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Electron Microscopy of the Microbial Populations Present and Their Modes of Attack on Various Cellulosic Substrates Undergoing Digestion in the Sheep Rumen

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Cotton fibers and various cell wall preparations from grass leaves and from the feces of sheep fed on dried grass were placed in the sheep rumen in bags made from 5-μm-mesh nylon cloth. After periods of from 3 to 48 h, bags were removed, and the contents were fixed, embedded, sectioned, and stained for electron microscopy. Some of the bacteria present were seen to be closely associated with the cell walls, either tunneling within them or making very close contact. Evidence was obtained for differential digestion of cell walls and of the layers within them. Distinct differences were noticed between bacterial populations attacking the more susceptible wall types and those attacking feces cell walls and cotton fibers. Among the latter, the dominant form was a long, thin rod with a typical gram-negative cell wall structure, different from that described for *Bacteroides succinogenes* S85 or for *Butyrivibrio fibrisolvens*.

Thirty years ago Baker and Nasr wrote of a radically new approach to the digestibility of feeding stuffs, using direct microscopy, the aim being "to restate the problem of digestibility in structural terms and to correlate the gross- and fine-structural organization of the cellulosic materials fed to the animals with the degree of maturity, regional characteristics and manurial status, of different fodder plant species" (6). Baker and his collaborators had demonstrated that some rumen bacteria establish a close association with the fibrous components of the feed. The resolving power of the light microscope was not sufficient to show this association clearly, and, perhaps for this reason, little further research was done for many years, although the transmission electron microscope, with continually improving techniques for specimen preparation, was available.

In 1973 Akin and his colleagues published the results of electron microscope studies of grass leaf sections undergoing digestion by rumen organisms in vitro (3). Their subsequent publications have described some of the features of microbial attack on plant cell walls under these conditions (1, 4) and also in the rumen (2). Emphasis has been placed by them and others (20, 26) on the adhesion of the bacteria to insoluble substrates.

We have been using the nylon bag technique to study the digestion of the polysaccharides in grass cell wall preparations in vivo (24). Here we report some observations on ultrathin sections made through samples of grass cell walls and other materials incubated for various lengths of time in the sheep rumen.

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MATERIALS AND METHODS

Materials. Ryegrass (*Lolium perenne* or *L. multiflorum*) was taken from pastures, and the cells were broken mechanically by grinding in liquid nitrogen in a mortar or without freezing in a roller mill, which gave essentially similar results. The cell contents were washed away by elutriation (5) or on the roller mill (15), using an aqueous medium containing a nonionic detergent (0.1% Triton X-100) and a mild antibacterial agent (0.1% Givgard DXN). The walls were further washed with water and either freeze-dried or dehydrated with a sequence of ethanol, acetone, and light petroleum. Yields and full chemical analyses of the products (15), as well as the digestibilities of various constituents, including sugars, in the sheep rumen, as measured by the nylon bag technique (24), have been described previously. Cell walls were also isolated by similar procedures from the feces of sheep fed on a dried grass diet (24).

Whatman no. 1 filter paper was treated for 15 s (dry) in an homogenizer-type mill (J & K IKA analytical mill A10; Janke & Kunkel KG, Staufen i. Breisgau, West Germany) to disperse the fibers.

In vivo digestion. Samples of cell walls (200 to 500 mg) were placed in bags made from 5-μm-mesh nylon cloth (A. J. Polak Ltd., London, United Kingdom). A strip of this cloth 200 mm wide was folded lengthwise, and the edges were sealed with a latex adhesive to form a tube; the top was left open, and the bottom was sealed in a semicircle to give a maximum depth of

200 mm. In use, the open end was gathered and fastened with a rubber band. Polypropylene string, sufficient to give a free length of 300 mm, was tied securely around this and attached to a freely rotating wire ring in a screw cap on a 40-mm rumen cannula. The bags were wetted, introduced into the rumen, submerged in the digesta, and left for periods of 3 to 72 h. After removal, they were washed externally with cold water from the tap and opened, and a sample for microscopy was immediately suspended in glutaraldehyde solution.

