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Rumen Bacterial and Fungal Degradation of *Digitaria pentzii* Grown With or Without Sulfur

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Sheep fed the forage *Digitaria pentzii* fertilized with sulfur were compared with those fed unfertilized forage for the rumen microbial population involved with fiber degradation. No differences were detected in the bacterial population as determined by anaerobic cultures on a habitat-simulating medium, xylan, or pectin, by ^{35}S labeling techniques for microbial protein, or by transmission electron microscopic studies of bacterium-fiber interactions. Rumen volume and water flow from the rumen were not different for sheep fed each of the forages. Rumen fungi were prevalent in sheep fed sulfur-fertilized *D. pentzii* as shown by sporangia adhering to forage fiber and by colonies developing from zoospores in roll tubes with cellobiose plus streptomycin and penicillin. Fungi were absent or in extremely small numbers in sheep fed unfertilized forage. Nylon bag digestibility studies showed that the fungi preferentially colonized the lignified cells of blade sclerenchyma by 6 h and caused extensive degradation by 24 h. In the absence of bacteria in in vitro studies, extensive hyphal development occurred; other lignified tissues in blades (i.e., mestome sheath and xylem) were attacked, resulting in a residue with partially degraded and weakened cell walls. Nonlignified tissues were also degraded. Breaking force tests of leaf blades incubated in vitro with penicillin and streptomycin and rumen fluid from sheep fed sulfur-fertilized forage or within nylon bags in such sheep showed a residue at least twice as fragile as that from sheep fed unfertilized forage. In vitro tests for dry matter loss showed that rumen fungi, in the absence of actively growing bacteria, could remove about 62% of the forage material. The response of rumen fungi in sheep fed sulfur-fertilized *D. pentzii* afforded a useful in vivo test to study the role of these microbes in fiber degradation. Our data establish that rumen fungi can be significant degraders of fiber and further establish a unique role for them in attacking and weakening lignocellulosic tissues. The more fragile residues resulting from attack by fungi could explain the greater intake consistently observed by sheep eating sulfur-fertilized compared with unfertilized *D. pentzii* forage.

Rees and co-workers (32, 33) showed that sulfur fertilization (+S) reduced retention time of forage in the rumen and increased voluntary intake and digestibility compared with unfertilized (−S) *Digitaria* sp. forage. The authors suggested that depressed microbial activity due to a sulfur deficiency could have caused poor animal response to −S forage. Similar studies with +S and −S *Digitaria pentzii* (31) indicated that S fertilization increased voluntary intake and reduced rumen ammonia levels, indicating enhanced microbial activity in the rumen.

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In a companion paper to the present one (D. E. Akin and J. P. Hogan, Crop Sci., in press), samples of +S and −S *D. pentzii* from the same harvest were studied for variations in forage digestibility by rumen microorganisms. Results indicated no differences in digestibility between treatments when tested with a microbial population from sheep fed a diet of alfalfa-oats (60:40). However, in nylon bag studies, sheep fed +S *D. pentzii* digested about four times more dry matter after 24 h than did sheep eating −S forage. Additionally, microscopic studies (Akin and Hogan, in press) suggested a possible role in the sheep fed +S forage for rumen fungi similar to those reported by Orpin (24) and Bauchop (6).

Our objectives were to evaluate rumen microbial populations from sheep fed +S or −S *D.*

pentzii for microbial types involved in fiber digestion and to investigate the possible role of rumen fungi in degrading plant tissue.

MATERIALS AND METHODS

Forages. *D. pentzii* (Stent), a warm-season forage in the same genus as digitgrass and kindly supplied by M. C. Rees (CSIRO, Division of Tropical Crops and Pastures, St. Lucia, Queensland, Australia), was grown at Beerwah, near Brisbane, Queensland, Australia. Forages were grown on S-deficient soil without (−S) or with (+S) 60 kg of S per hectare. Seven-week regrowths of each were cut, chopped, and dried at 90 to 100°C. The +S forage contained 0.17% S, and the −S contained 0.11% S. The forages were similar in N (1.2%), P, K, Ca, Mg, Na, Cu, Zn, cellulose, hemicellulose, and lignin.

For fresh samples, *D. pentzii* grown with a complete fertilizer in a pot and harvested just before flowering was supplied by D. J. Minson, Division of Tropical Crops and Pastures, St. Lucia, Queensland, Australia. Plants were frozen and used as needed in in vitro experiments with antibiotics.

Animals and their feeding. Merino wethers approximately 1.5 years old and maintained on a diet of alfalfa-oats (60:40) were prepared with permanent fistulae in the rumen and abomasum. Sheep chosen for study were adapted to either −S or +S *D. pentzii* and maintained on the respective diet for the entire study. The sheep were fed equal amounts of hay each 3 h by interval feeders. They received no additional supplements, and water was available at all times. Three intake trials with one or two sheep per trial on each diet confirmed other work with this forage (31) in that +S hay was eaten in greater amounts. Studies on microbial variations with diets were conducted during intake trials. For in vitro digestion studies, rumen digesta was collected in quantities of 100 to 150 ml per sheep and placed into vacuum bottles maintained at 39°C for transport to the laboratory. Digesta samples were mixed in a blender for 1 min with constant CO_2 gassing and filtered through single layers of nylon cloth with approximate pore sizes of 200 × 140 μm. The filtrate was then used as inoculum.

Estimation of microbial nitrogen, rumen volume, and flow. The estimation of the proportion of microbial nitrogen in digesta leaving the abomasum was based on the ^{35}S technique of Mathers and Miller (J. C. Mathers and E. L. Miller, Proc. Nutr. Soc. **36**:7A, 1976). An infusion of ^{35}S as sodium sulfate was made at approximately 6 μCi/h for 48 h with infusion for 24 h to ensure that the ratio of ^{35}S to nitrogen was the same in bacteria and protozoa (9). The volume of liquid in the rumen and the flow of liquid from the rumen were estimated with ^{51}Cr complexed with EDTA by the method of Weston and Hogan (35).

Tests for microbial attack on intact plant structures. For nylon bag studies, leaf blade portions 2 to 3 mm in length from +S or −S hay were sectioned with a new, cleaned razor blade. Ten to thirty blade sections were placed into Nylon 25T bags with an aperture size of 45 μm (Swiss Screens Propriety, Ltd., Seven Hills, New South Wales, Australia). Bags were anchored with 75-g iron weights and suspended in the rumens of cannulated sheep. After digestion for selected times, bags were retrieved from the rumen and washed with water to remove debris, and the leaf blades were removed from the bags.

