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Adhesion of Bacteria to Epithelial Cell Surfaces Within the Reticulo-Rumen of Cattle

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Blocks of tissue were removed from various locations in the bovine digestive tract and fixed and processed for transmission and scanning electron microscopy by techniques that retained adherent bacteria. The distribution of bacteria on the surface of epithelial cells was examined by scanning electron microscopy. This showed intermittent colonization of the epithelia with the formation of occasional microcolonies of morphologically similar bacterial cells. Transmission electron microscopy of ruthenium red-stained material showed the presence of both the glycocalyx of the bovine epithelial cells and fibrous carbohydrate coats surrounding adherent bacteria. The carbohydrate coats appeared to mediate the attachment of bacteria to the epithelium, to food particles, and to each other so that microcolonies were formed. Careful examination of the bacterial colonization of keratinized cells in the process of being sloughed from the surface of the stratified squamous epithelium of the rumen showed that these dead cells were digested by adherent bacteria of a limited number of morphological types. The spatial relationship of this mixed, adherent, microbial population to living and dead epithelial cells and to food particles indicates that digestive processes of some importance may be accomplished by this stationary component of the microbial flora of the digestive tract.

Bacterial populations attached to gut mucosa and food particles have been noted in many animal studies (2, 3, 7, 10, 13, 14) with the mechanism of attachment being attributed to: bacterial capsular material of either a proteinaceous (14) or carbohydrate nature (11); or pili (10); or host factors (14). The ability of a bacterium to adhere can influence its pathogenicity (10) and can increase its access to potential food sources (1, 3).

By scanning electron microscopy (SEM), both the average numbers and morphological types of bacteria as well as the tissue surfaces they occupy have been explored. However, SEM by itself cannot offer information about the surface characteristics of the bacteria and contributes scant information about mechanisms of adhesion.

Transmission electron microscopy (TEM) combined with appropriate stains has been used to provide information about the surface characteristics of bacteria (3, 5, 6, 11) and the mechanisms of bacterial adhesion (3, 7).

With the exception of the SEM observations of the rumens of sheep by Bauchop et al. (2), very little attention has been paid to populations of bacteria attached to the ruminant gut.

Through the use of both SEM and TEM, we investigated the bacterial populations attached to the epithelia of the bovine digestive tract and we discuss here possible mechanisms of attachment as indicated by stained material.

MATERIALS AND METHODS

Animals. Animals used in this study were seven yearling Hereford bulls. As calves, they grazed with their mothers on Russian wild rye pasture until 15 August and on native short-grass prairie until weaning 1 November. During the subsequent 168-day feedlot period, four of the bulls (D, E, F, and G) were self-fed a ration consisting of 60% barley, 10% oats, 10% beet pulp, and 20% chopped alfalfa hay (high plane diet), and the other three (H, I, and J) were fed chopped alfalfa hay (low plane diet).

At slaughter, the tracts of the bulls were rapidly exposed. Tissue samples were removed from the digestive tract and washed twice by rapid agitation in 0.85% sodium chloride solution. Each sample was then divided into two portions (about 2 by 2 cm), one for SEM and the other for TEM. Both samples were then placed in their respective prefixatives. Less than 3 min elapsed between excision of the sample and placement in prefixative, and the samples were taken within 5 min of the animal's death.

Preparation of TEM materials. Tissues were left in the prefixative solution of 0.015% ruthenium red and 0.5% glutaraldehyde in 0.067 M cacodylate buffer (pH 7.2) for 2 h, and then placed in a 5% solution of

glutaraldehyde in 0.067 M cacodylate buffer with 0.05% ruthenium red for 2 h at room temperature. They were then washed in 0.05% ruthenium red in the cacodylate buffer five times for 1 h each time, with one overnight wash included, before being post-fixed for 2 h in 2% osmium tetroxide in ruthenium red-cacodylate buffer. After five further 1-h washes in ruthenium red-cacodylate buffer, samples were subjected to an acetone dehydration series of 30 min each in steps of 15, 30, 50, 70, 90, and 100% acetone. The freshly redistilled acetone was diluted by ruthenium red-cacodylate buffer up to the 70% acetone solution, with the 90% acetone solution being diluted with double-distilled water. The sample was washed three times in 100% propylene oxide for 20 min and embedded in Vestopal W.

