirconium oxide (T.A.M. Division of National Lead Inc., Niagara Falls, N.Y.) at pH 6.7 with one volume of the sample in aqueous solution, and then placing a drop of the mixture on a formvar-coated 400 mesh copper grid. The grid was then blotted dry.

Critical point drying. Critical point drying of samples taken directly from the cellulose culture medium was done in a Freon 13 medium by the method of Cohen et al. (7). After critical point drying, the preparation was shadowed with platinum in a Balzers BA 360 freeze-etching unit.

Electron microscopy. All preparations for electron microscopy were examined by using an A.E.I. 801 electron microscope at an accelerating voltage of 60 kV.

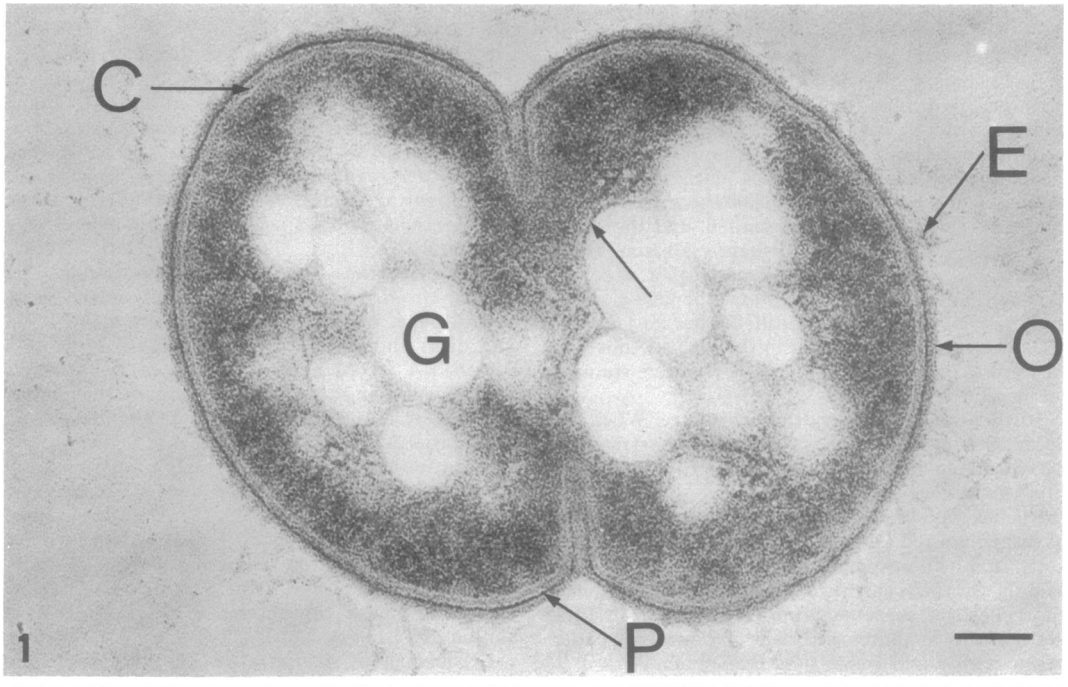
Light microscopy. Live cells were examined in wet mounts by differential interference microscopy by using a Leitz Dialux microscope.