Preparation for microscopy. Material was fixed for 3 h at 4°C in a solution of 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) before postfixation in Palade osmium tetroxide solution for 1 h; other samples were treated with ruthenium red during fixation (22). All material was rinsed and centrifuged in molten agar at about 50°C; the pellet, now held in agar gel, was cut into 1-mm cubes, dehydrated with ethanol, and embedded in Araldite or Spurr resin.

Microscopy. Survey sections (1 μm) were stained with toluidine blue and examined by light microscopy. Silver/gold sections cut from selected areas with an LKB Ultratome III and stained with uranyl formate and lead citrate were examined in an AEI 801A electron microscope and in a Philips EM400 microscope with an eucentric goniometer stage.

RESULTS

Examination of sections through undigested cell wall preparations showed that almost all of the cells were devoid of contents. Wall thickness varies greatly in the grass leaf, from 0.1 μm in the mesophyll cells (15) to 2.0 μm or more in the fiber elements. All of the walls were seen to be composed of two or more layers, distinguished by staining or by the orientation of the microfibrils. Cotton fibers, from which Whatman no. 1 filter paper is made, have walls of up to 10 μm in thickness.

The majority of bacteria present in digested samples were spheres or rods, with diameters between 0.3 and 1.0 μm and ratios of length to breadth of as high as 10.

A number of possible modes of attack of bacteria on the fiber can be visualized. Here we confine our attention to two.

Tunneling action. Bacteria were repeatedly observed in cavities within the depth of the secondarily thickened walls in grass cell wall preparations (Fig. 1); the type of plant cell could not always be identified. The innermost layer of these walls and the layers adjacent to the middle lamella seemed to resist digestion (Fig. 2 and 3). The middle lamella was also resistant in pits (Fig. 4), but apparently not where spiral or annular thickening was present (Fig. 5).

The organisms responsible for the tunneling action were usually cocci, with dimensions of 0.85 by 0.55 μm and with complex wall structures resembling those illustrated in bacteria taken from the rumen (11) and seen in some pure cultures of ruminococci grown for us by C. S. Stewart.

Erosion of surfaces. The wall texture created by the parallel orientation of cellulose microfibrils makes it possible to distinguish the shallow cavities produced by overlying bacteria from mere flexure of the wall (Fig. 6 and 7). This kind of attack was predominant when cotton fibers or feces cell walls were being digested. At least three different morphological types of bacteria were seen.

By far the most abundant form (a) associated with erosive action was a thin rod, 0.3 to 0.4 μm in cross section. These organisms were rarely seen in their entirety; lengths of 5 μm or longer sometimes lay within sections. Each organism was closely associated with a cotton fiber, a small but variable gap between the fiber and the bacterium (up to 0.1 μm) being crossed by occasional strands of material stainable with ruthenium red (Fig. 6). Groups of the bacteria lay side by side, their long axes parallel to the long axis of the plant fiber (Fig. 7). In the many sections examined, no signs of cell division were ever seen. These particular bacteria were never seen when grass cell wall preparations were being digested. Their very characteristic cell wall structure (see below) would have made them very easy to recognize.

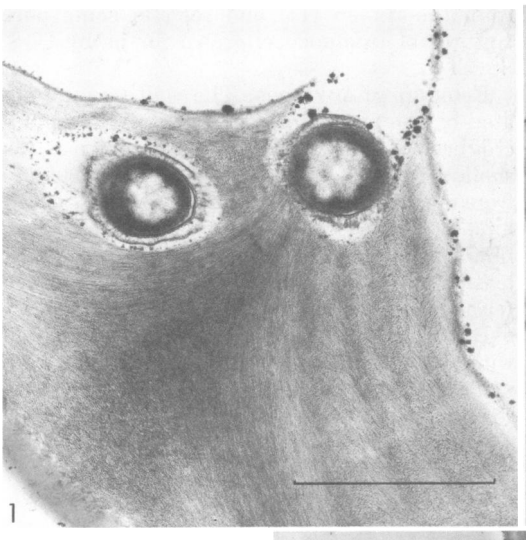
A less abundant type (b), circular in cross section (0.4 μm), was seen in contact with the fibers (Fig. 8).