Microtomy. Sections were cut on an LKB III ultramicrotome, mounted on 200-mesh grids, stained with uranyl acetate and lead citrate (12), and subsequently carbon-coated using a Balzers BA 360 M apparatus.

Electron microscopy of the specimens was carried out on an A.E.I. 801 electron microscope at an accelerating voltage of 60 kV.

Preparation of SEM samples. Tissues were left in 0.5% glutaraldehyde in 0.067 M cacodylate (pH 7.2) for 2 h followed by 2-h fixation in 5% glutaraldehyde in cacodylate. They were then subjected to five washes in cacodylate buffer and carried through the modified thiocarbohydrazide (TCH) procedure of Malick and Wilson (9). This was followed by dehydration through a graded series of 15, 30, 50, 70, 90, and 100% ethanol diluted with double-distilled water. The samples were then carried through a second graded series of Freon 13, in the same gradient steps, using ethanol as the diluent. They were critical-point dried in Freon 13 (4) and mounted on metal stubs with silver glue.

As a control for the TCH method, samples in the fifth wash after 5% glutaraldehyde were placed in 2% osmium tetroxide for 2 h, washed five times in the cacodylate buffer, and carried through the same dehydration steps and critical-point procedure as described above. The non-TCH-treated tissue was gold coated while rotating in an evacuated evaporation unit. TCH was found to yield superior results. Charging was encountered only with the gold-coated specimens and not with the TCH-treated ones. In addition, because TCH does not involve coating of the sample, detail cannot be lost by burying.

Samples for scanning were observed on a Cambridge Stereoscan 180 at an accelerating voltage varied between 20 and 25 kV.

RESULTS

Ruthenium red stains both the extracellular slime of the bacteria and the glycocalyx of the epithelial cells and thus reveals the contours of the epithelial cell and external characteristics of the bacteria (Fig. 1). Since the reticulo-rumen is nonsecreting (15), the slime network observed was of bacterial origin. This slime took the form of extracellular fibers of various patterns, which anchored the bacteria to the surface. Thus, the examination of ruthenium red-stained material indicated that bacterial slime was an important agent in facilitating bacterial adherence to the squamous epithelial cells of the rumen.

The adherent bacterial population of the bovine digestive tract is a complex mass of cells that is held together by slime fibers and attached to the epithelial surface of the gut by the interaction of slime fibers with the glycocalyx of the epithelial cells (Fig. 3). Within this adherent population, microcolonies of morphologically similar cells, such as curved rods (Fig. 4) or cocci (Fig. 5 and 6), are sometimes seen, and individual slime capsules of considerable complexity (Fig. 3) are often seen. Attachments may be to the adherent bacteria or to the epithelium, which do not lie in the plane of the section. Our observation that large numbers of gram-positive bacteria, as recognized on the basis of ultrastructure, are seen in adherent populations (Fig. 3) contrasts with the rare occurrence of these organisms in rumen contents of cattle fed concentrate diets (Cheng and Costerton, unpublished data) and suggests that the adherent population may differ from that of the rumen fluid.

SEM yields a topographic perspective of the tissue surface and reveals the morphology of the adherent bacteria. The adherent bacterial population is usually morphologically heterogeneous (Fig. 2) and often contains highly characteristic and recognizable forms (e.g., spiral-shaped bacteria; Fig. 2). The fine slime fibers that mediate cell-cell adhesion are below the limit of resolution of the SEM and are not seen unless they are aggregated (F, Fig. 2).