Anaerobic culturing of rumen microorganisms. The procedures, media (including xylan and pectin roll tubes), and material for viable colony counts and relative cellulose (filter paper) activity were as described (3) for rumen bacteria. Medium 10-X (cellobiose, starch, glucose, and xylan; 17) was used as a habitat-simulating medium in place of medium 10. Two sheep each on −S or +S forage were sampled four times for total viable counts. For culturing rumen fungi, the medium and antibiotics (benzyl penicillin, 2 × 10^4 IU/ml; and streptomycin sulfate, 2 mg/ml) were as described by Joblin (18) in Hungate tubes with the modification that cysteine-sodium sulfide (11) was included as the reducing agent. Sheep on −S or +S forage were sampled in three trials for the role of +S forage in stimulating fungi.

Fungal attack on intact blade tissues. Leaf blade sections in Hungate tubes containing 4 or 5 ml of complete fungal medium including antibiotics were inoculated with 0.1 or 0.2 ml of strained rumen fluid. After selected times, blades were retrieved and prepared for microscopic examination or tests for force required to break the blade (i.e., tensile strength).

Breaking force tests. Matched blades were prepared to test the force required to break blades. Individual blades from frozen plants were selected to include the midportion of blades. These sections were then further cut into equal segments and randomly placed into groups. Five to ten such blades of 3 or 4 cm were used in each test to make a matched set. Matched blades were then incubated in nylon bags in sheep fed +S or −S forage or in Hungate tubes with fungal medium plus antibiotics. After incubation for selected times (see Tables 5 and 6), blade segments were washed with distilled water and then frozen in distilled water. For stress tests, blades were thawed and then subjected to breaking force stress by clamping each end of the blades in an Instron Universal Testing Instrument (Instron Ltd., High Wycombe, Bucks, England) with a load cell of 5 or 50 N of maximum force. The force at which the blade pulled apart (i.e., the tensile strength) was automatically recorded. Blades pulled apart at random areas and were not preferentially weakened at the points of clamping.

Dry matter loss by rumen fungi. Matched groups of blades similar to those above, but cut at 2-cm lengths from five blades, were tested in vitro in each of two trials. Blades were incubated in Hungate tubes with 4 ml of Caldwell and Bryant (11) basal, semisynthetic medium without carbohydrates, but with antibiotics, as described by Joblin (18). Matched blades were incubated for 48 h with 0.1 ml of the following: (i) rumen fluid from sheep fed +S forage; (ii) same as (i), but autoclaved; (iii) rumen fluid from sheep fed −S forage. A fourth, control set was not inoculated. The blades were then washed with distilled water and freeze-dried, and the dry weight loss relative to the uninoculated control sample was calculated.

Preparation for SEM. Plant fractions were placed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C and fixed for 1 to several days. Plant specimens were washed in buffer and then postfixed in 1 to 1.5% OsO_4 buffered as above at 4°C for 4 h. Specimens were washed in buffer and dehydrated 5 min each in 25, 50, 75, 95, and 100% (3×) ethanol. Specimens were then

TABLE 1. Total viable counts of rumen bacteria and rumen flow parameters for sheep fed +S and −S *D. pentzii*

Inoculum from sheep fed:	Colonies ($\times 10^8$/ml) developing on[a]:			Water flow from rumen[b] (liters per day)	Vol of liquid rumen[b] (liters)
	10-X (n = 4)	Xylan (n = 3)	Pectin (n = 3)		
+S forage	8.91 ± 3.28	5.06 ± 2.10	3.74 ± 1.68	10.75 ± 0.78	3.93 ± 0.19
−S forage	7.88 ± 1.91	5.11 ± 3.46	4.18 ± 1.76	9.32 ± 0.58	3.78 ± 0.20

[a] Counts are averages and standard deviations from duplicate samples in four trials.
[b] Estimated by dilution of single dose of [^{51}Cr]EDTA into rumen; average and standard deviation.

critical point dried in liquid CO_2 without transitional solvents, with return to atmospheric pressure over a 30- to 45-min period. Dried specimens were adhered with conducting cement to scanning electron microscopy (SEM) stubs and sputter coated with gold for 4 to 5 min.

Preparation for transmission electron microscopy (TEM). Plant fractions were fixed and postfixed as above. After fixation, specimens were dehydrated for 20 min each with 25, 50, 75, (overnight), 95, and 100% (3×) ethanol at 4°C. Specimens were washed in propylene oxide for 20 min at room temperature and infiltrated with 1:2, 1:1, and 2:1 proportions of Spurr epoxy resin-propylene oxide for 2 h each with constant mixing at room temperature. Specimens were infiltrated in 100% Spurr without mixing at room temperature overnight and then cured in fresh Spurr for 24 h at 70°C. After hardening, thin sections were cut with a diamond knife and stained with uranyl acetate (saturated, aqueous) and Reynold's lead citrate (34) for 15 min each. Sections 1 μm thick were cut and prepared for examination by staining with a 1:1 solution of 1% azure II in 1% Borax and 1% methylene blue in 1% Borax at 60°C for 30 to 120 s.

Fungal sporangia counts by light microscopy. Leaf blade sections from nylon bags or in vitro vessels were stained for 30 to 60 s with lactophenol cotton blue made by diluting 5 ml of stock solution (1 g of cotton blue in 100 ml of lactophenol) with 100 ml of lactophenol. Blades were then rinsed twice with distilled water and mounted on microscope slides. Sporangia gave a dark blue color, as did rumen protozoa, so care was used in evaluating the presence of colonizing fungi. For estimating relative numbers of sporangia, a standard area was chosen, and all attaching sporangia within the defined area of the 40× objective lens (0.75 mm^2) were counted.

RESULTS

Bacterial numbers and activity from sheep fed +S or −S *D. pentzii*. Total viable counts of rumen bacterial colonies developing on habitat-simulating (10-X), xylan, or pectin media from rumens with similar volumes and rates of fluid movement (Table 1) indicated that bacterial populations were not different between sheep fed the two diets. Similarly, from data on ^{35}S uptake, bacterial nitrogen represented almost the same proportions (66 and 63%, respectively, for +S and −S diets) of the nitrogen in digesta leaving the stomach. Further, relative cellulolytic activity (based on breakage of filter paper strips) showed no differences with the average breaking time in two trials of 10.1 days for +S and 9.9 days for −S rumen fluids.