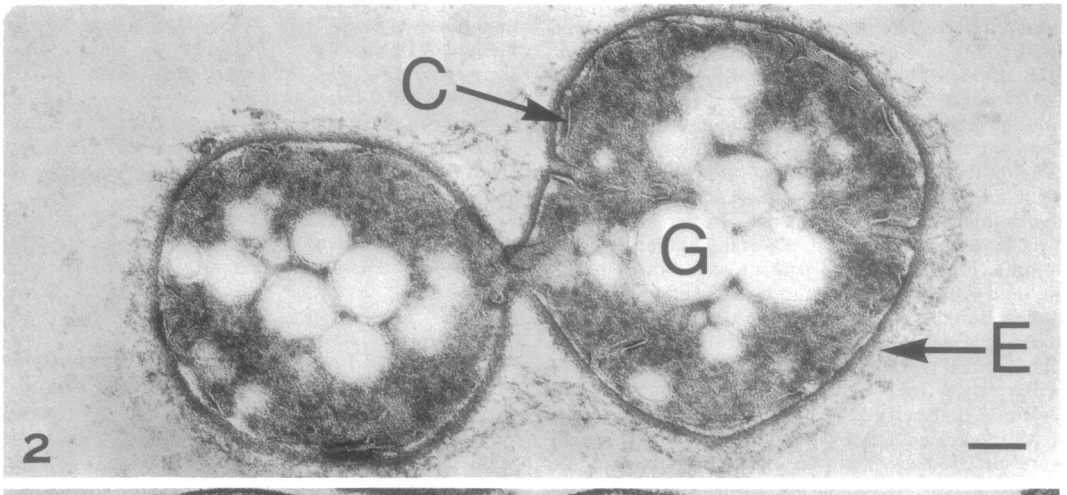
RESULTS

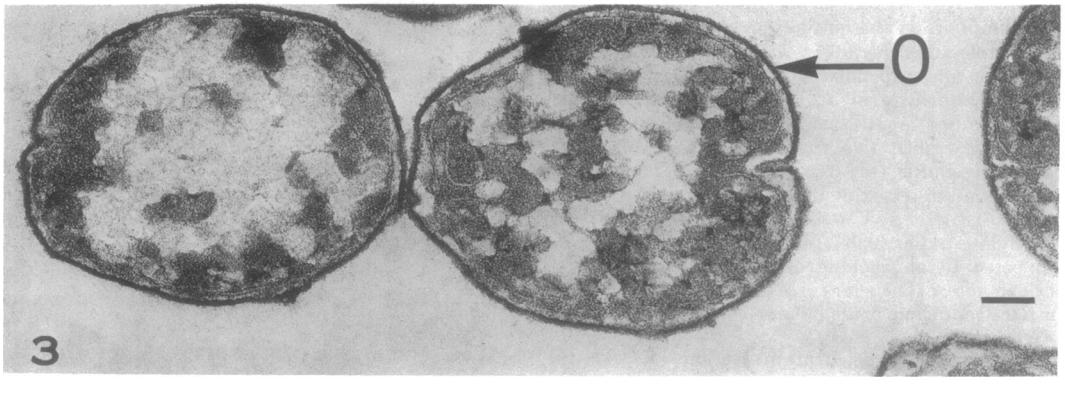
The general morphology of *R. albus* includes both cocci and diplococci as seen by differential interference light microscopy (see Fig. 12), and by electron microscopy of thin sections (Fig. 1-3), freeze-etching (see Fig. 9-11), and critical point drying (see Fig. 14 and 15). The cocci are from 700 to 800 nm in diameter and the diplococci measure roughly 800 nm by 1200 nm.

Electron-transparent amylopectin granules (Fig. 1 and 2, G) (13; unpublished results) occupy a large part of the cytoplasm of the cells of these organisms, especially in younger cells. Each of these deposits is enclosed by a single electron-dense layer (Fig. 1, arrow). The normal nucleoid and particulate cytoplasmic elements are often displaced to the cellular periphery by the presence of these carbohydrate deposits (Fig. 1 and 2).

The cytoplasmic membrane is clearly re-







FIGS. 1-3.

solved in thin sections (Fig. 1) and is also demonstrated in freeze-etched material where protein studs are visible on both aspects of the membrane (see Fig. 8 and 9). The electron-dense peptidoglycan of the cell envelope of cells of this organism is sufficiently thick to be easily resolved and is seen to participate in formation of a distinct septum in dividing cells (Fig. 1). The outer membrane is clearly resolved in sectioned material after boiling and ruthenium red staining (Fig. 3), but it is barely distinguishable in preparations made by more usual methods (Fig. 1). A cleavage plane in the outer membrane was not observed in freeze-etch preparations.

The treatment of live cells with ruthenium red before fixing gives excellent staining of the polysaccharides of the outer coat but clearly widens the space between the cytoplasmic and outer membranes of the bacteria (Fig. 2). Cells stained in this way show a loose fibrous coat between 40 and 100 nm in thickness (Fig. 2), and slightly thicker sections (Fig. 4) show a uniform electron-dense mat of fibers at the cell surface, and much thicker sections (Fig. 5) show a system of radiating fibers in addition to this fibrous capsule. These radiating fibers extend as much as 600 nm from the cell surface.

That this arrangement of radiating fibers is found in the rumen as well as in pure culture is indicated by ruthenium red staining of raw rumen contents, which shows this surface structure in a large number of unidentified cells (Fig. 6). The coat material is electron-dense in these ruthenium red-stained cells even when the usual stains (uranyl acetate and lead citrate) were not applied to the sectioned material (Fig. 7), and uranyl acetate and lead citrate staining does not add much electron density to the coat material in the absence of ruthenium red (Fig. 1). Ruthenium red staining of cells after the removal of the outer coat material by heating showed that the electron-dense outer material had been removed from the cell surface (Fig. 3).

