Rumen Bacterial Degradation of Forage Cell Walls Investigated by Electron Microscopy

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The association of rumen bacteria with specific leaf tissues of the forage grass Kentucky-31 tall fescue (*Festuca arundinacea* Schreb.) during in vitro degradation was investigated by transmission and scanning electron microscopy. Examination of degraded leaf cross-sections revealed differential rates of tissue degradation in that the cell walls of the mesophyll and phloem were degraded prior to those of the outer bundle sheath and epidermis. Rumen bacteria appeared to degrade the mesophyll, in some cases, and phloem without prior attachment to the plant cell walls. The degradation of bundle sheath and epidermal cell walls appeared to be preceded by attachment of bacteria to the plant cell wall. Ultrastructural features apparently involved in the adhesion of large cocci to plant cells were observed by transmission and scanning electron microscopy. The physical association between plant and rumen bacterial cells during degradation apparently varies with tissue types. Bacterial attachment, by extracellular features in some microorganisms, is required prior to degradation of the more resistant tissues.

The microorganisms comprising the rumen bacterial population vary in morphology and metabolic activity (7, 19). Symbiotic relationships for the degradation and utilization of plant cell wall constituents have been shown by using known cultures of rumen bacteria (10, 13–16). Investigations of the metabolic activities of these bacteria have helped clarify the complex nutritional system in the rumen. However, the initial steps in tissue digestion involving the mode of association with and degradation of intact plant cell walls by rumen bacteria have not been investigated extensively.

Electron microscopic investigations have revealed certain interesting ultrastructural features of rumen bacteria (3, 11, 23, 28). Leatherwood (23), using the scanning electron microscope (SEM), observed "tube-like appendages" on the cellulolytic coccus *Ruminococcus albus* only when grown on cellulose-containing media. Costerton et al. (11), using the transmission electron microscope (TEM), reported coats external to the outer membrane of three gram-negative rumen bacteria, *Bacteroides ruminicola*, *Bacteroides succinogenes*, and *Megasphaera elsdenii*. Earlier TEM observations from our laboratory of the tropical forage Coastal Bermuda grass degraded by rumen bacteria in vitro revealed attachment, apparently by an extracellular matrix, of large cocci to the thick forage cell walls (3).

In addition, observations of intact leaf sections by using the SEM have shown differences in the ease and extent of forage tissue digestion by rumen microorganisms (1, 2). Investigations using the SEM at the level of microbial attachment should be useful in adding a new perspective to the complex problems of bacterial attachment and degradation of forage tissues by rumen bacteria. The objective of the work reported here was to examine the mode of bacterial attachment to and degradation of intact cell walls in tissues of the temperate forage grass Kentucky (Ky)-31 tall fescue (*Festuca arundinacea* Schreb.) by TEM and SEM.

MATERIALS AND METHODS

Substrate. Leaf blades of Ky-31 tall fescue were harvested after 4 weeks of summer regrowth, frozen immediately, and stored at –30 C until used. Sections 2 to 5 mm in length were cut from the midportion of the frozen blades and used as substrate for the bacteria.

Microbial inoculum. To obtain a preparation relatively free from particulate debris which would obstruct TEM observation, whole rumen contents were squeezed through four layers of cheesecloth, centrifuged at 250 × *g* for 1 min, and prepared as previously described (1). Leaf sections were placed in

flasks with 250 ml of the bacterial buffer suspension and continuously bubbled with CO_2 at 39 C for a maximum of 72 h. Control leaf sections were incubated in buffer (9) with constant CO_2 bubbling at 39 C for 72 h.

For observation of degraded forage tissue by the SEM, rumen microorganisms were prepared by two methods. (i) Whole rumen fluid was strained through four layers of cheesecloth and diluted with an equal volume of McDougall's carbonate-phosphate buffer (24). (ii) Strained, whole rumen fluid was mixed with an equal volume of phosphate buffer (20) and incubated in a separatory funnel for 1 h at 39 C to permit sedimentation of heavy feed particles and large protozoa. Leaf sections were placed in 250 ml of each of these inocula and continuously bubbled with CO_2 at 39 C for 4 or 6 h.

TEM. Leaf sections incubated with the bacterial inoculum and with control buffer (without microorganisms) were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.0 for 2 to 3 days and postfixed in 1.5% buffered osmuim tetroxide for 4 h. Leaf sections were prepared for the TEM as previously described (3).