TEM of bacterial attack on plant tissues. Leaf blades in nylon bags incubated for 6, 24, and 48 h

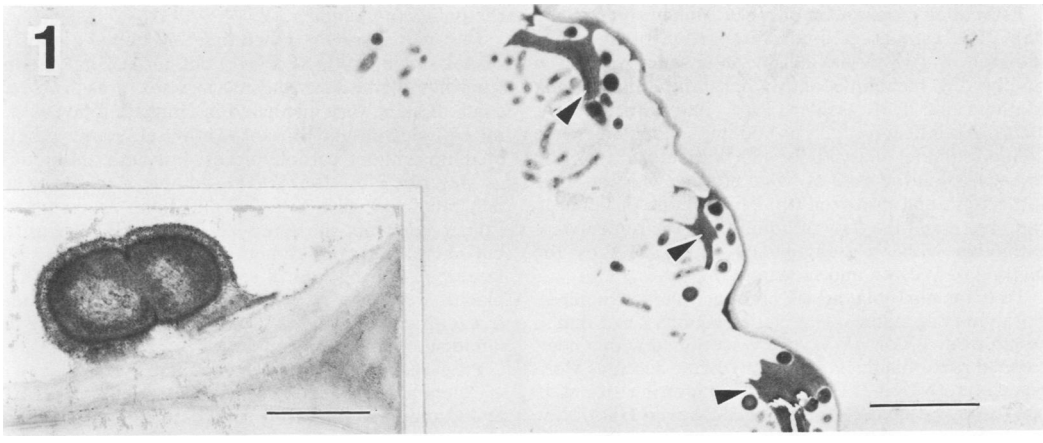

FIG. 1. Leaf blade incubated for 24 h in a nylon bag within the rumen of a sheep fed +S forage. Shown is the attack on thicker-walled parts of epidermis (arrows). Bar, 5 μm. Inset, Encapsulated cocci attached to plant cell walls. Bar, 0.5 μm.

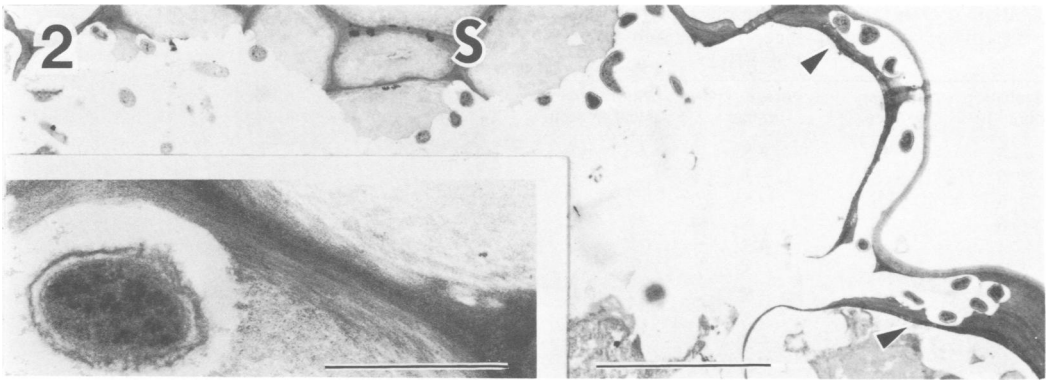

FIG. 2. Leaf blade incubated for 48 h in a nylon bag within the rumen of a sheep fed +S forage. Shown is the attack on the periphery of sclerenchyma (S) tissues and thicker portions of epidermal cell walls (arrows). Bar, 5 μm. Inset, Irregularly shaped bacteria not intimately associated with zones of degradation in the cell wall. Bar, 0.5 μm.

within the rumens of sheep fed +S or −S forage were examined for bacterial morphotypes attacking plant cell walls. No differences in bacterial types or in patterns of attack were found between treatments. After 6 h, few bacteria were observed in thin sections, and no bacteria adhered to fiber. After 24 h, encapsulated cocci were present, but these were few in number, and they mostly adhered to the periphery of residual sclerenchyma and the thicker portions of the epidermis adjacent to the cuticle (Fig. 1). After incubation for 48 h, few adherent bacteria were observed, and the predominant morphotype was an irregularly shaped bacterium often not directly attached at clearly defined zones of erosion in the plant cell walls (Fig. 2). Most adherence or close association was to sclerenchyma and epidermal cells as observed after 24 h. In general, even the most prevalent adhering morphotypes, i.e., encapsulated cocci and irregularly shaped bacteria, were present only in small numbers in electron micrographs.

Relative numbers of rumen fungi in the rumens of sheep fed +S or −S *D. pentzii*. Light microscopy revealed that fungi with sporangia of diverse shapes were prevalent on the cut ends of leaf blades and were also present on the surfaces of leaves incubated in rumen fluid from sheep fed +S forage. Counts of sporangia within a standard area (40× objective lens) to compare fungi between treatments indicated substantially higher numbers for rumen fluid from +S sheep (Table 2).

Fungal colonies developing on cellobiose medium plus antibiotics provided an estimate of the free zoospore population in rumen fluid from animals on the two treatments. Based on preliminary work, 10^{-1} and 10^{-2} dilutions were used as inocula to obtain countable colonies, and numerous colonies were found only from +S sheep (Table 3). A second experiment with undiluted rumen fluid from a sheep fed −S forage showed only one fungal colony from four roll tubes. Several small pinpoint colonies, presumably bacteria, were observed, but these did not interfere with counting of the larger, filamentous fungal colonies.

Microscopy of fungal attack on plant tissue incubated in nylon bags within sheep fed +S or −S forage. SEM showed extensive colonization

TABLE 2. Sporangia on leaf blades incubated in vitro with antibiotics for 48 h after inoculation with rumen fluid from sheep fed +S or −S *D. pentzii*[a]

Inoculum from sheep fed:	Sporangia per microscopic field[b]	
	Trial 1	Trial 2
+S forage	3.3	11.0
−S forage	0.6	0.2

[a] Medium was that described by Joblin (18) containing cellobiose, penicillin, and streptomycin.
[b] Counts are the average of 10 sites from each blade incubated in duplicate. Microscopic field was that of a ×40 objective lens and was 0.75 mm².

TABLE 3. Fungal colonies developing on cellobiose medium plus antibiotics from rumen fluid of sheep fed +S or −S *D. pentzii*[a]

Inoculum from sheep fed:	Fungal colonies (10^3/ml)[b]	
	Trial 1	Trial 2
+S forage	3.55	4.53
−S forage	0[c]	0[c]

[a] Medium was that described by Joblin (18) containing penicillin and streptomycin.
[b] Average for two tubes each of duplicate samples.
[c] Number of colonies from 10^{-1} dilution (see the text).