The radial fibers were very clearly seen in freeze-etched preparations, where they are exposed by sublimation and seen to extend up to 800 nm from cell surface (Fig. 8-11). Cells grown in the absence of rumen fluid also showed the fibrous capsule and radiating fibers (Fig. 11). The abundance of this intercellular fibrous material was best demonstrated in replicas of a freeze-etched preparation of cells centrifuged from an 8-h culture of *R. albus* in which all of the extensive intercellular space is seen to be occupied by these fibers (Fig. 10). Sublimation reveals some of the structural detail of the fibrillar capsule in freeze-etched cells of the organism (Fig. 8-11), but often this detail is incompletely seen because of the formation of a eutectic (10) at the surface of the cells during freezing. Negative staining of purified coat material shows its basically fibrous nature.

When cellulose fibers were added to the growth medium of *R. albus*, examination by differential interference microscopy showed that very few cells were free in the medium, and that almost all the cells adhered to the cellulose fibers (Fig. 12). The cells were often lined up along a particular cellulose fiber (Fig. 13). This adherence was further demonstrated by examination by transmission electron microscopy of critical point-dried material from cellulose cultures in which all of the bacteria were clearly seen to be adherent to the cellulose fibers (Fig. 14). Examinations at higher magnifications (Fig. 15) showed the presence of fibers of varying thickness (Fig. 15, arrow), which could not be ascribed with any degree of certainty to the cell or the cellulose.

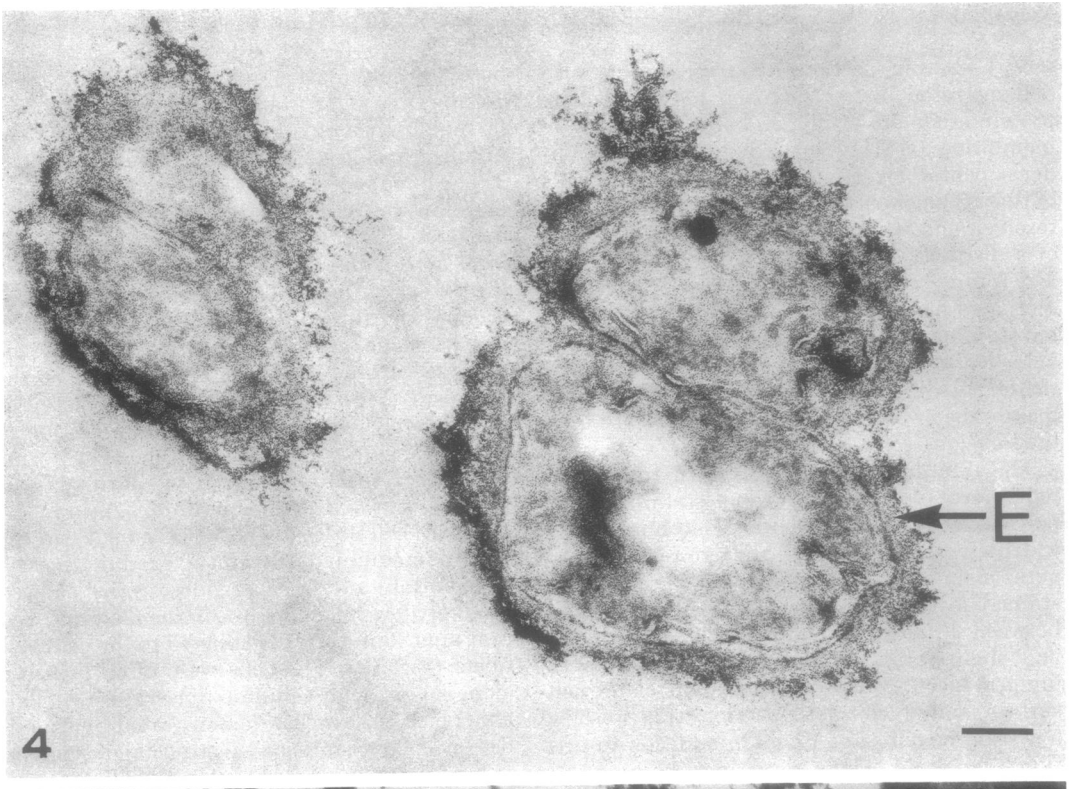
DISCUSSION

In cells of *R. albus*, the electron-transparent cytoplasmic inclusions, each of which is surrounded by a single electron-dense structure, are morphologically similar to the cytoplasmic glucan deposits found in the rumen organism *Megasphaera elsdenii* (6) and to the glucan deposits found in *Clostridium pasteurianum* (16). Cross-cleavage of frozen cells showed no cleavage planes in the cytoplasm, and we can

