Rumen Bacterial Interrelationships with Plant Tissue During Degradation Revealed by Transmission Electron Microscopy

DANNY E. AKIN, DONALD BURDICK, AND GENE E. MICHAELS

Richard B. Russell Agricultural Research Center, Athens, Georgia 30604, and Department of Microbiology, University of Georgia, Athens, Georgia 30602

Received for publication 18 January 1974

The mode of rumen bacterial degradation of cell walls in coastal bermudagrass [Cynodon dactylon (L) Pers.] differed with the plant tissue type. Bacteria degraded thin, primary cell walls of mesophyll and phloem apparently by extracellular enzymes and without prior attachment; thick-walled bundle sheath and epidermal cells apparently were degraded after bacterial attachment, in some types by an extracellular substance, to the plant cell walls. Rumen bacteria split the nondegraded cuticle from the epidermis by preferentially attacking the cell just underneath the cuticle. The propensity for bacterial attachment to lignified cells of the vascular tissue was low, and bacterial degradation of these cells did not occur after 72 h of incubation.

In providing energy and protein to the rumen, bacteria in the rumen degrade tissues of forages. The various tissues, however, differ in the rate and extent to which they are degraded. For example, the protective cuticle, which covers the epidermis, and the thick-walled lignified tissues of the plant resist degradation (3, 8). Conversely, the thin-walled mesophyll and phloem tissues are readily degraded by rumen bacteria prior to other tissues (1, 10).

Although the digestibility of empirically-isolated fractions of cell walls has been investigated using pure (6) and mixed (2, 7) cultures of rumen bacteria, investigations of sufficient resolution and magnification to reveal differences in the physical interrelationship of rumen bacteria to various plant tissues have not been reported. Information of this nature would be useful in explaining the mode of bacterial degradation of various plant tissues and the limitations imposed on bacterial degradation by resistant tissues. The present study was undertaken to investigate the physical interrelationship between rumen bacteria and degradable and nondegradable tissues of the forage grass coastal bermudagrass [Cynodon dactylon (L) Pers.] by transmission electron microscopy.

MATERIALS AND METHODS

Microorganisms. To obtain a representative rumen bacterial population, rumen contents were removed 4 h after feeding from two permanently fistulated steers maintained on a diet of coastal bermudagrass hay. The rumen contents were squeezed through four layers of cheesecloth until 3 liters of rumen fluid were collected in thermos bottles preheated to 39 C. The rumen fluid was centrifuged at 250 x g for 1 min to remove large feed particles and diluted with an equal volume of CO₂-saturated, deionized water. Bacteria were then sedimented from the rumen fluid using continuous-flow centrifugation at 16,000 x g. The microorganisms were resuspended in the CO₂-saturated phosphate-carbonate buffer of Cheng, Hall, and Burroughs (5) so that the final volume was one-half that of the original rumen fluid.

Substrate. Leaf sections of coastal bermudagrass frozen in dry ice immediately after harvesting and stored at -30 C were cut into 5-mm lengths. The sections were incubated with 250 ml of the rumen bacterial-buffer suspension at 39 C while the contents were continually flushed with CO₂. Leaf samples were removed after 6, 12, or 72 h of rumen bacterial degradation. Control leaf samples were incubated as previously described for 72 h in the buffer without rumen microorganisms.

Transmission electron microscopy. Leaf samples removed from the incubation and control flasks were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer for 3 to 5 days, washed in buffer, and postfixed in 1.5% buffered osmium tetroxide for 4 h. Samples were then washed with buffer and dehydrated in a graded series of ethanol-water washes (25, 50, 75, 95, and 100% vol/vol), followed by washing in 100% propylene oxide. They were then infiltrated with Spurr epoxy resin (20) for 2 to 3 h before embedding in fresh epoxy resin. Samples were polymerized at 65 to 70 C for 8 to 12 h. Ultrathin sections were cut at 60 to 80 nm and poststained with 2% aqueous uranyl acetate and lead citrate (21). Sections were observed in a transmission electron microscope at 75 kV.