TABLE 4. Fungal colonization of *D. pentzii* placed in nylon bags within sheep rumens and then observed by SEM

Incubation time (h)	Sheep diet	Forage type in bag	Sporangia on leaf or stem
6	+S	+S	+
6	−S	−S	−
6	+S	−S	+
6	−S	+S	−
24	+S	+S	+
24	−S	−S	−
24	+S	−S	+
24	−S	+S	−[a]
48	+S	+S	+
48	−S	−S	−
48	+S	−S	+
48	−S	+S	−[a]

[a] Only one to few sporangia observed.

by rumen fungi of leaf blades and stems in sheep fed +S forage. In one experiment, the various combinations of forage type in sheep on each diet were scored for the presence of sporangia. These data (Table 4) showed that rumen fungi were present in sheep fed +S forage regardless of the forage type in the nylon bag, thus indicating that rumen fungi were not contaminants of feed.

After 6 h, sporangia of uniform size and shape selectively occurred on sclerenchyma patches (Fig. 3). By 24 h, larger sporangia were present on blades, often adhering to sclerenchyma cells. Sporangia were not prevalent on blades after 48 h of incubation, but they occurred extensively on the lignified epidermis, sclerenchyma ring, and vascular cells of stems. Fungal association with stomata of leaf blades was frequently observed (Fig. 4) and appeared to be a prevalent way of fungal entry into plant tissue of *D. pentzii* leaf blades.

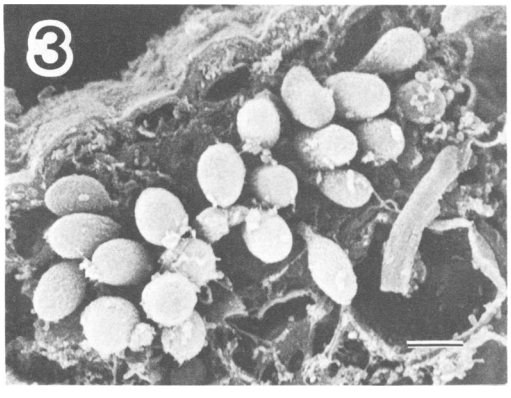

FIG. 3. Leaf blade incubated for 6 h in a nylon bag within the rumen of a sheep fed +S forage. Shown are the uniform sporangia preferentially associated with sclerenchyma. Bar, 5 μm.

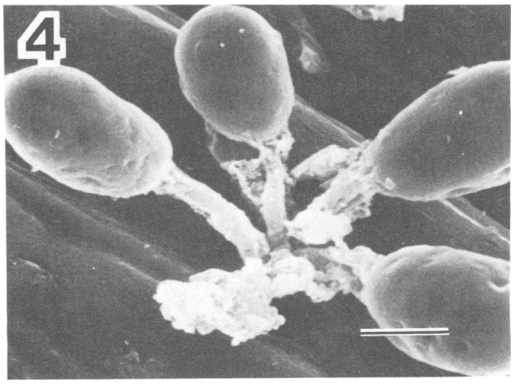

FIG. 4. Sporangia associated with stoma of leaf blade. Bar, 10 μm.

TEM of thin sections cut at the edge of the blade portion after 6 h of incubation showed colonization of sclerenchyma by fungi and penetration into cell walls by rhizoids (Fig. 5). Filamentous structures, possibly thalli or rhizoids similar to those reported by Munn et al. (22), were prevalent in zones of erosion in the cell wall below the end of the blade cross section. After 24 h, hyphae devoid of cytoplasm were prevalent in extensively degraded sclerenchyma regions.

Electron microscopy of rumen fungal attack in the presence of streptomycin and penicillin. SEM showed that, in the presence of antibiotics, fungal hyphae were produced to such an extent that the leaf was overgrown and plant tissue could not be observed (Fig. 6). TEM showed the degradation by fungi of specific plant tissues and further indicated that negligible numbers of bacteria were present in thin-sections. Epidermis and mesophyll were extensively degraded, whereas the forage residue consisted of cuticle and vascular tissue. Sclerenchyma was extensively degraded and was associated with pleomorphic fungal bodies (Fig. 7). Although considerable lignified vascular tissue was left, some attack resulting in disruption and weakening of these tissues (e.g., metaxylem vessel) occurred. Cell wall structure appeared to be disrupted by fungal attack. Fungal entry through leaf stomata was frequently observed (Fig. 8) and appeared to be one route of quick access to tissues within intact leaf blades. Often cross sections of developing sporangia showed packets of flagella and structures resembling zoospore nuclei (22) within degraded blades (Fig. 9).

Measure of fungal attack on intact leaf blades. Rumen populations with (sheep fed +S forage) or without (sheep fed −S forage) fungi afforded an in vivo test system to evaluate the role of rumen fungi in degrading and weakening plant structure in fresh frozen leaf blades. Breaking

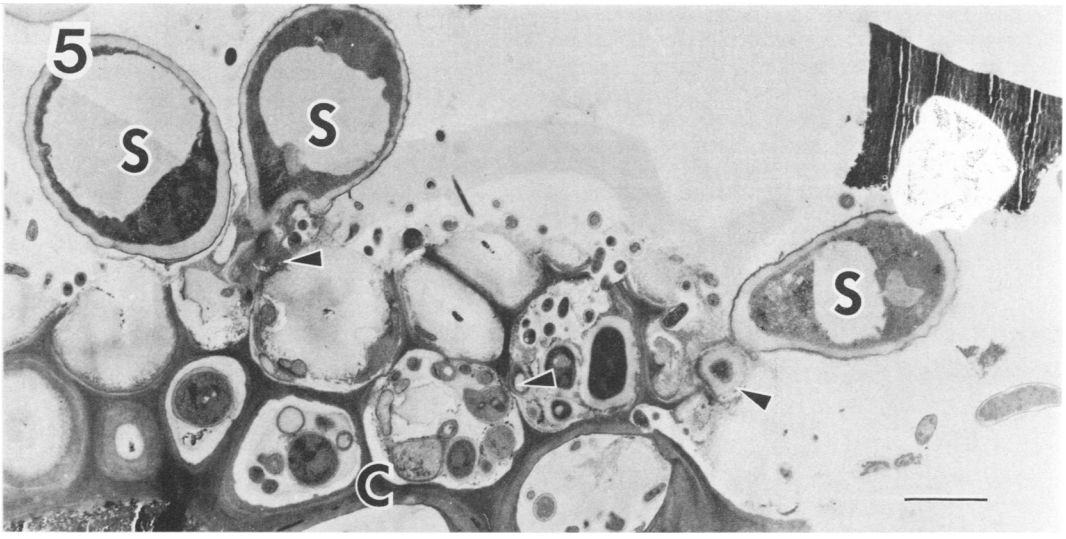

FIG. 5. Sporangia (S) associated with sclerenchyma cells (C) after incubation for 6 h in nylon bags within the rumen of a sheep fed +S forage. Shown are zones of degradation in cell walls near fungal structures (arrows). Bar, 2 μm.

forces were always less for blades within sheep fed +S forage (Table 5). Similarly, matched blades incubated in vitro with antibiotics and rumen fluid from sheep on each diet further showed that rumen fungi caused a considerable weakening of intact blades (Table 6).