SEM. Leaf sections incubated in the microbial inoculum for 4 to 6 h were fixed in 4% buffered glutaraldehyde for 30 h and postfixed in 1.5% buffered osmium tetroxide for 4 h. The degraded leaf sections were blotted free of excess moisture and affixed to SEM stubs so that the degraded edge of the cross-section was prominently displayed in a vertical position.

The stubs were quick-frozen in dry ice-isopropanol or liquid nitrogen and allowed to reach room temperature as the specimens were dried in a vacuum evaporator before coating with gold-palladium (60:40) alloy wire. The specimens were then observed in a field emission SEM at about 15 kV.

RESULTS

Control leaf sections observed with the SEM revealed that the buffer did not solubilize the tissues after 72 h of incubation (Fig. 1). As observed with the TEM, even the easily digested phloem cell walls were intact after 72 h of incubation in buffer (Fig. 2). However, an intact leaf section incubated with rumen microorganisms and observed with the SEM revealed differential tissue destruction after 4 h (Fig. 3). Mesophyll (M) and phloem (P) cell walls were removed to a point beyond the depth of focus of the SEM (at ×416); remnants of the outer bundle sheath (B) remained although this tissue had lost structural integrity. The epidermis (E) appeared to be partially degraded (arrow), whereas the sclerenchyma (S) and rigid vascular tissue (V) resisted microbial digestion.

The bacterium-plant cell wall association investigated at higher magnifications revealed differences dependent on the tissue type. Non-
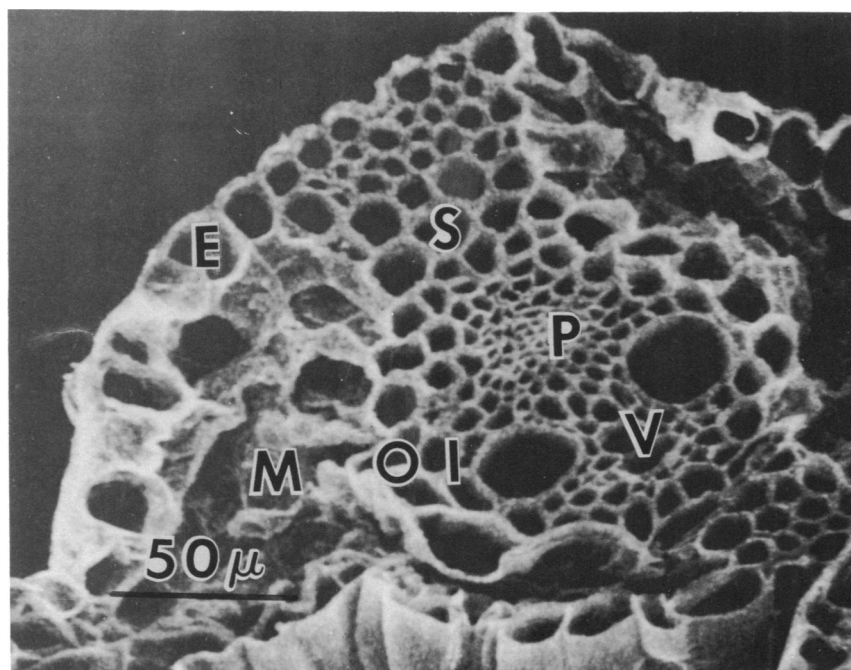