RESULTS

Micrographs of sections from control samples
(Fig. 1 and 2) indicate that none of the tissues have been solubilized or removed by buffer after 72 h.

The physical relationship of rumen bacteria to plant cell walls during degradation varies with plant tissue type. Bacteria appear to degrade portions of the thin-walled mesophyll cells and at times the phloem tissue without prior attachment to the plant cells (Fig. 3 and 4).

Conversely, rumen bacteria appear to attach to the thick cell walls of the epidermis and bundle sheath prior to the degradation of these tissues (Fig. 5, 6, and 7). An undefined, extracellular substance that is electron dense in the micrographs appears to connect some of the large coccoid to the plant cell wall (Fig. 6 and 7, arrows). However, the extracellular substance is not observed between plant cell walls and the small coccoid or bacilli (Fig. 5 and 6). Rumen bacteria lose their uniform coccolid or rod-like morphology at times when they are closely adhering to the plant cell walls, and the point of junction in many cases is indistinguishable (Fig. 5, arrow).

In degrading epidermal cells, rumen bacteria preferentially first degrade the region immediately below the cuticle (Fig. 7). Eventually, the bacteria degrade all the epidermal cell wall except the protective cuticle. The bacteria do not attach to the cuticle, which is dissociated from the epidermis during degradation.

Large numbers of rumen bacteria do not attach to the nondegradable tissues containing lignin (polymers of substituted phenylpropanes) in the vascular bundles (Fig. 8). The connective, extracellular substance is present at times between coccoid and lignified plant cells, but although the bacteria apparently attach to the plant cell walls, the adherence is not such that the bacteria lose their uniformity of shape (Fig. 8, arrow). Even after 72 h, when all degradable tissues have been removed, large numbers of rumen bacteria have not readily attached to or degraded the lignified cells of the vascular bundle.

Rumen bacteria do appear to attach to and degrade the peripheral cells of the sclerenchyma, which are thick-walled supportive cells containing lignin (Fig. 9). However, this tissue does not appear to be easily degraded and bacteria in the plant cell lumen do not readily attach to the cell wall (Fig. 9, arrow).

DISCUSSION

Previous light (10) and electron microscope (1) observations have shown that rumen bacteria degrade the mesophyll and phloem prior to the other tissues in forage grasses. In the present study, it was observed that the cell walls of

Fig. 1. Control leaf section incubated for 72 h in buffer without rumen microorganisms. Note the intact bundle sheath cell wall (B), mesophyll (M) and sclerenchyma (S). x9,800.
Fig. 2. Control leaf section incubated for 72 h in buffer without rumen microorganisms. Note the intact phloem cell walls showing no loss in cell wall integrity due to buffer alone. ×12,600.

Fig. 3. Rumen bacterial degradation of cell walls of the mesophyll without bacterial attachment at 12 h of incubation. Bacteria are near mesophyll cell walls which have become diffuse and have lost structural integrity (arrow), but bacteria are not attached to these plant cell walls. Large, thick-walled bundle sheath cells show no evidence of degradation. ×10,080.
Fig. 4. Rumen bacterial degradation of cell walls of the phloem without bacterial attachment at 12 h of incubation. Cleared zones (arrows) along the plant cell wall are present but nearby bacteria have not attached to the phloem cells. ×28,800.

Fig. 5. Rumen bacterial attachment and degradation of thick-walled bundle sheath cells at 12 h of incubation. The association between bacteria and plant cell wall is so close that the point of juncture between bacterial and plant wall is indistinguishable at times (arrow). ×17,000.
Fig. 6. Rumen bacterial attachment and degradation of bundle sheath cells at 12 h of incubation. The large cocci appear to attach to the plant cell wall by means of an extracellular substance (arrow). Other bacteria maintain close association to the plant cell wall without evidence of such a substance. ×12,000.

Fig. 7. Rumen bacterial attachment and degradation of thick-walled epidermal cells underneath the cuticle (C) at 12 h of incubation. The large coccus appears to attach to the plant cell wall by an extracellular substance (arrow) and bacteria of various shapes are located in the zone of degradation. ×30,000.
**FIG. 8.** Rumen bacterial association with lignified tissue in the vascular bundle at 12 h of incubation. A large coccus appears to be loosely attached to the lignified cell walls (arrow); no degradation of the lignified tissue has taken place although the degradable tissues have been removed. ×9,000.