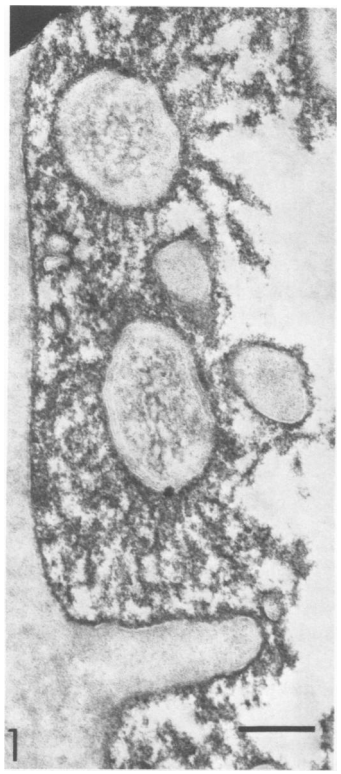
The squamous epithelium consists of several

FIG. 1. *Electron micrograph of bacteria encased in a confluent mass of ruthenium-red-positive slime adherent to the squamous epithelium (bull J, rumen, ventral surface of the ventral sac, low plane diet). The bar in this and subsequent transmission micrographs = 0.2 μm.*

FIG. 2. *Scanning micrograph of a mixed population of bacteria on the tissue surface. The bacteria adhere to each other as well as to the tissue surface (bull E, cranial surface of the cranial pillar, high plane diet). Note the spiral-shaped bacteria and the fibers and amorphous strands (F) surrounding many of the bacteria. The bar in this and other scanning micrographs = 0.5 μm.*

FIG. 3. *Longitudinal section of the epithelial surface indicating the heterogeneous bacterial population as well as the varying ruthenium red-staining slime types. A thin layer of ruthenium red-stained material (arrow) reveals the contours of the glycocalyx (bull G, left surface of ventral sac, high plane diet).*