FIG. 1. *Control leaf section observed by the SEM after incubation for 72 h in buffer without rumen microorganisms. The rigid vascular tissue (V), phloem (P), inner (I) and outer (O) bundle sheaths, mesophyll (M), epidermis (E), and sclerenchyma (S) are intact. ×480.*
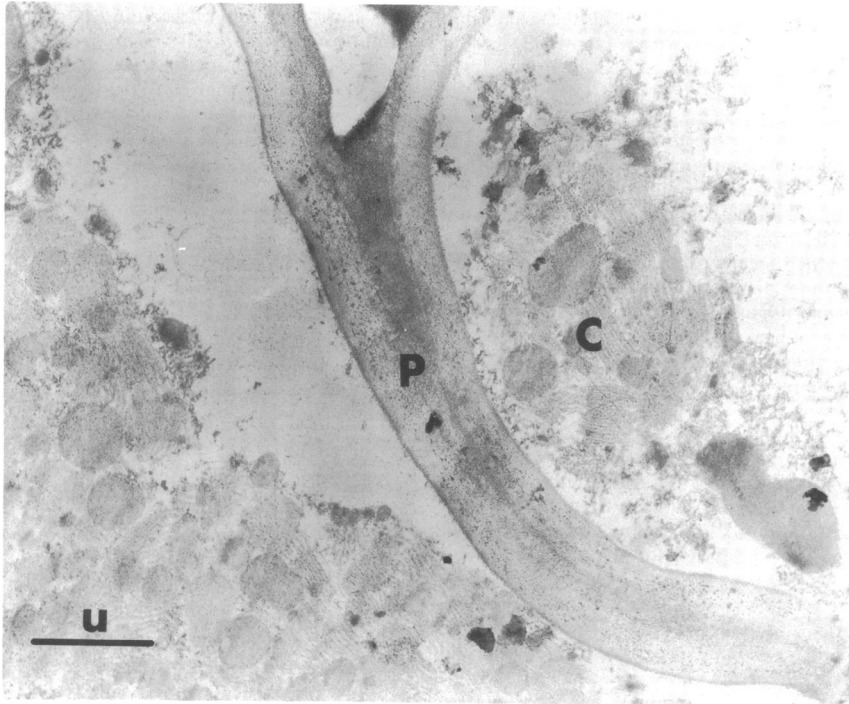
FIG. 2. *Control leaf section observed by the TEM after incubation for 72 h in buffer without rumen microorganisms. The phloem cell wall (P) is intact, and chloroplasts (C) are seen inside the plant cells.* ×16,000.
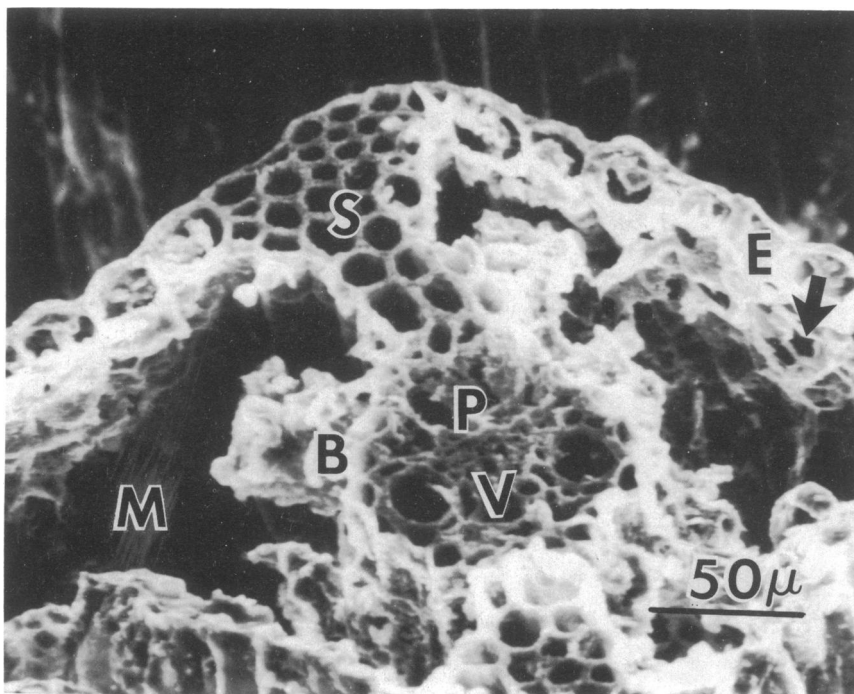

FIG. 3. *Leaf cross-section observed by the SEM after 4 h of incubation with rumen microorganisms. Mesophyll (M) and phloem (P) cells have been degraded. A portion of the outer bundle sheath (B) remains and the epidermis (E) is being degraded (arrow). The sclerenchyma (S) and rigid vascular tissue (V) are structurally intact, showing no signs of tissue destruction.* ×416.

descript areas of degradation were evident in the mesophyll cell walls near the bacteria but not necessarily in the location of bacterial association with the cell wall (Fig. 4, arrows). Similar phenomena were revealed by the TEM in that bacteria were seen near but not necessarily attached to the degraded areas in both mesophyll (Fig. 5, C) and phloem cells (Fig. 6, arrow). However, bacteria appeared at times to be attached by a dense extracellular substance to the mesophyll cell wall (Fig. 5, arrows).