Other tests of matched leaf blade portions incubated in vitro with antibiotics showed a substantial loss in dry matter with rumen fluid from sheep fed +S, but not −S, forage (Table 7). These losses represent minimum values since residues were not corrected for contributing fungal biomass.

DISCUSSION

No differences were observed in bacterial populations between sheep fed either +S or −S *D. pentzii* when examined either by anaerobic culture or TEM. Microbial protein as measured by ^{35}S incorporation also represented a similar proportion of the crude protein leaving the stomach with both diets. However, marked differences in the prevalence of sporangia and zoospores typical of rumen fungi (6, 22–26) were observed between diets. Evidence of rumen fungi was always found with sheep fed +S forage, but few or no fungi were seen with −S forage even though all sheep initially had fungi when fed alfalfa-oats.

The levels of sulfur in forage needed to meet microbial needs cannot readily be defined (21). Levels of cysteine and methionine in different rumen bacteria can vary more than twofold (30), whereas the availability of sulfur for rumen microbes may also vary between forages. Additional S frequently enhances the digestibilities of cellulose and lignocellulose (10, 14) and perhaps even that of lignin (32). Unfortunately, insufficient data are available to show which specific microorganisms are stimulated by S. Although our results suggest that ingestion of +S forage stimulates rumen fungi, definitive data as to the specific stimulation from sulfur fertilization are not yet available. Indeed, in some studies elemental S as an additive to animal feed has not shown the same benefits as Na_2SO_4 (31).

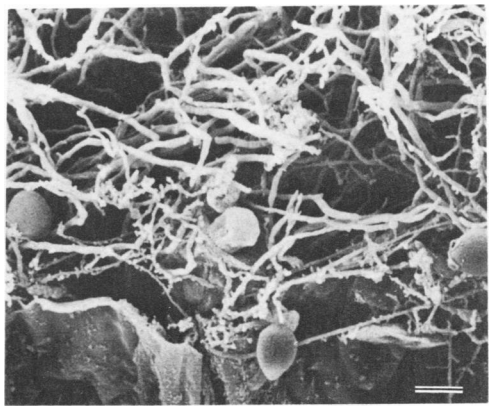

FIG. 6. Overgrowth of leaf blade by hyphae after incubation in vitro for 72 h with rumen fluid from sheep fed +S forage in the presence of penicillin and streptomycin. Bar, 10 μm.

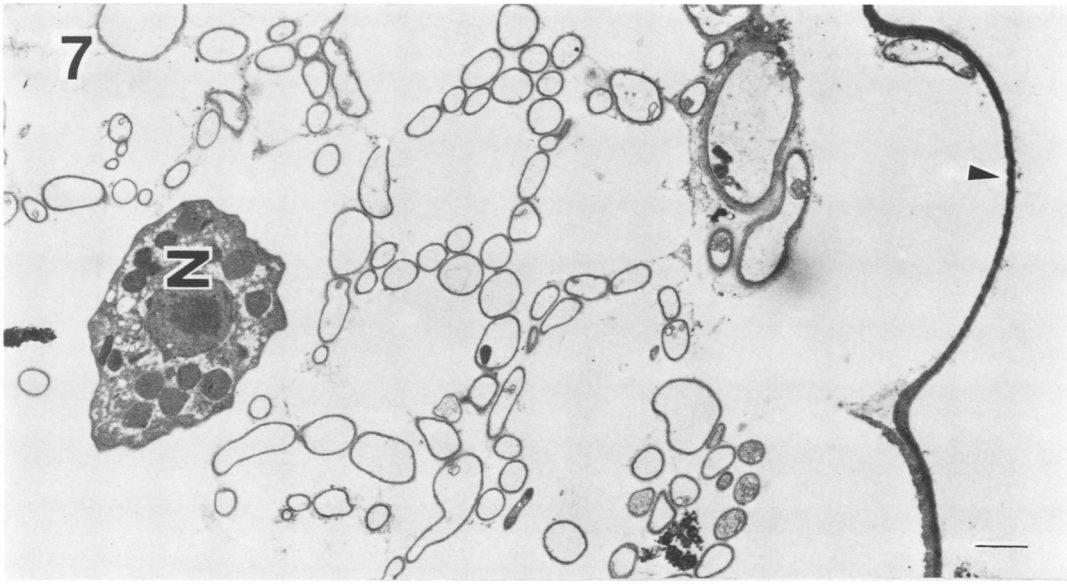

FIG. 7. Total loss of sclerenchyma tissue and presence of structures devoid of cytoplasm in leaf blades incubated for 72 h with the rumen fluid from a sheep fed +S forage in the presence of penicillin and streptomycin. Note the structure resembling a zoospore (Z) and the lack of degradation of the cuticle (arrow). Bar, 1 μm.

The predominant morphotypes of rumen bacteria in mixed populations that adhere to fiber have been examined by TEM and are identical to *Ruminococcus* spp. and *Bacteroides succinogenes* (2). Generally, TEM can demonstrate that many bacteria adhere to fiber undergoing digestion, although variations in morphotypes occur for various plant species (3). It may be significant that few bacteria from rumen populations of sheep fed +S or −S *D. pentzii* were observed to adhere to fiber, and few of the predominant, adhering morphotypes observed previously (3)