FIG. 4. *Scanning micrograph of bacteria adherent to the tissue. These bacteria are morphologically very similar and may represent a nearly pure microcolony (bull E, caudo-dorsal blind sac, high plane diet).*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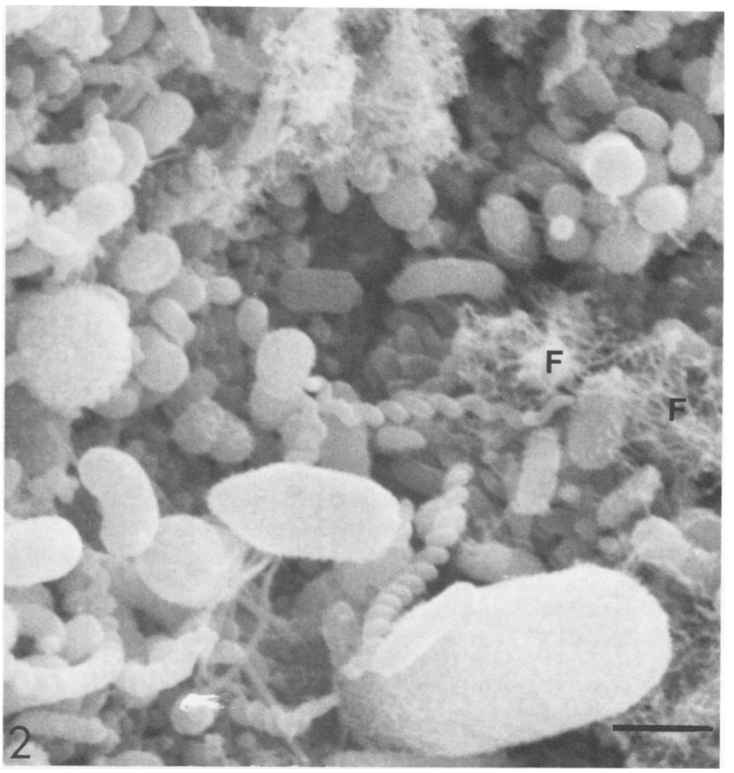
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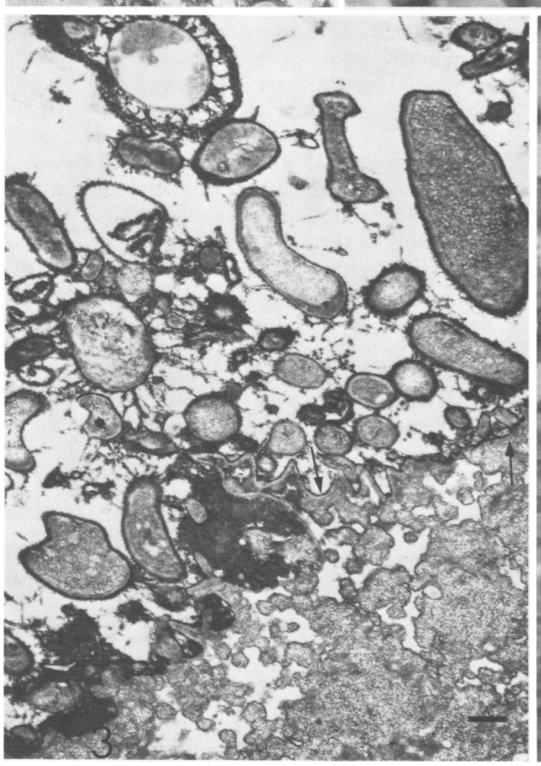
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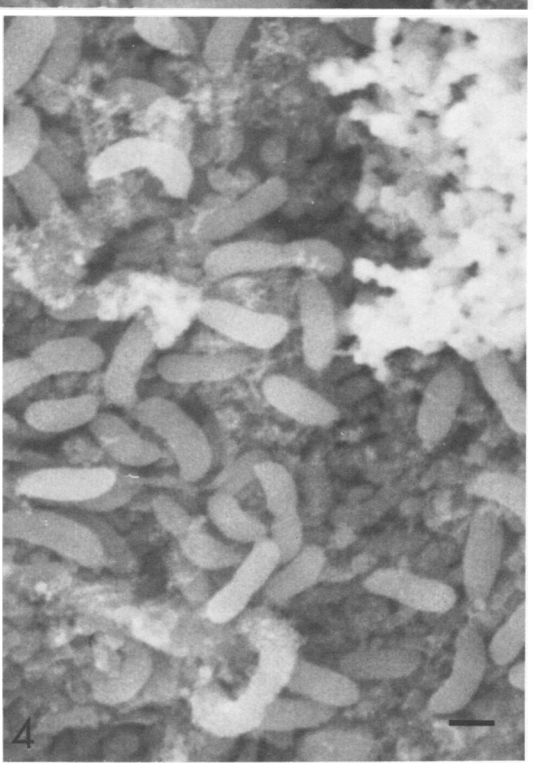
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stratified cell layers that are constantly being shed from epithelial surfaces (Fig. 6 and 7). The multiple cell layers forming the mucosal surface are normally heavily keratinized, rigid structures (Fig. 8). As the surface cells slough off, bacteria colonize the newly exposed underlying cells, often before the desquamating cell has been totally shed. Figure 6, which typified this situation, reveals a microcolony of cocci with a few other morphological types colonizing a new epithelial surface, whereas Fig. 7 indicates that the bacteria also adhere to the surface of the desquamating cell. The bacterial adhesion to the undersurface of the desquamating cell suggests that bacteria may play a role in the digestion and degradation of these sloughed cells and that adhesion facilitates this process.