FIG. 1. *Electron micrograph of sectioned cells of R. albus. These cells have been fixed in glutaraldehyde-osmium and the sections were stained with uranyl acetate and lead citrate. Note the electron-transparent cytoplasmic glucan deposits (G) with their electron-dense bordering layer (arrow), the cytoplasmic membrane (C), and electron-dense peptidoglycan layer (P) of the cell envelope. The outer membrane (O) is only poorly resolved, and the extracellular coat (E) is only lightly stained. The bar on this and on subsequent micrographs indicates 0.1 μm.*

FIG. 2. *Electron micrograph of a section of cells treated with ruthenium red before fixation. Note the electron density of the extracellular coat (E) surrounding these cells and the separation of the cytoplasmic membrane (C) from the cell wall.*

FIG. 3. *Electron micrograph of a section of cells fixed and stained as for Fig. 2 after removal of the extracellular coat layer by boiling. The outer membrane (O) is resolved in these cells.*



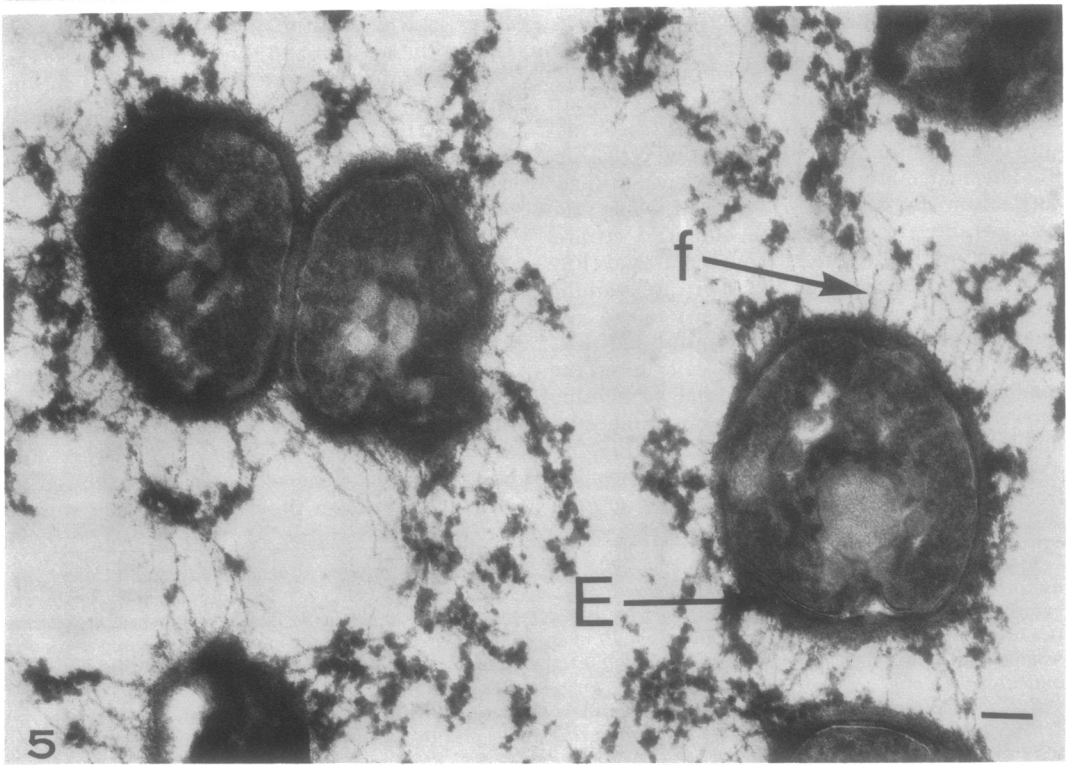
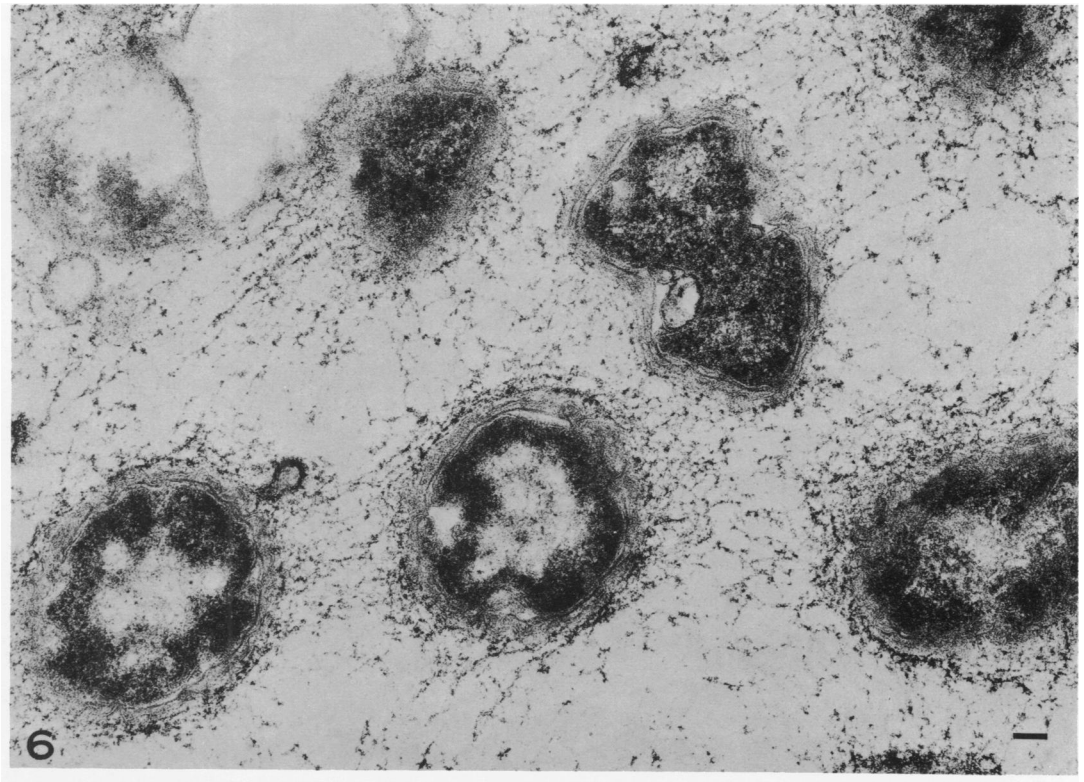