The tissues digested after longer incubation times (i.e., the outer bundle sheath and epidermal cells) appeared to require bacterial attachment prior to tissue degradation. Rod-shaped bacteria, lying on the epidermal cell wall, were surrounded at times by an apparent zone of degradation (Fig. 7, arrows). The TEM revealed similar conditions with bacterial degradation preceded by attachment of bacterial cells apparently by an extracellular substance to the epidermal wall (Fig. 8). The zones of degradation were sharply defined in contrast to the diffuse zones in the mesophyll and phloem cells (Fig. 5 and 6). In addition, bacteria had tightly adhered to the outer bundle sheath cells (Fig. 9, B), and zones of hydrolysis surrounded rod-shaped bacteria in the inner bundle sheath cell

(Fig. 9, arrow). Attachment to the outer bundle sheath by a large coccus as shown by the TEM appeared to be mediated by an extracellular substance (Fig. 10, arrow); similar electron-dense substances were not apparent with the other three attached bacteria. Observations by SEM revealed a large coccus that appeared to be attached to the epidermal cell wall by rod-like appendages (Fig. 11, arrow).

DISCUSSION

That rumen microorganisms degrade the mesophyll and phloem cell walls more rapidly than other tissues has been shown for various forages (2). The current observations by the TEM and SEM, as well as previous ones by the TEM (3) on a grass of lower digestibility (i.e., Coastal Bermuda grass) (1), indicated that rumen bacteria at times degrade these tissues without prior attachment. The degradation of mesophyll and phloem cell walls by enzymes free from the surfaces of a specific microbe or group of microbes cannot be ruled out at this time. However, bacteria near degraded areas were diverse in morphology, indicating that no one species alone was involved. In addition, the bacterial attachment prior to degradation of bundle sheath and epidermal cells appeared to
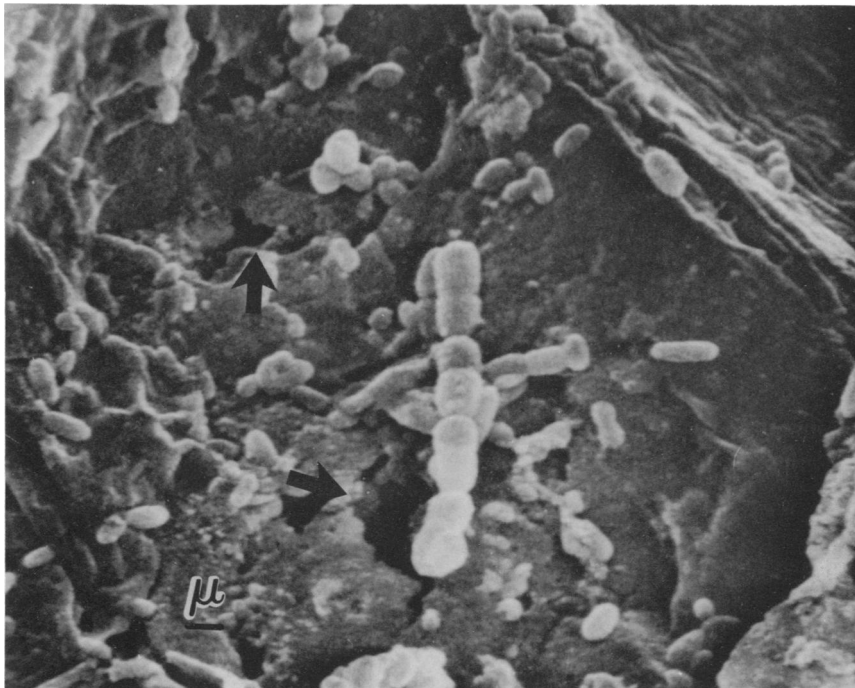