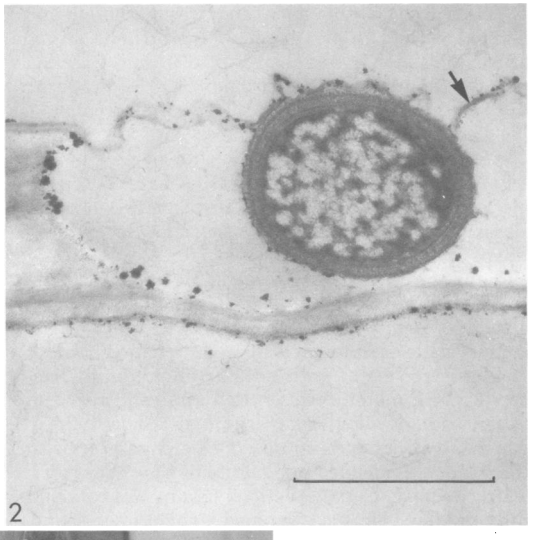
Another less abundant form (c), associated with the others but apparently not causing erosion, consisted of sharply curving cells (0.15 μm in cross section) of indefinite length, with a loose outer envelope (Fig. 9).

In addition, particularly after longer periods of digestion, many other types could be seen in the sections, usually at a distance from the fibers. Cocci like those present during grass cell wall digestion were rarely seen.

Morphology of cellulose colonizers. The rodlike organism (a) described above had a characteristic speckled appearance by either uranium-lead or ruthenium red staining. This was due to short protrusions from the surface, not long enough to make contact with the substrate; in tangential sections the effect was very striking (Fig. 10).

In sections stained with ruthenium red, the cytoplasmic membrane and a double outer membrane were clearly differentiated (Fig. 11). The zone between them (about 20 nm wide) was subdivided by a single, well-defined electron-dense layer running parallel to the cytoplasmic membrane. The outer membrane had few inflections, and the speckled effect was due to the





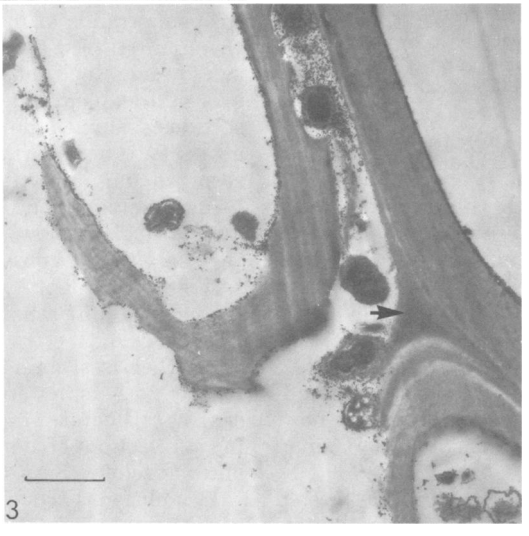


FIG. 1. *Thin section through grass cell wall digested for 7 h. The cavities do not seem to be influenced by the orientation of the fibrils and have well-defined boundaries. Bar marker = 1 μm.*

FIG. 2. *Thin section through grass cell wall digested for 6 h. The extent of erosion is indicated by the unattacked portion. A thick wall (0.9 μm) has been attacked, leaving only the tertiary wall as a thin distorted layer (arrow), while the contiguous wall (0.1 μm) is not touched. Bar marker = 1 μm.*

FIG. 3. *Thin section of grass cell wall digested for 12 h. Bacteria are seen attacking one wall from within, but the middle lamella, which has a characteristic triangular section at the cell junction (arrow), protects the contiguous wall. Bar marker = 1 μm.*

periodic occurrence of densely stained regions in its outer layer.

The second type (b) had a simple wall structure which could be interpreted as gram positive. It was always circular or slightly oval in sections and was further distinguished from the rod (a) by a halo of fibers which seemed to attach it to the substrate.