FIG. 4. *Electron micrograph of a moderately thick section (ca. 100 nm) of ruthenium red-stained cells of R. albus showing the mat-like mass of fibers that make up the extracellular coat layer (E).*

FIG. 5. *Electron micrograph of a very thick (about 300 nm) section of ruthenium red-stained cells of R. albus showing extensive radial fibers (f) projecting outward from the extracellular coat layer (E).*



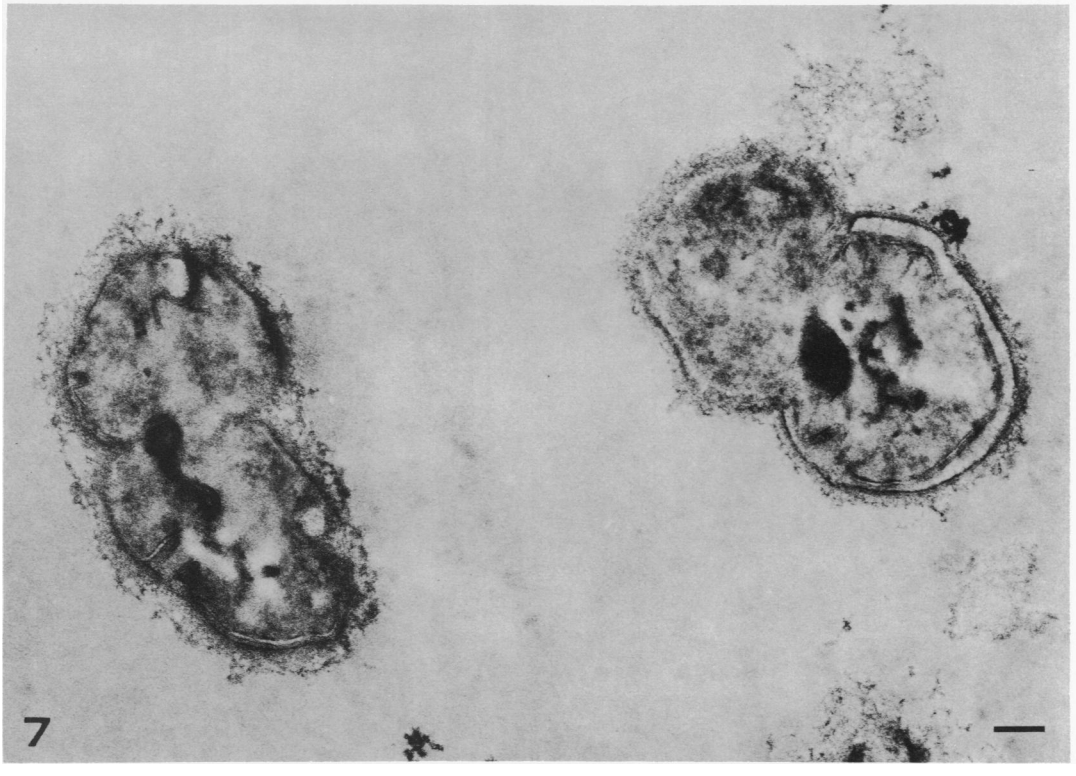
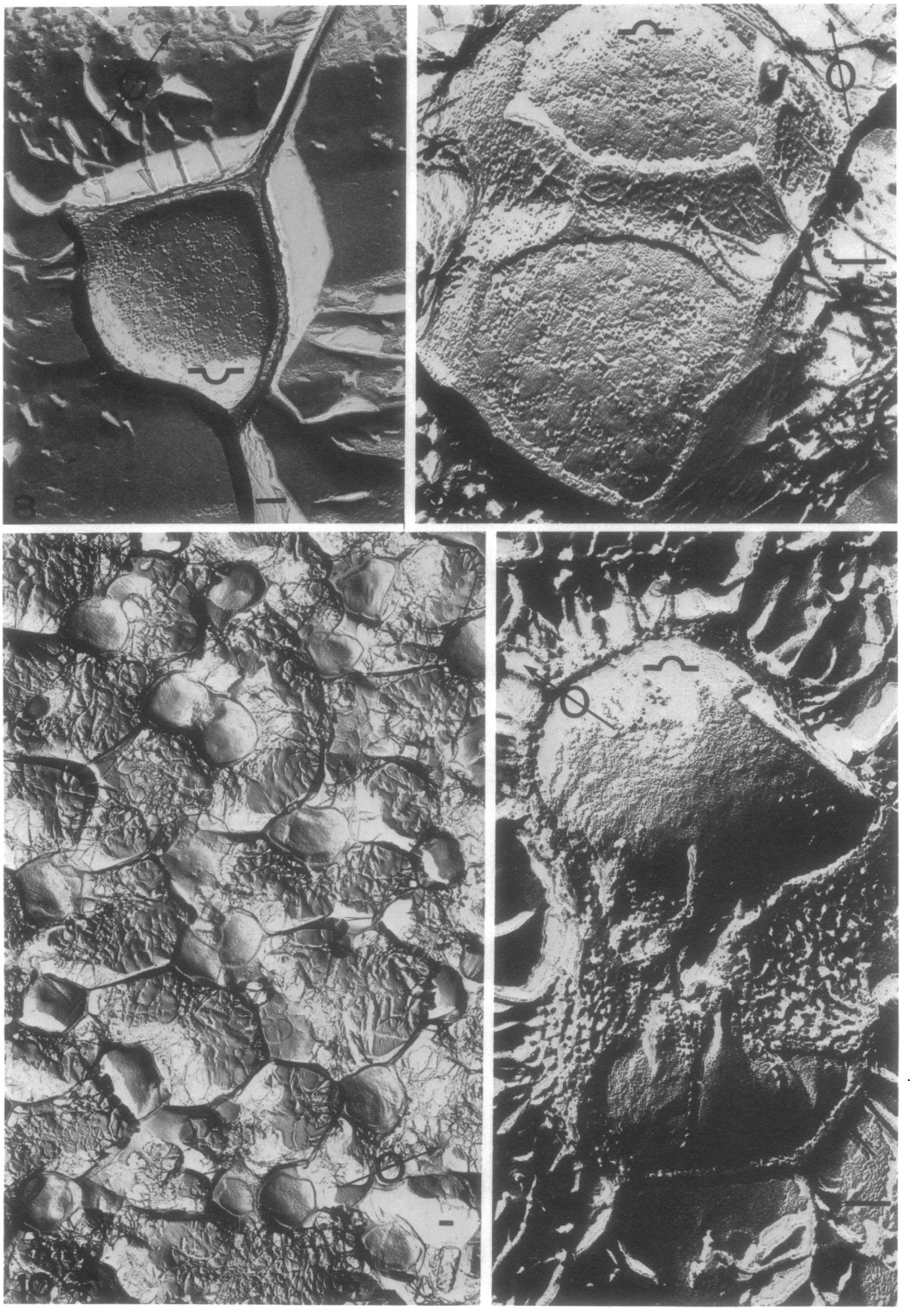


FIG. 6. *Electron micrograph of a section of unidentified ruthenium red-stained bacteria in a normal rumen fluid sample showing the production of radial extracellular fibers of these cells.*

FIG. 7. *Electron micrograph of a section of ruthenium red-stained cells of R. albus that had not been stained with uranyl acetate or lead citrate.*



FIGS. 8–11.

conclude that the electron-dense enclosing strutures of these deposits are not true membranes. The amount of this material varies in different preparations indicating that, like the glucan of *M. elsdenii* (6), it is related to the physiological condition of the cells (8).