The appearance of sectioned material supports this assumption, because bacteria infiltrate epithelial cells at points where the cell membrane has ruptured and attach themselves to this potential substrate (Fig. 9). Because the membranes of the squamous cells are reinforced by keratinization, they often remain partially intact and functionally associated with the underlying cells by maintaining the desmosomal or apical bar links that existed between the cells when they were intact (Fig. 8 and 10). Bacteria may attach to both the membrane remnants and extruded cytoplasmic material (Fig. 10). The bacteria found associated with the extruded cytoplasmic material are usually of one morphological type, suggesting that a few bacteria may occupy a specific, narrow niche related to digestion of the desquamating cells. The invasion of the ruptured desquamating cells by bacteria as well as the slime fibers maintaining them there was also best observed with sectioned material.

DISCUSSION

The stratified squamous epithelium in the reticulo-rumen of cattle maintains a large stationary population of adherent bacteria. Our morphological data suggest that these bacteria recolonize the underlying squamous cells as the superficial cells are sloughed off.

The adherent bacteria may derive several advantages from attachment to the tissue surface. The bacterial adherence to the epithelial surface may protect the bacteria from digestion by ciliate protozoans (2). Bacteria that are present in low numbers, or that are incapable of surviving free in the rumen, may be able to avoid being washed out of the rumen by adhering to the mucosa. This may be true of the gram-positive organisms that formed in a higher proportion of bacteria adhering to the epithelial surface than of those found free in the rumen fluid of cattle fed concentrate diets.

By adhering to the epithelial wall, the bacteria have access to a number of potential substrates. Due to the rhythmic contraction of the reticulorumen, metabolites in the rumen fluid are continually made available to the adherent bacteria while they also have access to metabolites transported through the epithelium. Of special note is the fact that at least some of the bacteria are capable of invading and digesting the degenerating squamous epithelial cells. Just as Akin and Amos (1) found in the adherence of cellulolytic rumen bacteria to plant cell wall material, adhesion of bacteria to dead and keratinized epithelial cells may be an important and necessary step in their degradation.

The ruminant may also benefit from this association by using the bacteria as an additional selective barrier for specific metabolites passing through the wall, or as a means of blocking adherence of pathogenic bacteria. McGavin and Morrill (8) suggested that the adsorptive capacity of the squamous epithelium was decreased by thick layers of keratinized cells. Bacteria capable of digesting the keratinized cells would induce their removal and facilitate movement of metabolites through the tissue.

Our studies have indicated that acid polysaccharides (6) are involved in the adhesion process. They may be in the form of pure polysaccharides, glycoproteins, or other mixed polymers and may either function as major structures responsible for adhesion or serve a minor role as part of a progressive sequence involved in the binding.

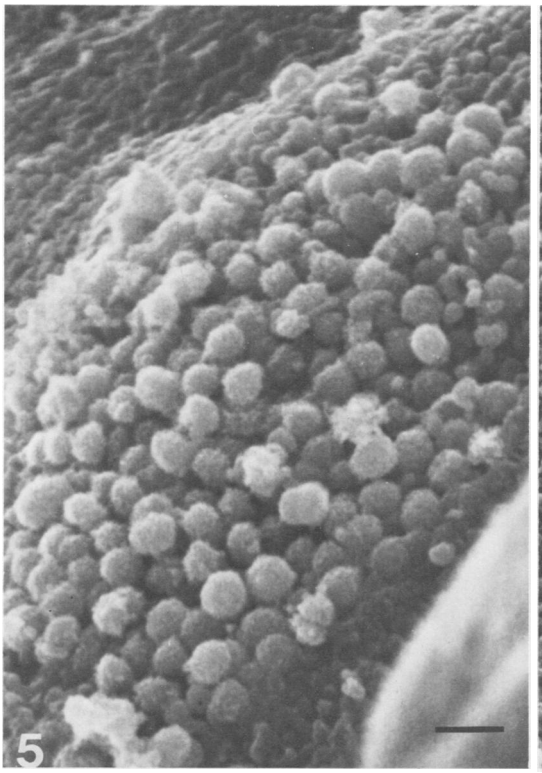
Indigenous bacteria have been found to ad-