**FIG. 9.** Rumen bacterial association with sclerencyma at 12 h of incubation. The bacteria attach to and degrade cell walls in the periphery of the sclerenchyma, but bacteria (arrow) in the lumen of the cells appear inactive. ×10,080.
these tissues were degraded in many cases without bacterial attachment of nearby bacteria. It is not likely that previously attached, degrading bacteria were removed or displaced from the mesophyll or phloem cells during preparation. Such displacement would result in cavities or zones of hydrolysis resembling the bacterial morphology in the cell walls as Baker and Harriss (3) reported. Instead of cavities, the degraded portions of the thin cell walls had no uniform shape but extended along the cell wall. Furthermore, bacterial displacement with the resulting cavities was not prevalent in other types of plant cell walls as shown by the numbers of attached bacteria (Fig. 5 and 6).

Conflicting results have been reported from attempts to determine the presence of extracellular enzymes in rumen fluid free of rumen bacteria (9, 10, 14, 19). Our transmission electron micrographs indicate that enzymes free from bacterial surfaces can partially degrade the primary cell walls of mesophyll and phloem but not the thick-walled cells of other tissues. Recently, Smith, Yu, and Hungate (19) showed the effect of adsorbence by cellulse from Ruminococcus albus on the hydrolysis of cellulose. The degradation of the thin-walled cells may be caused by greater enzyme adsorption due to the increased surface area or to the particular arrangement of the plant cell wall carbohydrates. Shafizadeh and McGinnis (17) indicated that the microfibrillar framework of cellulose in the primary cell walls was loose, with the fibrils having a low and non-uniform degree of polymerization. Therefore, it is possible that extracellular enzymes may readily penetrate and hydrolyze these cell wall carbohydrates.

Attachment of rumen bacteria to thick-walled cells prior to degradation appeared to be mediated by a dense, extracellular matrix that firmly bound the large cocci to the plant cell wall. Degradation of plant tissue occurred subsequent to attachment. Baker and Harriss (3), using light microscopy, observed rumen bacteria surrounded by clear zones resulting from degraded plant tissues. Hungate (12) reported that cellulolytic rumin bacteria attach to plant particles in the rumen digesta but the extent of occurrence is unknown. Other research indicated that cellulase is bound to rumen bacterial cell surfaces as shown by the cellulolytic activity that resulted from subsequent lysis of particulate particles of rumen bacteria treated in pressure cells (13). Perhaps the extracellular matrix is important in securing the bacterial cells close enough to the rigid plant cell wall for surface-bound, hydrolytic enzymes to degrade plant wall carbohydrates.

The preferential attack by rumen bacteria of the epidermis just beneath the plant cuticle was consistent in this study. Previous reports indicated that this region of the epidermis is higher in pectin content in some plants (16) whereas in other species the microfibrillar arrangement of cellulose differs (4). Similar phenomena could exist in bermudagrass that would facilitate bacterial degradation of the epidermal cells. The electron microscope observations confirmed the results of others (3, 8, 15) that the cuticle resists degradation by rumen microorganisms and established that rumen bacteria do not attach to it.

Baker and Harriss (3) and Drapala et al. (8), using light microscopy, reported that lignified tissue is not degraded in the rumen by bacteria. However, release of phenolic compounds from grass cell walls by fungal cellulases has been reported (11). Gravimetric determinations of ground forage samples, furthermore, have indicated loss of lignin after extended digestion times (7, 18), but this may be related to solubilization rather than digestion. The present study revealed that rumen bacteria for the most part do not attach to the lignified tissue of the vascular bundle and confirmed previous research (1) that degradation did not occur even after 72 h in coastal bermudagrass. In instances where bacteria appeared to attach to lignified cells, the association was not so close that the uniformity of bacterial shape was lost such as occurred with the degradable tissue.