The peptidoglycan layer of *R. albus* is thick enough (4 nm) to be clearly resolved in sectioned material and, as in *M. elsdenii* (8), distinct septa are formed in dividing cells. The outer membrane of the cell envelope of *R. albus* is discernible but difficult to resolve in sectioned material, and it does not constitute a cleavage plane in frozen cells. This failure to form a distinct double-track artifact, and the failure to cleave in frozen cells, has also been noted in smooth strains of both *Escherichia coli* and *Salmonella typhimurium* (unpublished data).

The extensive extracellular coat layer, which lies outside the outer membrane of these cells, is barely visible in material stained with uranium and lead salts, but it stains very heavily with ruthenium red, indicating that it is a polysaccharide (17, 28). Biochemical studies of the purified coat material have established that it contains essentially glycoprotein (unpublished data). The examination of negatively stained material and of thick sections of ruthenium red-stained cells indicate that this extracellular coat comprises a dense layer of material which would be expected to protect the cell by excluding particles and molecules, and by conditioning the ionic and molecular environment within the cell envelope (9).

Extracellular polysaccharide has been implicated in the adhesion of bacterial cells to surfaces (11, 15, 18) and we have examined the adhesion of cells of *R. albus* to cellulose fibers that were added to its medium to replace soluble cellobiose as a carbon source. Examination of these cultures by differential interference microscopy showed that many bacteria were firmly adherent to the cellulose fibers but that a distinct space separated them from this substrate. This suggested that the extracellular coat material could be involved in the adherence of these cells to the fibers. Akin et al. (1) have also observed that rumen bacteria are attached to plant cell walls with consequent degradation of these structures. Electron microscopy of critical point-dried material showed a similar degree of adherence. While it is difficult to determine whether the finest fibers seen in Fig. 14 and 15 are of bacterial origin and therefore constitute the extracellular coat, or are derived from the layer of cellulose fibers, it is clear from these micrographs that these fine fibers mediate the attachment of the cells to the cellulose.

Work by Smith et al. (27) on the cellulolytic activity of *R. albus* has shown that most of the cellulases of this organism are released into the medium during growth and are subsequently bound to the cellulose fibers, but that digestion is facilitated by the proximity of the cells to the cellulose fibers. For this reason, the attachment of these cells to their polymeric substrate, by means of their extracellular coats, would facilitate their growth in this environment.

FIG. 8. *Electron micrograph of freeze-etched cells of R. albus showing the fibrillar nature of the extracellular coat layer and the pattern of protein globules in the concave-convex cleavage of the cytoplasmic membrane. Direction of shadow is indicated* ♂ *in this and subsequent electron micrographs.*

FIG. 9. *Electron micrograph of freeze-etched cells of R. albus showing the particle-studded convex cleavage plane of the cytoplasmic membrane and the very large amounts of fibrillar extracellular coat material between the cells.*

FIG. 10. *Low-power electron micrograph of freeze-etched cells of R. albus showing the very extensive amounts of fibrillar extracellular coat material which fills a large proportion of the intercellular space.*

FIG. 11. *Electron micrograph of freeze-etched cells of R. albus grown in an artificial media which does not contain fibers. This micrograph shows that the fibrillar extracellular material is a product of these bacteria and is not adsorbed from the culture medium.*

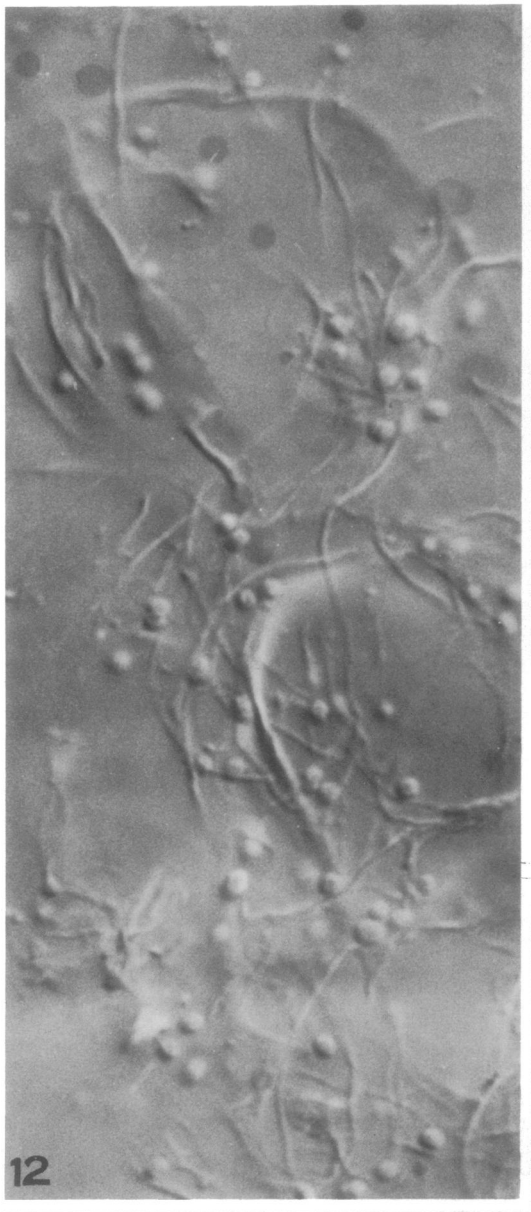
FIGS. 12-15 on page 286.