FIG. 4. *Mesophyll cell wall observed by the SEM after 6 h of incubation with rumen microorganisms. Zones of degradation (arrows) are seen in areas free from bacteria. The zones do not have a uniform shape which would be indicative of degradation with bacterial attachment. ×4,160.*
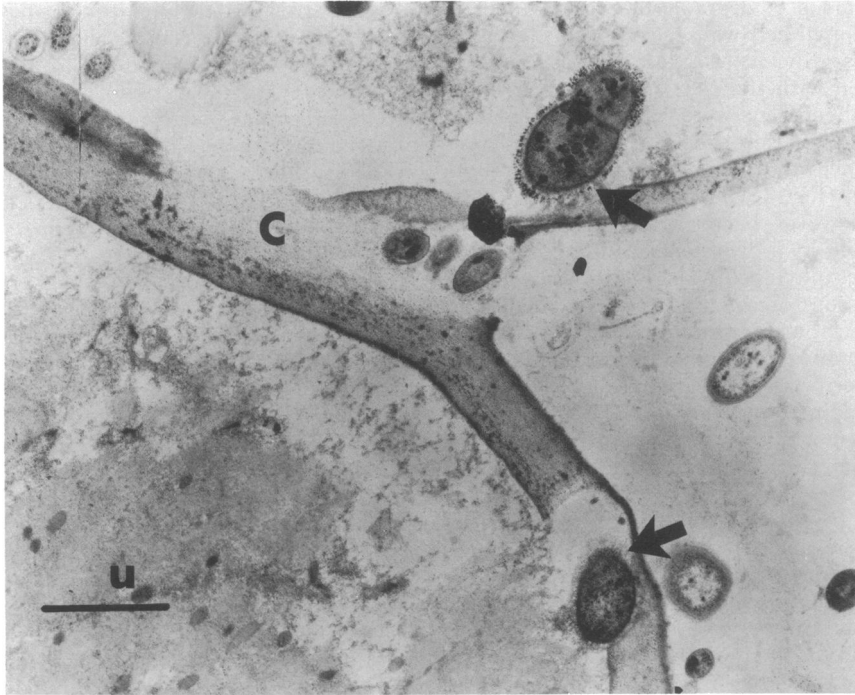
FIG. 5. *Mesophyll cell wall observed by the TEM after 4 h of incubation with rumen microorganisms. Diffuse, clear (C) areas where the plant cell wall has been degraded are seen but attachment by an extracellular substance to the plant wall appears to have taken place with two bacteria (arrows).* ×16,000.