The curved organisms (c) passed in and out of the ultrathin sections; therefore, their total lengths were difficult to determine. A trilamellar structure was visible in the cytoplasmic membrane and in the very loose outer membrane. This organism has the morphological features of a spirochete and appears to be that first described by Hungate (17) and later isolated and identified by Bryant (9) as a treponeme (*Borrelia*) characteristically associated with cultures of cellulolytic bacteria, but not itself cellulolytic. The protoplast is wound in a very tight helix, and the outer membrane is well separated. Such a wide separation has been shown to be an

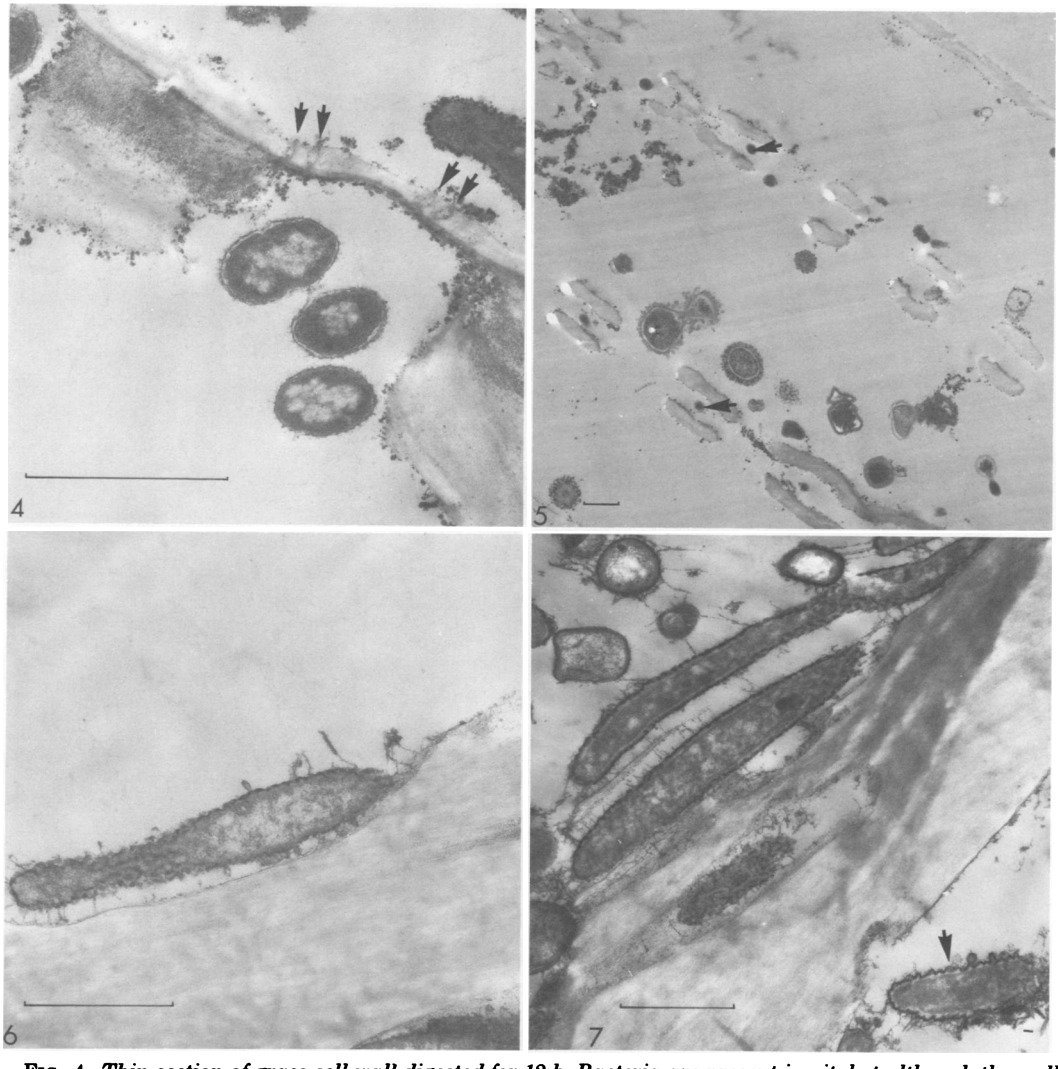


FIG. 4. *Thin section of grass cell wall digested for 12 h. Bacteria are present in pit, but although the wall is eroded elsewhere the pit lining and the contiguous wall are unattacked. (Arrows indicate plasmodesmata, which confirm the interpretation of wall structure.) Bar marker = 1 μm.*

FIG. 5. *Thin section of grass cell wall digested for 24 h. A wall with annular or spiral thickening has lost the primary walls and associated structures. Bacteria with very small cross sections are seen in this region (arrows). Bar marker = 1 μm.*