FIG. 5. *Scanning micrograph of a morphologically homogeneous colony of cocci (bull E, edge of cranial pillar, high plane diet).*

FIG. 6. *Scanning micrograph of a gold-coated specimen showing desquamating epithelium. A microcolony of cocci with a few other morphological types have colonized the new epithelial cell underlying the shedding cell (S) (bull E, caudo-dorsal blind sac, high plane diet).*

FIG. 7. *Scanning micrograph of a desquamating epithelial cell being colonized on both sides by a mixed bacterial population (bull E, caudo-ventral blind sac, high plane diet).*

FIG. 8. *Electron micrograph of sectioned epithelial surface. The upper cells of the stratified epithelium are heavily keratinized. Rigid remnants of a degenerated epithelial cell (arrow) are still linked to the intact squamous cell by desmosomes (bull D, cranial surface of reticulum, high plane diet).*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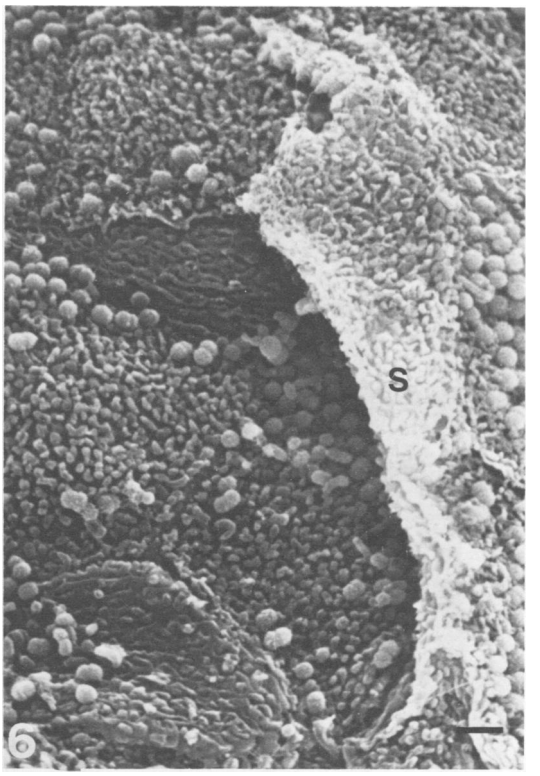
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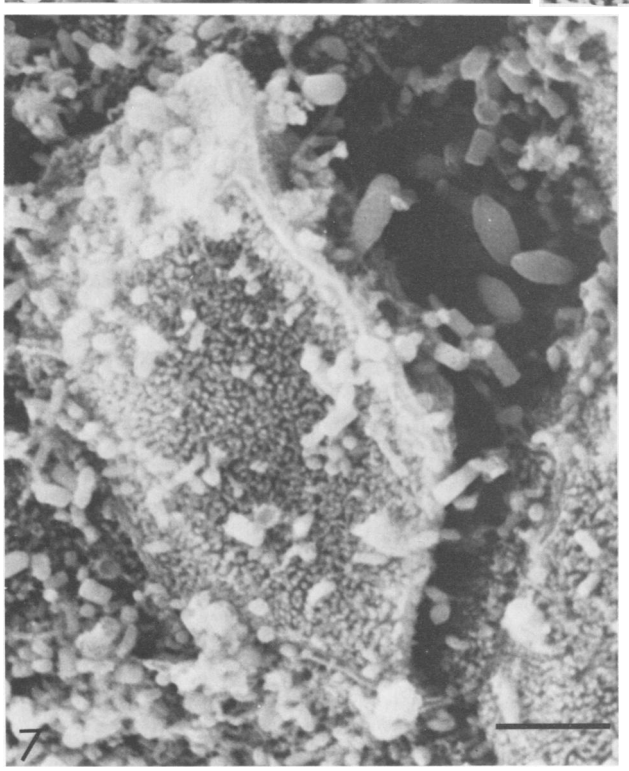
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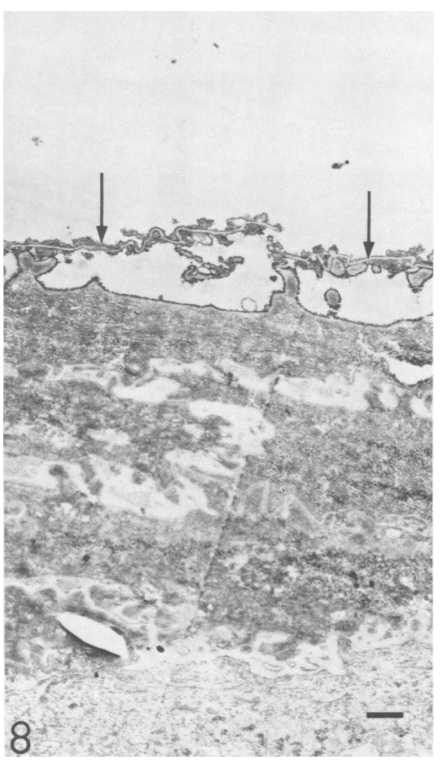
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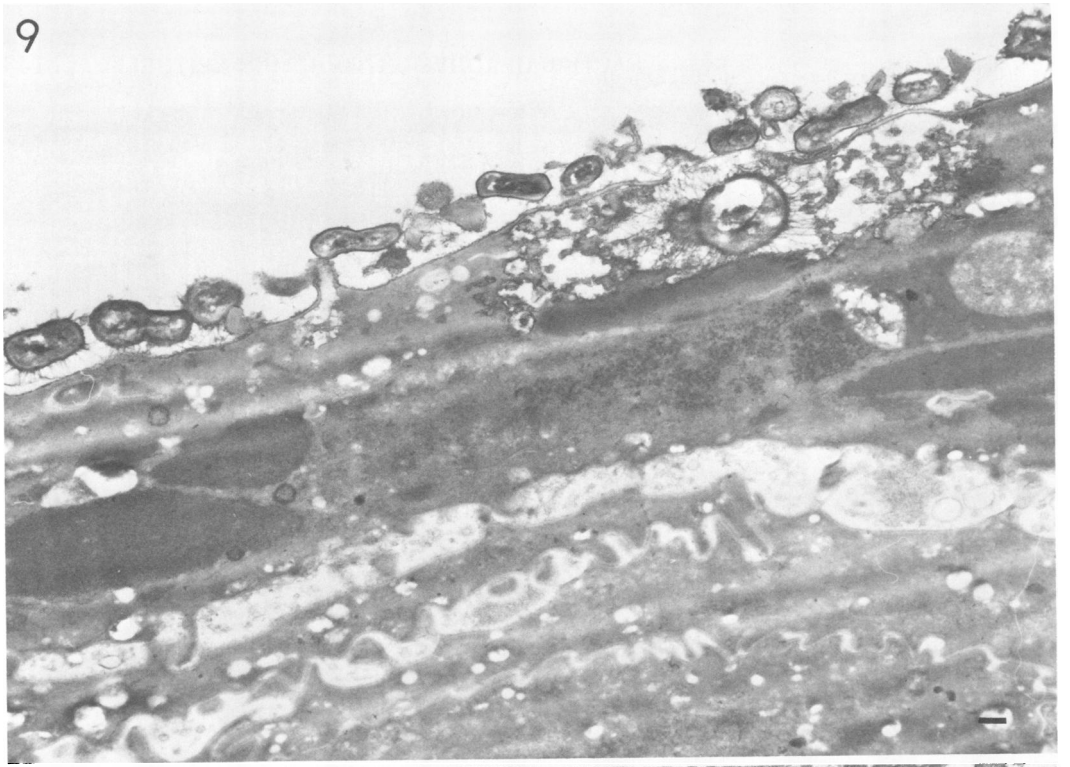