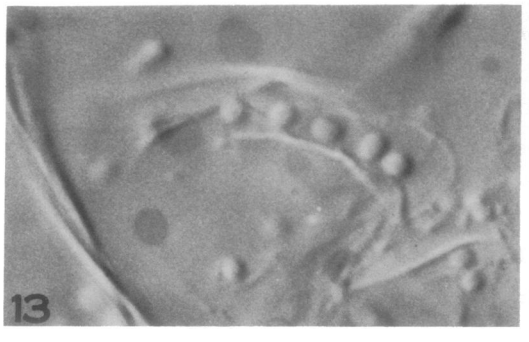
FIG. 12. *Light micrograph using differential interference optics which show the distribution of cells of R. albus grown in the presence of cellulose fibers. Note that the bacterial cells are all adherent to the fibers.*

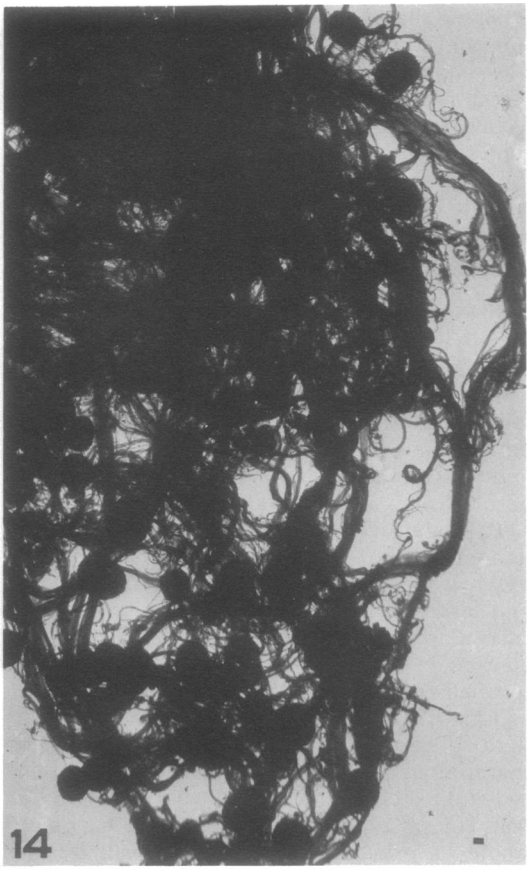
FIG. 13. *Light micrograph shows five bacterial cells lined up along a cellulose fiber.*

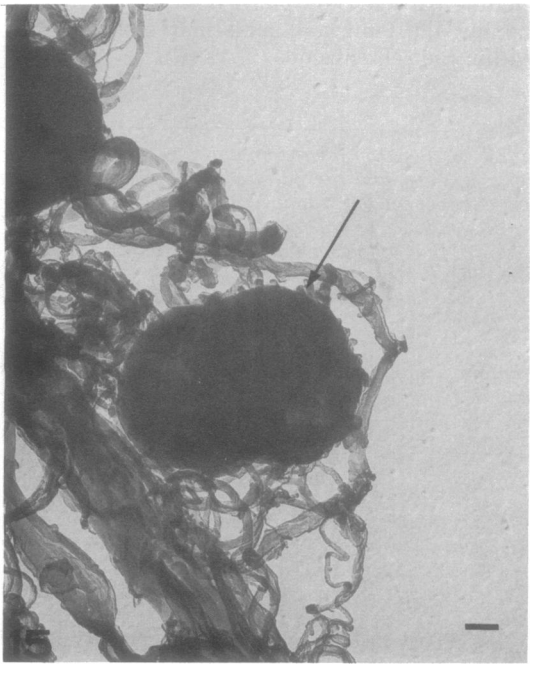
FIG. 14. *Electron micrograph of a critical point-dried preparation of cellulose fibers with adherent cells of R. albus.*

FIG. 15. *Electron micrograph of a critical point-dried preparation of cellulose fibers with adherent cells of R. albus showing the fine fibrils (arrow) that appear to mediate the attachment of the bacteria to the cellulose.*









FIGS. 12–15.

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