FIG. 6. *Phloem cell wall observed by the TEM after 6 h of incubation with rumen microorganisms. The plant wall has been cleared (arrow) to a large extent; bacteria of diverse morphologies are near the area of degradation.* ×18,500.
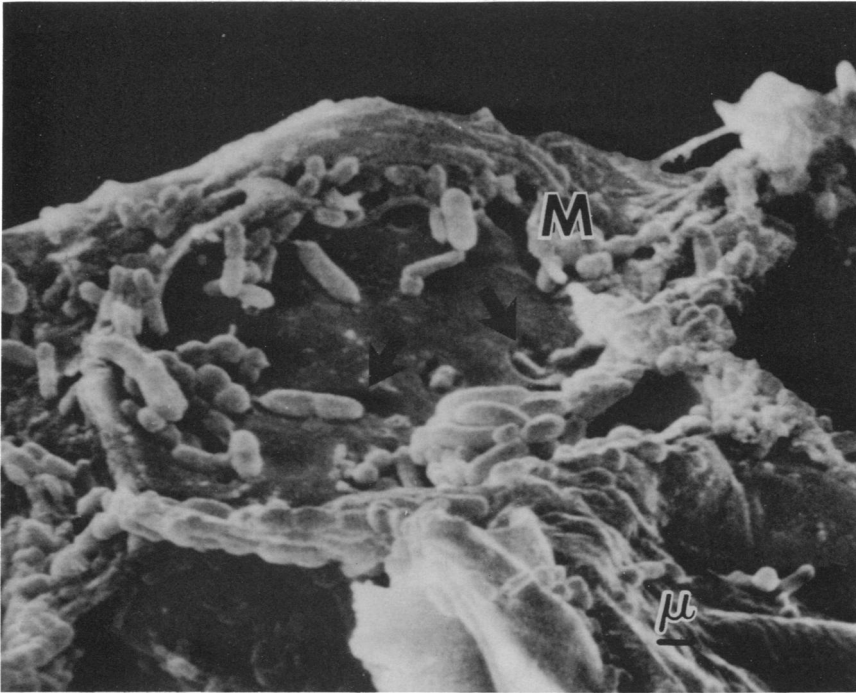
FIG. 7. *Epidermal cell observed by the SEM after 6 h of incubation with rumen microorganisms. Bacteria lying on the wall appear to be surrounded by depressed zones of degradation (arrows). Bacteria are localized en masse in regions (M) indicative of adhesion between cells.* ×4,160.
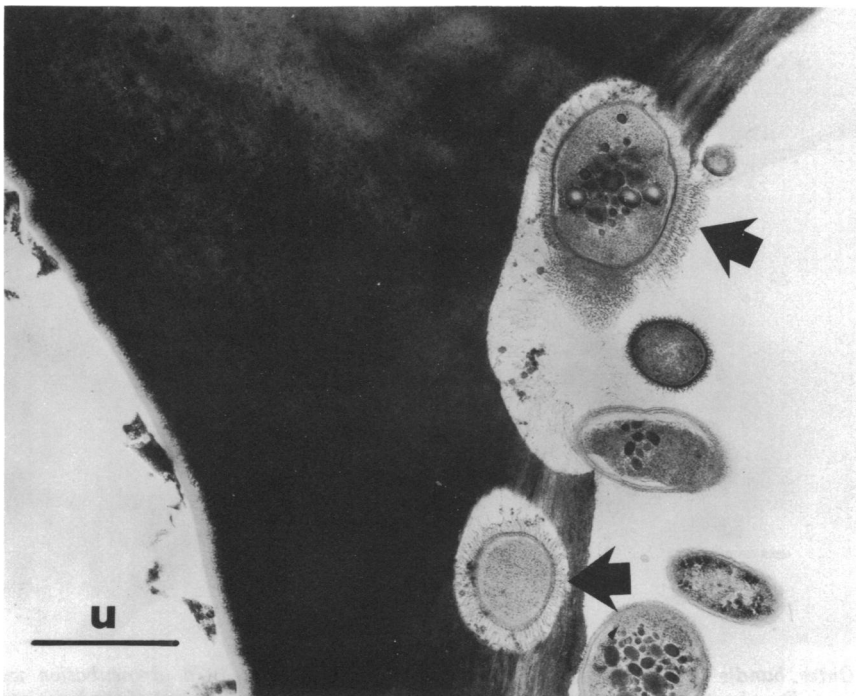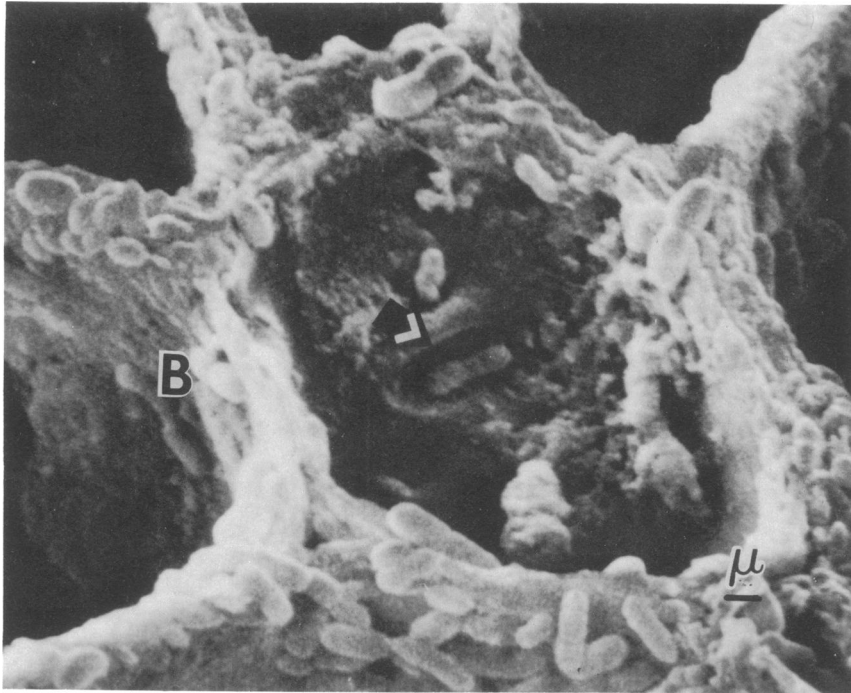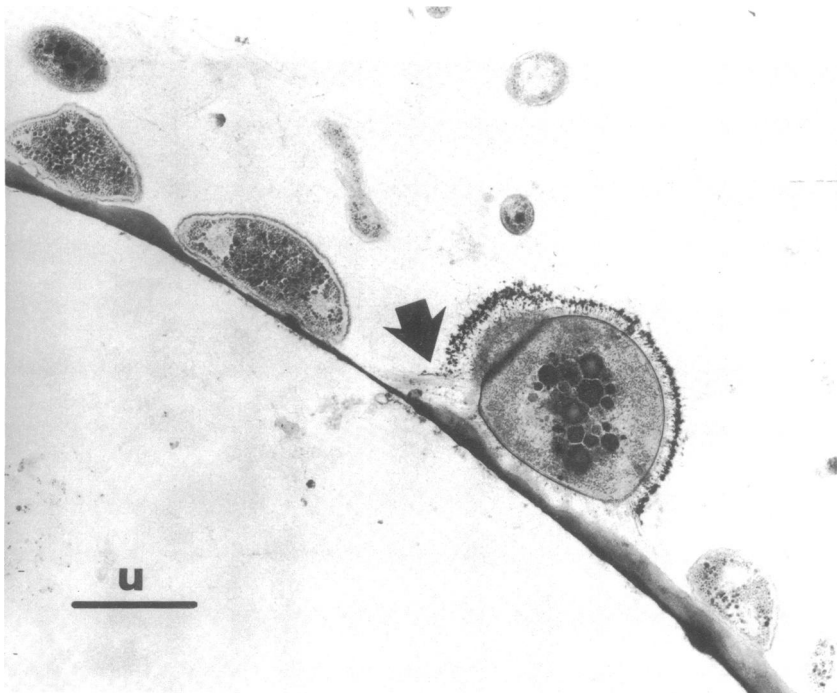