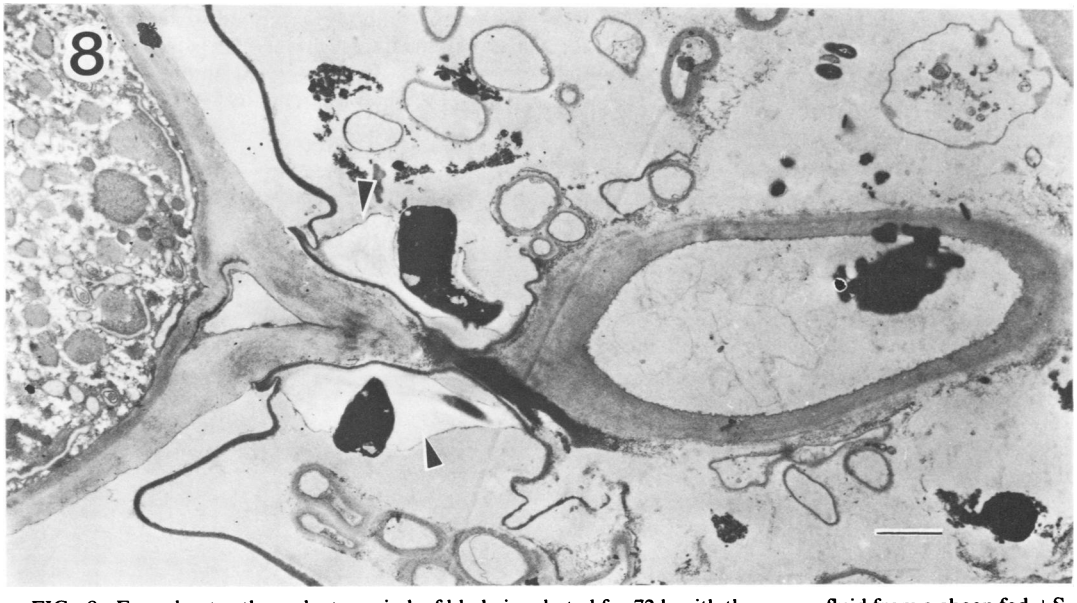

FIG. 8. Fungal entry through stoma in leaf blade incubated for 72 h with the rumen fluid from a sheep fed +S forage in the presence of penicillin and streptomycin. Note the loss of plant cell wall material and loss of electron denseness in the guard cells (arrows). Bar, 2 μm.

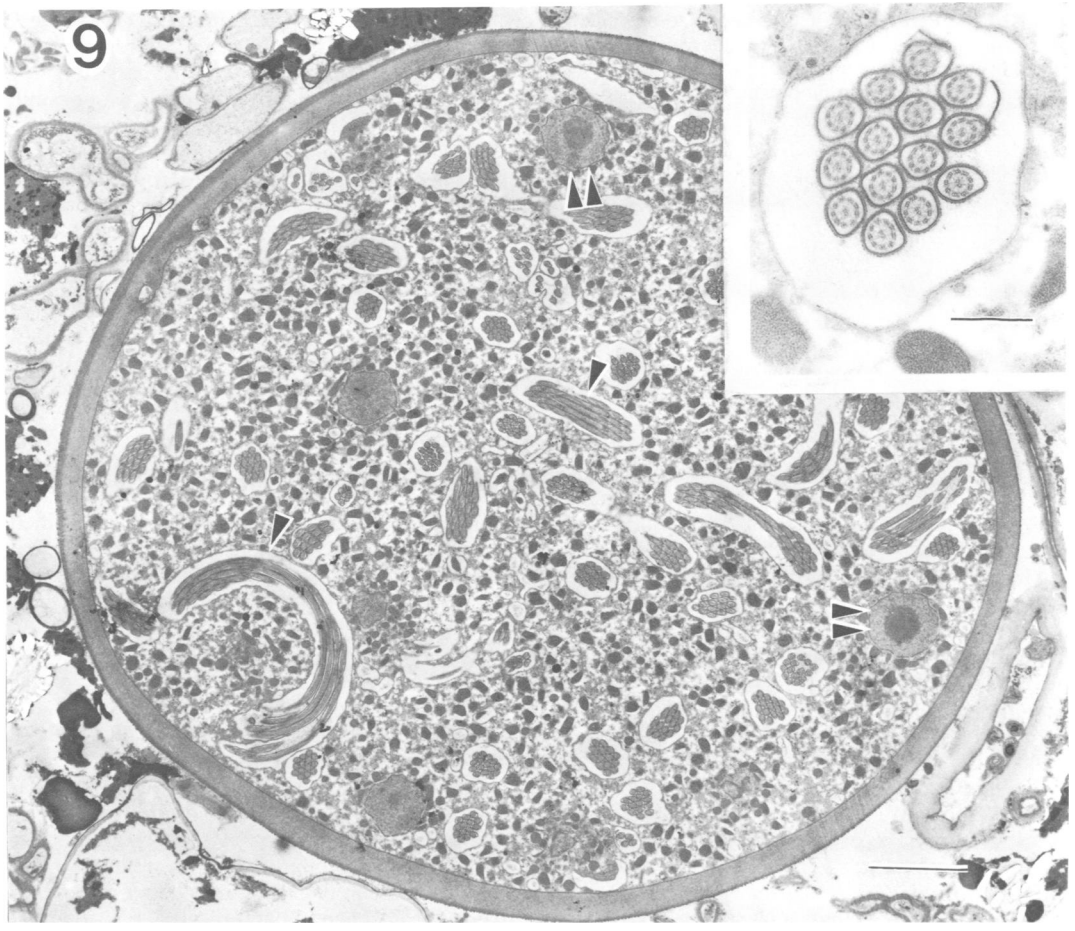

FIG. 9. Sporangium from rumen fluid of sheep fed +S. Shown are bundles of flagella (arrows) and circular bodies resembling zoospore nuclei (double arrows). Bar, 5 μm. Inset, Enlargement of flagella showing "9 + 2" internal organization. Bar, 0.5 μm.

were even seen at all in thin sections observed by TEM. Most attachment occurred to the more resistant tissues, including the thicker parts of the epidermis adjacent to the cuticle and the periphery of the sclerenchyma. A shift from encapsulated cocci to other morphotypes from 24 to 48 h was observed with nylon bag studies as had been reported in in vitro tests (3). Although encapsulated cocci in this study did resemble ruminococci (19), few bacteria resembled *Bacteroides succinogenes*, the other major fiber digester that tightly adheres to plant fiber.

TABLE 5. Breaking strength of matched leaf blades suspended in nylon bags in rumen of sheep fed +S or −S *D. pentzii*[a]

Incubated in sheep fed:	Force required to break blades (N)[a]		
	Trial 1 (6 h)[b]	Trial 2 (6 h)[b]	Trial 2 (18 h)[b]
+S forage	4.1 ± 2.8	4.2 ± 2.2	1.0 ± 0.7
−S forage	8.6 ± 1.0	4.9 ± 1.4	3.6 ± 1.7

[a] Average and standard deviation.
[b] Incubation period in nylon bags.