FIG. 6. *Thin section through filter paper digested for 72 h (ruthenium red stained). Erosion is demonstrated by the microfibrillar structure in the cotton cell wall. Bar marker = 1 μm.*

FIG. 7. *Thin section of filter paper digested for 48 h (ruthenium red stained). Several rodlike bacteria of type (a) described in the text are eroding the cotton fiber. An organism (arrow) has a wall structure similar to B. succinogenes S85 and may have produced the small cavity adjacent to it. Bar marker = 1 μm.*

artifact in some myxobacteria (25), but is probably not so here, because in our sections the outer membranes of other gram-negative organisms accompanying it were always closely applied to the cell body. It is interesting that this organism is very frequently associated with the cellulolytic rods in the rumen, just as it is in culture.

DISCUSSION

The technique that we have used permits the insertion into the rumen of small quantities of substrate which need not be representative of the diet undergoing digestion (13); in all of our experiments, the sheep were receiving a good-quality dried grass. Withdrawal of the bags at

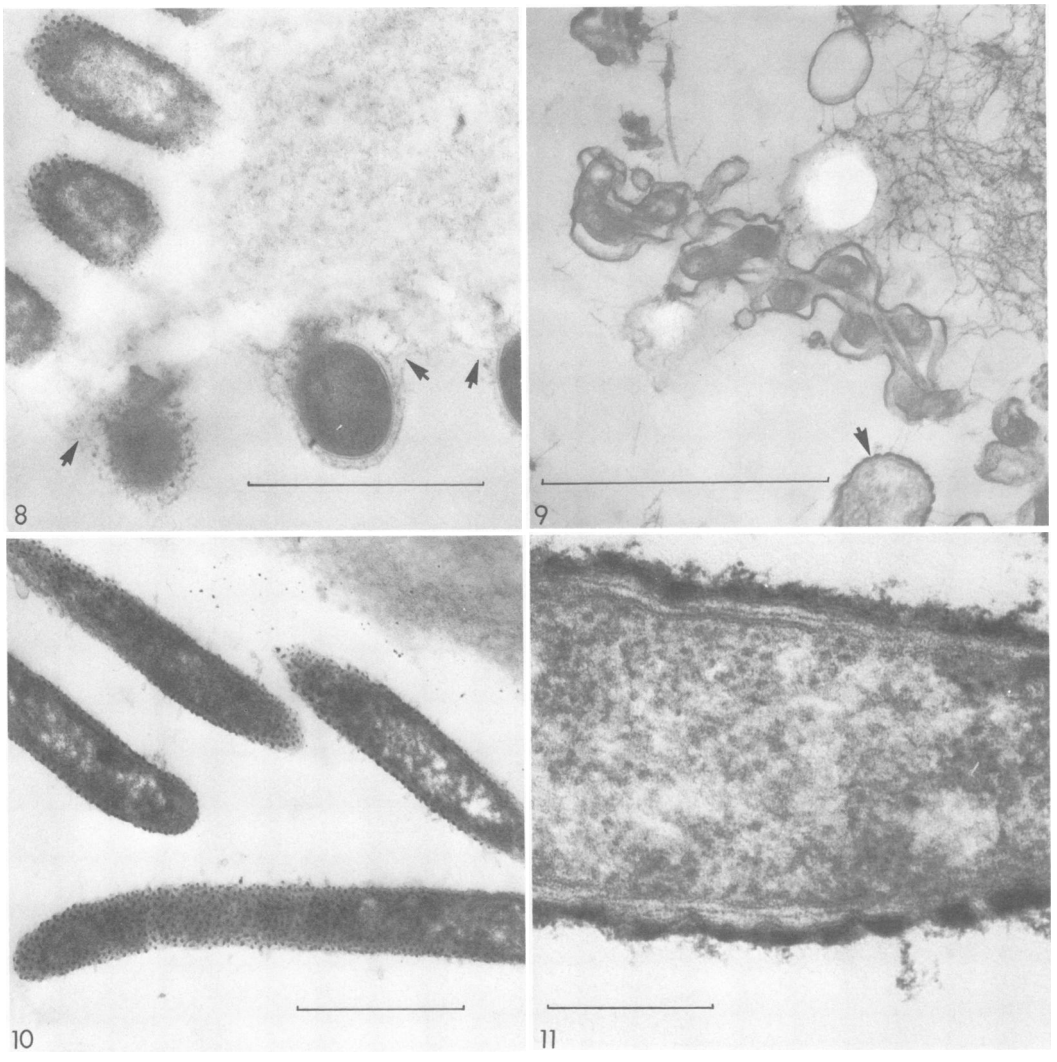


FIG. 8. *Thin section of filter paper digested for 48 h. The bacteria on one side of the cotton fiber have the speckled appearance of type (a), despite the omission of ruthenium red stain. The organisms on the other side are type (b) and have coats adhering to the substrate (arrows). Bar marker = 1 μm.*

FIG. 9. *Thin section of filter paper digested for 72 h. An organism of type (c), presumed to have a helical form, with a loose outer membrane can be seen. Organisms of type (a) can also be seen (arrow). Bar marker = 1 μm.*

FIG. 10. *Thin section through filter paper digested for 48 h. Four organisms of type (a) with eroded substrate. All show speckling of outer wall, despite the omission of ruthenium red staining. Bar marker = 1 μm.*

intervals makes it possible to follow the time course of the process. By using cell wall preparations, one can eliminate the effects of the plant cell contents on the microbial population in the bag, and insoluble materials such as chemically modified cell walls or, in the present case, cotton fibers can be used to increase still further the selectivity of the substrate.

The most serious technical difficulty that we have encountered is caused by the cotton fibers in our samples which make it difficult to cut sections sufficiently thin to establish the cell wall structure of the accompanying bacteria; this may be due to incomplete infiltration of the fibers. Therefore, although the general morphology of the various bacterial types can be recognized in the majority of sections, wall structure is clearly displayed in only a few.

A serious gap in our knowledge of cell wall digestion in the ruminant is that so far no one