ACKNOWLEDGMENT

Appreciation is expressed to Henry E. Amos, Animal Physiologist, Russell Research Center, for assistance with the collection and preparation of the rumen microbial inoculum.

LITERATURE CITED

1. Akin, D. E., H. E. Amos, F. E. Barton II, and D. Burdick. 1973. Rumen microbial degradation of grass tissue revealed by scanning electron microscopy. Agron. J. 65:825–828.
2. Bailey, R. W., and D. I. H. Jones. 1971. Pasture quality and ruminant nutrition. III. Hydrolysis of ryegrass structural carbohydrates with carbohydrates in relation to rumen digestion. N.Z. J. Agr. Res. 14:847–857.
3. Baker, F., and S. T. Harriss. 1947. Microbial digestion in the rumen (and caecum), with special reference to the decomposition of structural cellulose. Nutr. Abstr. Rev. 17:3–12.
4. Chafe, S., C., and A.B. Wardrop. 1972. Fine structural observations on the epidermis. I. The epidermal cell wall. Planta 107:269–278.
5. Cheng, E. W., G. Hall, and W. Burroughs. 1965. A method for the study of cellulose digestion by washed suspensions of rumen microorganisms. J. Dairy Sci. 38:1225–1230.
6. Coen, J. A., and B. A. Dehority. 1970. Degradation and utilization of hemicellulose from intact forages by pure cultures of rumen bacteria. Appl. Microbiol. 20:362–368.
7. Dekker, R. F. H., G. N. Richards, and M. J. Playne. 1972. Digestion of polysaccharide constituents of tropical herbage in the bovine rumen. I. Townsville Stylo (Stylosanthes humilis). Carbohydr. Res. 22:173-185.
8. Drapala, W. J., L. C. Raymond, and E. W. Crampton. 1947. Pasture studies. XXVII. The effects of maturity of the plant and its lignification and subsequent digestibility by animals as indicated by methods of plant histology. Sci. Agr. 27:36-41.
9. Gill, J. W., and K. W. King. 1957. Characteristics of free rumen cellulases. J. Agr. Food Chem. 5:363-367.
10. Hanna, W. W., W. G. Monson, and G. W. Burton. 1973. Histological examination of fresh forage leaves after in vitro digestion. Crop Sci. 13:98-102.
11. Hartley, R. D., E. C. Jones, and T. M. Wood. 1973. Comparison of cell walls of Lolium multiflorum with cotton cellulose in relation to their digestion with enzymes associated with cellulolysis. Phytochemistry 12:763-766.
12. Hungate, R. E. 1966. The rumen bacteria, p. 8-90. In R. E. Hungate (ed.), The rumen and its microbes. Academic Press Inc., New York.
13. King, K. W. 1959. Activation and cell-surface localization of certain β-glucosidases of the ruminal flora. J. Dairy Sci. 42:1848-1856.
14. Krishnamurti, C. R., and W. D. Kitts. 1969. Preparation and properties of cellulases from rumen microorganisms. Can. J. Microbiol. 15:1373-1379.
15. Monson, W. G., J. B. Powell, and G. W. Burton. 1972. Digestion of fresh forage in rumen fluid. Agron. J. 64:231-233.
16. Roelofson, P. A. 1959. Cell-wall substances of lipid character, p. 86-90. In The plant cell wall. Gebrüder Borntraeger, Berlin-Nikolassee.
17. Shafizadeh, F., and G. D. McGinnis. 1971. Morphology and biogenesis of cellulose and plant cell walls, p. 297-349. In R. S. Tipson (ed.), Advances in carbohydrate chemistry and biochemistry, vol. 26. Academic Press Inc., New York.
18. Smith, L. W., H. K. Goering, and C. H. Gordon. 1972. Relationships of forage compositions with rates of cell wall digestion and indigestibility of cell walls. J. Dairy Sci. 55:1140-1147.
19. Smith, W. R., I. Yu, and R. E. Hungate. 1973. Factors affecting cellulolysis by Ruminococcus albus. J. Bacteriol. 114:729-737.
20. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-45.
21. Venable, J. H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407-408.