TABLE 6. Breaking strength of matched leaf blades incubated in vitro in fungal medium plus antibiotics and inoculated with rumen fluid from sheep fed +S or −S *D. pentzii*[a]

Inoculum from sheep fed:	Incubation time (h)	Force required to break blades (N)[b]	
		Trial 1	Trial 2
+S forage	48	0.9 ± 0.6	1.0 ± 0.5
+S forage	96	0.2 ± 0.2	0.2 ± 0.2
−S forage	48	3.8 ± 1.2	4.6 ± 0.6
−S forage	96	1.1 ± 0.6	2.9 ± 0.8

[a] Medium was that described by Joblin (18) containing cellobiose, penicillin, and streptomycin.
[b] Average and standard deviation.

TABLE 7. Percent dry matter degraded by rumen fungi from matched, intact leaf blades incubated in vitro in fungal medium plus antibiotics for 48 h and inoculated with rumen fluid from sheep fed +S or −S *D. pentzii*

Inoculum from sheep fed:	Residue dry wt (mg)		Loss compared with control (%)	
	Trial 1	Trial 2	Trial 1	Trial 2
+S forage	4.5	3.8	61.9	63.1
−S forage	11.5	10.0	3.5	2.9
+S forage (autoclaved)	11.8	10.0	0	2.9
Control (no inoculum)	11.8	10.3		

TEM studies indicated that much fiber digestion (e.g., mesophyll and portions of epidermis) occurred with enzymes free from microbial cells, but this phenomenon has not been studied by other methods.

Molds and yeasts have been found in the rumen (20). However, initial research by Orpin (23, 24) and further studies by Bauchop (6, 7) have indicated that fungi resembling aquatic phycomycetes can occur in large numbers in the rumen. In particular, Bauchop (7) has shown extensive colonization of forage fiber by these fungi and has found them in ruminants, hindgut fermenters, and members of the Kangaroo tribe. To date only three species of these sporangial-bearing fungi have been named: *Neocallimastix frontalis, Piromonas communis*, and *Sphaeromonas frontalis* (26).

Studies indicate that anaerobic fungi from rumen and horse cecum can degrade structural carbohydrates of plant cell walls (6, 8, 29). Cellulose and hemicellulose I have been shown to be particularly susceptible to fungal enzymes. Orpin and Hart (Abstr. Meet. Soc. Appl. Microbiol. **49**:X, 1980) reported 45% dry matter loss in perennial ryegrass with pure cultures of *P. communis* and 42 and 31% dry matter loss with the other two fungal species. We found a loss of 63% from intact leaf sections of *D. pentzii* incubated with whole rumen fluid from animals fed +S forage in the presence of penicillin and streptomycin. Related research on plant aspects of −S and +S *D. pentzii* (Akin and Hogan, in press) indicated that rumen fluid from sheep maintained on +S forage as in this study degraded about 20% dry matter of the +S forage after 24 h in nylon bags; rumen fluid from sheep fed −S forage degraded only 5% at this time. The pattern of tissue digestion and the amount digested (55% after 48 h) for −S and +S forage were similar with rumen fluid from sheep fed alfalfa-oats. These data (Akin and Hogan, in press) along with those presented herein indicate that the microbes stimulated by the feed play a major role in improved animal gains with +S forage, and that fungi are important in this regime.

The heavy colonization by rumen fungi of lignified cell walls (Fig. 5) (6) suggests a possible role in degrading lignocellulosic structures. Orpin (27) showed that similar fungi isolated from horse cecum could remove up to 16% of the lignin from grass. However, such loss does not prove lignin fermentation, for Gaillard and Richards (15) reported that 50% of forage lignin could be solubilized in the rumen and the reprecipitated in the abomasum. Other researchers have also reported the solubilization of nondegradable phenolic acid-carbohydrate complexes from plant cell walls (16). Frequently, aerobic microorganisms able to attack lignocelluloses attack mostly the carbohydrate moieties of the complexes (12).

Our results indicate that rumen fungi in mixed populations can extensively degrade the lignified sclerenchyma tissues and weaken intact leaf blades. Such attack on sclerenchyma does not occur extensively with rumen bacteria. Further, in nylon bag studies of related work (Akin and Hogan, in press), rumen fluid from sheep fed −S *D. pentzii* (i.e., no fungi) left almost twice as much sclerenchyma as that in sheep eating +S forage (i.e., fungi present). In the absence of actively growing bacteria, fungi can degrade and weaken the more resistant lignified vascular structures. The extent of activity in these bacterium-free systems probably overestimates the attack and weakening of plant tissue in vivo, for hyphal development appears to be more extensive in the absence of bacteria. It is possible that extensive mycelial development permits growth into remote areas of the plant (7) and disrupts tissues and thereby weakens intact leaves. Interestingly, though, Bauchop and Mountfort (8) found a synergistic effect on cellulose fermentation between *Neocallimastix frontalis* and methanogenic rumen microorganisms.

Increased fragility (as shown by breaking force tests) occurred for intact leaves incubated in vitro or in vivo when rumen fungi were present. Although Bauchop (7) reported that fungal penetration through stomata was of minor importance in grass and legume stems, our studies with *D. pentzii* indicated that cuticular rupture was not frequent in leaves, but that hyphal penetration through stomata occurred frequently (Fig. 6) (12) and could result in a greatly weakened leaf after only 6 h.

In the past we have often observed degradation of sclerenchyma in association with pleomorphic structures (1). It now appears possible to conclude that these structures are thin sections of thalli or rhizoids (22) of rumen fungi that are anchored at these sites and have digested surrounding cell walls. Chemotactic responses

of the rumen fungal zoospores have been found for several soluble carbohydrates (28), especially in response to carbohydrate mixtures from awn and inflorescence tissue. However, it is not known whether similar mechanisms attract the zoospores to the sclerenchyma for initial colonization. Although rumen fungi do colonize other types of lignified fiber, notably the epidermis and sclerenchyma ring of stems and the vascular tissue of blades and stems (Fig. 5) (6), sclerenchyma was more susceptible to breakdown, as has also been observed in other studies (4, 5).

Since the function of lignified tissues is for plant support, it is possible that fungal breakdown of sclerenchyma and weakening of other support tissue could result in forages being more easily reduced in particle size or made more fragile. Such a role for rumen fungi could help explain other results with *Digitaria* species which had been fertilized in a similar way to this study (31–33) where reduced fiber retention time in the rumen and increased forage intake were observed with +S compared with −S forages.