has been able to isolate and characterize enzymes capable of attacking highly ordered cellulose from rumen bacteria, although such enzymes have been extensively studied in filamentous fungi. Bacteria capable of attacking cotton fibers have been isolated from the rumen, notably, *Bacteroides succinogenes* and *Ruminococcus flavefaciens* (16, 20), but the presence of the enzyme responsible could not be demonstrated in culture filtrates or in cell extracts.

Several explanations are possible. (i) The bulk of the cellulose in cell walls of forage species has only a moderate degree of order (7), and in the most digestible cell type (mesophyll), it has a very low degree of order (15); rumen digestion could, therefore, be quite extensive, without much of the highly ordered cellulose being attacked. The cotton-degrading organisms present would be only minor elements in the cellulolytic population and would attract correspondingly little attention.

(ii) The enzymes responsible could be insoluble by virtue of their attachment to the outer surface of the bacteria in question. This would explain the need for close adhesion between the bacteria and the plant cell walls.

(iii) Some of the bacteria responsible may have been overlooked because on isolation they had lost their ability to attack highly ordered cellulose as a consequence of the changed conditions of culture.

Our present observations permit the following comments. Suggestion (i) may be partly true, in the sense that the majority of cellulose-decomposing organisms present when a good-quality forage is being digested may be forms unable to attack highly ordered cellulose. Ruminococci have been shown to possess enzymes of the C_X type (28), and they seem to be among those most directly engaged in cell wall digestion. (In addition, it may be noted that because they often burrow in the middle layers of the wall, the enzymes that they secrete will not diffuse rapidly from their sites of action). A corollary to this hypothesis is that the organisms capable of attacking cotton will be present in relatively small numbers on good-quality diets. This could explain the lag phase in the digestion of cotton samples placed in the rumen in nylon bags (8), compared with the extremely rapid disappearance of other plant cell walls.

The population of organisms associated with the digestion of cotton fibers in our sheep is so different from that seen attacking grass cell walls that it encourages the hope that some of the organisms present may be recognized by their appearance in ultrathin sections.

The predominant type, which has appeared on cotton fibers taken from four different sheep (all fed on dried grass diets), was never seen on our grass cell wall preparations, nor can we recognize it in the micrographs published by Akin and his colleagues. In conformity with suggestion (ii) above, it appears to erode the cellulose that is in contact with it. Whether it belongs to any of the types so far described in the literature cannot be determined with any certainty.