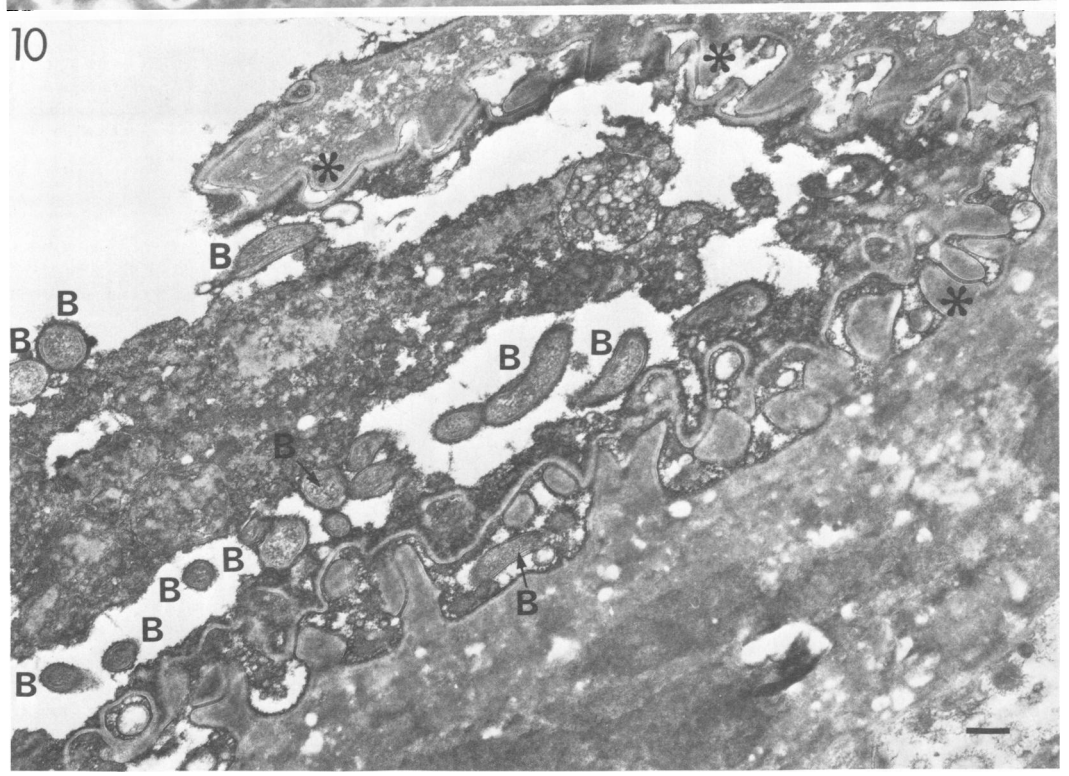

FIG. 9. *Electron micrograph of stratified squamous epithelium. Bacteria adhere to the surface of the cell and also infiltrate the cell where it has ruptured. Parts of the keratinized cell membrane are still intact. Radial slime fibers anchor the bacterium to the disrupted cell (bull J, caudal surface of cranial pillar, low plane diet).*

FIG. 10. *Electron micrograph of a degenerating epithelial cell. The bacteria (B) adherent to the ruptured epithelial cell membrane and contents are of a similar morphological type. The residual epithelial membrane still adheres to the surrounding intact cells by desmosomes and apical bars (*) (bull D, cranial surface of reticulum, high plane diet).*

here preferentially to the mucosal epithelia of their natural hosts in birds and mammals and are often unable to adhere to other hosts (7, 14). The fact that large numbers of bacteria of the gram-positive morphological type are present in adherent populations indicates a preference for a sessile rather than free-floating existence in the rumen and suggests a certain specificity.

Elucidation of the specificity of adhesion, and whether structures other than the slime coat are responsible for these interactions, will be the aim of our future investigations.

The variations in the adherent microbial populations seen at different locations, and variations in these populations caused by dietary factors, will be presented in subsequent publications.

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