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LITERATURE CITED

1. **Akin, D. E.** 1976. Ultrastructure of rigid and lignified forage tissue degradation by a filamentous rumen microorganism. J. Bacteriol. **125**:1156–1162.
2. **Akin, D. E.** 1979. Microscopic evaluation of forage digestion by rumen microorganisms—a review. J. Anim. Sci. **48**:701–710.
3. **Akin, D. E.** 1980. Evaluation by electron microscopy and anaerobic culture of types of rumen bacteria associated with digestion of forage cell walls. Appl. Environ. Microbiol. **39**:242–252.
4. **Akin, D. E.** 1980. Attack on lignified grass cell walls by a facultatively anaerobic bacterium. Appl. Environ. Microbiol. **40**:809–820.
5. **Barton, F. E., II, and D. E. Akin.** 1977. Digestibility of delignified forage cell walls. J. Agric. Food Chem. **25**:1299–1303.
6. **Bauchop, T.** 1979. Rumen anaerobic fungi of cattle and sheep. Appl. Environ. Microbiol. **38**:148–158.
7. **Bauchop, T.** 1981. The anaerobic fungi in rumen fibre digestion. Agric. Environ. **6**:339–348.
8. **Bauchop, T., and D. O. Mountfort.** 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. Appl. Environ. Microbiol. **42**:1103–1110.
9. **Beever, D. E., D. G. Harrison, D. J. Thomson, S. B. Cammell, and D. F. Osbourn.** 1974. A method for the estimation of dietary and microbial protein in duodenal digesta in ruminants. Br. J. Nutr. **32**:99–112.
10. **Bull, L. S., and J. H. Vandersall.** 1973. Sulfur source for *in vitro* cellulose digestion and *in vivo* ratio utilization, nitrogen metabolism, and sulfur balance. J. Dairy Sci. **56**:106–112.
11. **Caldwell, D. R., and M. P. Bryant.** 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. **14**:794–801.
12. **Crawford, D. L.** 1974. Growth of *Thermonospora fusca* on lignocellulosic pulps of varying lignin content. Can. J. Microbiol. **20**:1069–1072.
13. **Downes, A. M., and I. W. McDonald.** 1964. The chromium-51 complex of ethylenediamine tetraacetic acid as a soluble rumen marker. Br. J. Nutr. **18**:153–162.
14. **Evans, J. L., and G. K. Davis.** 1966. Influence of sulfur, molybdenum, phosphorus and copper interrelationships in cattle upon cellulose digestion *in vivo* and *in vitro*. J. Anim. Sci. **25**:1014–1018.
15. **Gaillard, B. D. E., and G. N. Richards.** 1975. Presence of soluble lignin-carbohydrate complexes in the bovine rumen. Carbohydr. Res. **42**:135–145.
16. **Hartley, R. D., E. C. Jones, and J. S. Fenlon.** 1974. Prediction of the digestibility of forages by treatment of their cell walls with cellulolytic enzymes. J. Sci. Food Agric. **25**:947–954.
17. **Henning, P. A., and A. E. van der Walt.** 1978. Inclusion of xylan in a medium for the enumeration of total culturable rumen bacteria. Appl. Environ. Microbiol. **35**:1008–1011.
18. **Joblin, K. N.** 1981. Isolation, enumeration, and maintenance of rumen anaerobic fungi in roll tubes. Appl. Environ. Microbiol. **42**:1119–1122.
19. **Latham, M. J., B. E. Brooker, G. L. Pettipher, and P. J. Harris.** 1978. *Ruminococcus flavefaciens* cell coat and adhesion to cotton cellulose and to cell walls in leaves of perennial ryegrass (*Lolium perenne*). Appl. Environ. Microbiol. **35**:156–165.
20. **Lund, A.** 1974. Yeasts and moulds in the bovine rumen. J. Gen. Microbiol. **81**:453–462.
21. **Moir, R. J.** 1975. The role and requirement for sulphur in ruminant nutrition, p. 109–116. *In* K. D. McLachlan (ed.), Sulphur in Australian agriculture. Sydney University Press, Sydney, Australia.
22. **Munn, E. A., C. G. Orpin, and F. J. Hall.** 1981. Ultrastructural studies of the free zoospore of the rumen phycomycete *Neocallimastix frontalis*. J. Gen. Microbiol. **125**:311–323.
23. **Orpin, C. G.** 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. J. Gen. Microbiol. **91**:249–262.
24. **Orpin, C. G.** 1977. Invasion of plant tissue in the rumen by the flagellate *Neocallimastix frontalis*. J. Gen. Microbiol. **98**:423–430.
25. **Orpin, C. G.** 1977. The rumen flagellate *Piromonas communis*: its life-history and invasion of plant material in the rumen. J. Gen. Microbiol. **99**:107–117.
26. **Orpin, C. G.** 1977. On the induction of zoosporogenesis in the rumen phycomycetes *Neocallimastix frontalis*, *Piromonas communis*, and *Sphaeromonas communis*. J. Gen. Microbiol. **101**:181–189.
27. **Orpin, C. G.** 1981. Isolation of cellulolytic phycomycete fungi from the caecum of the horse. J. Gen. Microbiol. **123**:287–296.
28. **Orpin, C. G., and L. Bountiff.** 1978. Zoospore chemotaxis in the rumen phycomycete *Neocallimastix frontalis*. J. Gen. Microbiol. **104**:113–122.
29. **Orpin, C. G., and A. J. Letcher.** 1979. Utilization of cellulose, starch, xylan, and other hemicelluloses for growth by the rumen phycomycete *Neocallimastix frontalis*. Curr. Microbiol. **3**:121–124.
30. **Purser, D. B., and S. M. Buechler.** 1966. Amino acid composition of rumen microorganisms. J. Dairy Sci. **49**:81–84.
31. **Rees, M. C., P. Davis, and J. P. Hogan.** 1982. The effect of fertilizer and supplementary sulphur on the digestion of *Digitaria pentzii* in sheep. Proc. Aust. Soc. Anim. Prod. **14**:681.
32. **Rees, M. C., and D. J. Minson.** 1978. Fertilizer sulphur as a factor affecting voluntary intake, digestibility and reten-

tion time of pangola grass (*Digitaria decumbens*) by sheep. Br. J. Nutr. **39**:5–11.

33. **Rees, M. C., D. J. Minson, and F. W. Smith.** 1974. The effect of supplementary and fertilizer sulphur on voluntary intake, digestibility, retention time in the rumen, and site of digestion of pangola grass in sheep. J. Agric. Sci. Camb. **82**:419–422.
34. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
35. **Weston, R. H., and J. P. Hogan.** 1967. The digestion of chopped and ground roughages by sheep. 1. The movement of digesta through the stomach. Aust. J. Agric. Res. **18**:789–801.