Of the four species presently considered to make the greatest contribution to cellulose digestion in the rumen (10), two are rodlike forms: *Butyrivibrio fibrisolvens* and *B. succinogenes*. There seems to be general agreement that gram-negative rodlike bacteria predominate on the less digestible forage diets (29), and a large number have been isolated in pure culture. Many have been identified as *Butyrivibrio*, but not many of these showed the ability to attack cellulose in pure culture.

Recently, Cheng and Costerton (12) published an account of two strains of *B. fibrisolvens*, showing that they do not have a typical gram-negative wall structure. We have examined three other strains: Nor37, an electron micrograph of which has been published by Sharpe et al. (27), and two strains isolated at this institute. None (Fig. 12 to 14) shows the knoblike extracellular structures found by Cheng and Costerton in their strain C_3 (12). It is a strange coincidence that the speckled effect shown by our rod (a) in tangential section resembles that seen by them in C_3. However, rod (a) has this appearance whether or not ruthenium red staining is used, and its wall structure is clearly of the gram-negative type, whereas that of *Butyrivibrio* is not. It therefore seems that rod (a) is not a *Butyrivibrio*.

From what is known of the cellulose-decomposing abilities of rumen organisms, *B. succinogenes* is a more likely candidate (10). We examined strain S85 of Bryant and confirm the features of its wall structure described by Costerton et al. (14). Although a rod when first isolated, in pure culture it is now more or less spherical and about 1.0 μm in diameter. Differences in growth conditions could account for these differences in shape and size, but might not alter the cell wall structures.

In *B. succinogenes* S85 the peptidoglycan layer is inconspicuous, but in our bacterium this can be clearly seen whenever the wall structure is fully resolved. A characteristic feature of the outer membrane of *B. succinogenes* S85 is its irregularity (Fig. 15), which extends to the formation of blebs like those illustrated by Lev (21) in *Fusiformis nigrescens* (*Bacteroides melaninogenicus*), another pleomorphic organism, and those seen when a lysineless mutant of *Escherichia coli* was grown on lysine-limiting medium