FIG. 8. *Epidermal cell observed by the TEM after 6 h of incubation with rumen microorganisms. Sharp zones of degradation are observed around the bacteria, two of which have an electron-dense, extracellular substance (arrows).* ×18,500.

FIG. 9. *Bundle sheath cells observed by the SEM after 6 h of incubation with rumen microorganisms. Bacteria (B) are closely associated with the outer bundle sheath cell wall, and rod-shaped bacteria are surrounded by depressed zones of hydrolysis in the inner sheath cell (arrow). ×4,500.*

FIG. 10. *Outer bundle sheath cell wall observed by the TEM after 12 h of incubation with rumen microorganisms. Four bacteria appear to be attached to the plant wall. Attachment of the large coccus to the plant wall is mediated by an electron-dense, extracellular substance (arrow), whereas no such structure is apparent with the rods. However, the attachment appears to be so close that the bacterial shape of the attaching side is modified. ×16,000.*
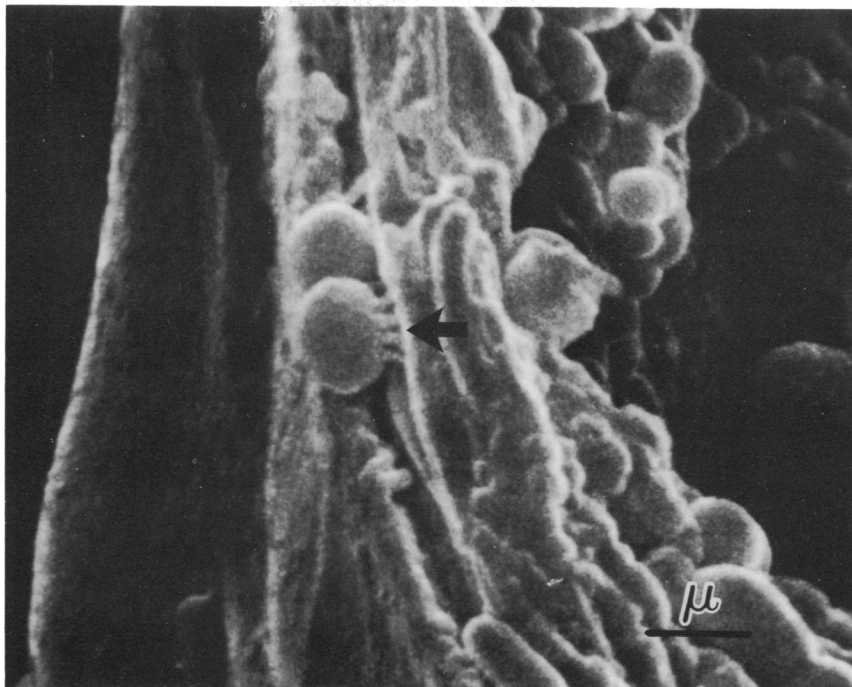
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FIG. 11. *Epidermal cell observed by the SEM after 6 h of incubation with rumen microorganisms. A large coccus appears to be associated with the plant wall by extracellular, rod-like appendages (arrow). Five such structures are observed between the bacteria and plant cell wall.* ×13,000.