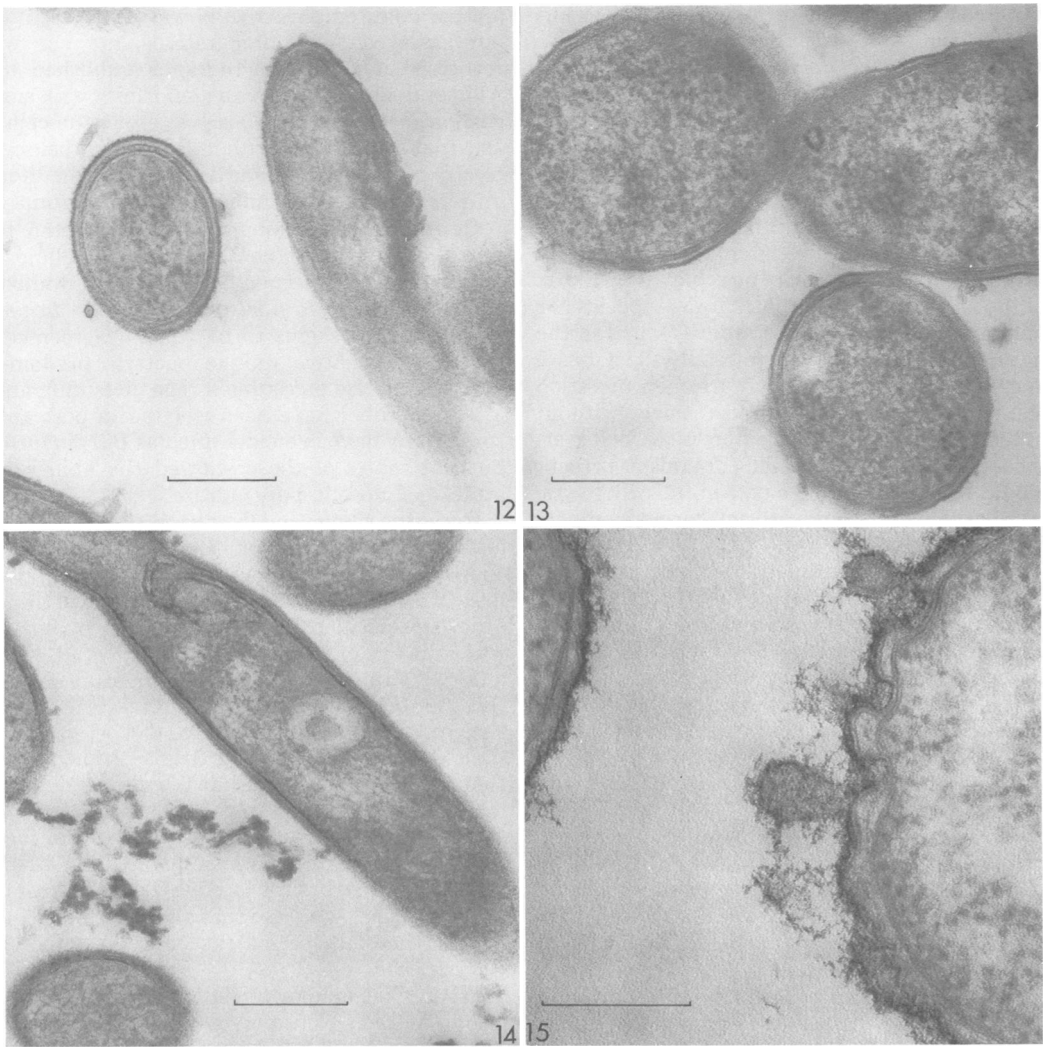


FIG. 11. *Thin section of type (a) organism on filter paper digested for 48 h (ruthenium red stained), showing gram-negative cell wall structure. Bar marker = 0.2 μm.*

FIG. 12 to 14. *Thin sections through cells of three strains of B. fibrisolvens (ruthenium red stained), showing, respectively, Nor37, B834, and D6-1. Bar markers = 0.2 μm.*

FIG. 15. *Thin section through cells of B. succinogenes S85 (ruthenium red stained). Bar marker = 0.2 μm.*

(19). We have searched our samples for bacteria with this wall structure and have found very few. One is shown in Fig. 7; it has a wall structure remarkably like *B. succinogenes* S85, the peptidoglycan layer being very thin and not always visible. It is only 0.4 μm in cross section and is probably a short rod, about 1.2 μm long. The other examples that we have found are similar in all of these respects.

The differences between this rarely seen organism and rod (a) are clearly shown in Fig. 7, and the close proximity of the two forms in this section makes it unlikely that the differences are due to variations in the conditions of specimen preparation.

There is very little published evidence about the occurrence of *B. succinogenes* in the sheep rumen. Hungate (18) mentions its presence there, but Van Gylswyk (29) was unable to find it among numerous isolates from sheep receiving diets in which one would expect the more resistant forms of cellulose to be present; so far it has not been isolated from sheep at this institute. M. P. Bryant informed us that it was the main cellulolytic bacterium in the rumen of sheep fed wood cellulose (Solka Floc) as the sole energy

source (unpublished data with G. T. Schelling).

Recently, Mackie et al. (23) have indicated that about half of the cellulolytic bacteria in sheep on a diet in which roughage was progressively replaced by concentrates were presumptively identified (to genus level) as *Bacteroides*.

It therefore remains a possibility that rod (a) is a form of *B. succinogenes* or perhaps could be a closely related organism occupying a niche in the sheep rumen similar to that occupied by *B. succinogenes* in the bovine rumen. It will be necessary to examine other strains of *B. succinogenes* as these become available and to isolate rod (a) in pure culture before these questions can be answered.

The significance of the present results for an understanding of forage digestion is that they show how complex the relationship between wall structure and digestibility can be; not only are different cell wall types digested at different rates, but also the layers within them. Moreover, the organisms involved differ according to the nature of the substrate. Therefore, the cell walls prepared from leaves of grasses seem to be digested mainly by cocci, whereas the resistant residues from the digestion of dried whole grass (feces cell walls) and cotton fibers elicit a population of rodlike organisms. This would confirm the conclusions based on the isolation of cellulolytic forms from animals on different diets.

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