occur with bacteria of diverse morphologies. Reports have shown that higher temperatures increase the cell wall constituents of tall fescue with a resultant decrease in in vitro digestibility (4, 17). Research should be undertaken to examine the bacterial attachment phenomenon relative to the cell walls of plants that have undergone environmental changes with resultant changes in the rate of digestibility.

The secretion of degradative enzymes has been reported for the rumen bacterium *R. albus* (23, 26). Smith et al. (26) reported that extracellular enzymes from *R. albus* digested up to 65% of a small quantity of ground or blended cellulose. The cell walls of the mesophyll and phloem may be so structurally different from bundle sheath and epidermal cell walls that cell-free enzymes, or fractions of the degradative enzyme complexes (22), can degrade the former tissues. In addition, Leatherwood (23) proposed that in *R. albus* an "affinity factor" may be necessary to hold the "hydrolytic factor" of cellulase in position to the insoluble cellulose for multiple attacks to occur. Such a phenomenon may indeed be required for hydrolysis of the cell walls more resistant to bacterial degradation where attachment precedes degradation.

Previously we reported that large rumen cocci possessed an electron-dense extracellular substance that appeared to adjoin the bacterial and plant cell walls (3). Leatherwood (23), using the SEM, reported tube-like appendages associated with *R. albus* cells only when grown on cellulose-containing media. We found similar structures in a large coccus that apparently was attached to the epidermal cell wall as shown by the SEM (Fig. 11). The rod-like appearance and amorphous, capsular-like appearance of this extracellular feature by the SEM and TEM, respectively, of the large cocci may be the same structure manifested in various ways by different drying techniques. Springer and Roth (27), using the TEM, had reported that the length and width of fibrils of capsules seen in *Klebsiella pneumoniae* varied with the dehydrating procedures. Perhaps electron microscopy of critical-point-dried (5) samples would elucidate these extracellular features in rumen bacteria as has been shown recently for other bacterial species (8).

Jones et al. (21) and Fletcher and Floodgate (18) have reported the attachment of bacteria to substrates by extracellular substances. In addition, Shilo (25) showed that close contact be-

tween myxobacteria and the blue-green algae was required for algal lysis, although no capsule-like material was reported. Berg et al. (6) reported that *Cellvibrio fulvus* and *Sporocytophaga myxococcoides* cells grown on different types of cellulose media adjoined the cellulose fibers with distinct depressions made in the substrate; *S. myxococcoides* produced extracellular substances interpreted as bacterial envelopes when grown on cellulose. Costerton et al. (11) reported that three gram-negative rumen bacteria, *B. ruminicola*, *B. succinogenes*, and *M. elsdenii*, possessed extracellular coats outside the cell envelope. Although these authors (11) concluded that the extracellular coats provided protection in a highly competitive environment, our current and previous (3) observations indicated that an extracellular substance may also mediate the attachment of rumen bacteria to particular plant cell walls so that degradation may occur. Although attachment of large cocci to substrate by capsule-like material was noted often, we have not observed extracellular substances in all attached bacteria. It is possible that the coats are thin in some cases (11) and not seen without specific staining (i.e., with ruthenium red).

Costerton et al. (12), reviewing the cell envelope of gram-negative bacteria, reported that the degradative enzymes associated with the cell wall provide a "facility unique among unicellular organisms in that complex food molecules are broken down into their component monomers in a zone immediately surrounding the cell." Our observations suggest that attachment to intact plant cell walls, mediated by extracellular substances in at least some rumen bacteria, is required before the hydrolytic fraction of the enzymes can degrade the complex organization of certain forage cell walls. Such a phenomenon would help explain why certain forages, whose microanatomy consists of a high ratio of bundle sheath and epidermal to mesophyll and phloem cells, are less rapidly digested than forages with a lower ratio of these tissues.

ACKNOWLEDGMENT

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