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Mode of Attack on Orchardgrass Leaf Blades by Rumen Protozoa

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Leaf blade sections of orchardgrass were incubated with rumen fluid and examined by scanning and transmission electron microscopy for the mode of attack on tissues by rumen protozoa. Rumen protozoa resembling *Epidinium ecaudatum* form *caudatum* degraded forage tissue in diluted, whole rumen fluid suspensions of microbes containing 1.6 mg of streptomycin per ml, which inhibited bacterial fiber-digesting activity. Cell walls of mesophyll, parenchyma bundle sheath, and epidermis became swollen and frayed to reveal a microfibrillar network and loss of electron density, indicating partial degradation. Then the protozoa ingested whole cells and fragments of cell walls with the aid of their cilia. Plant cells with partially degraded walls as well as chloroplasts without walls were present within the protozoa. These entodiniomorphs digested orchardgrass leaves by partially degrading the plant cell walls apparently by extracellular enzymes and then ingestion of the plant cells and cell wall fragments.

Although rumen bacteria are more active forage cell wall degraders than the protozoa, certain entodiniomorphs possess the ability to degrade cell wall constitutents (10). Yoder et al. (14) stated that cellulose digestion by rumen protozoa alone was about 7%. Bauchop and Clarke (9) reported that *Epidinium ecaudatum* form *caudatum* associated in high number with alfalfa stems during digestion in the rumen. Recently we found (6) that a rumen protozoan resembling *E. ecaudatum* form *caudatum* preferentially occupied degraded regions in cool-season grasses, as shown by light and electron microscopy, and digested in vitro (in the absence of rumen bacterial fiber-digesting activity) 13.6% of orchardgrass on a dry-matter basis; mesophyll was degraded first and parenchyma bundle sheath and epidermis later. *Epidinium* has been reported to have cellobiase and hemicellulase activity, but "do not ingest cellulosic plant parts in the same fashion as do the cellulolytic entodiniomorphs" (10).

The objective of the present work was to examine by ultrastructural methods the attack by rumen protozoa on orchardgrass tissues and to determine the manner in which forage cell walls are digested.

MATERIALS AND METHODS

Microbial inocula. The inocula used were (i) washed cell suspensions of rumen bacteria (3) and (ii) whole rumen fluid (containing both bacteria and protozoa) diluted 1:1 with McDougall carbonate buffer as described previously (6).

Substrate. "Boone" orchardgrass (*Dactylis glomerata* L.) was harvested at 28 days of regrowth, frozen in dry ice, and stored at −30°C until used. Sections of blades, 2 to 3 mm, from the center of the leaf were prepared for in vitro digestion studies.

In vitro digestion studies. Fifty sections of orchardgrass blades were placed into 50-ml tubes with 30 ml of inoculum as described (6), and 1.6 mg of streptomycin was added per ml, a concentration that had been shown by microscopy and dry-matter digestion tests to inhibit the fiber-digesting activity of rumen bacteria (6). Streptomycin was omitted from one tube containing substrate and the washed cell inoculum. The tubes were sealed with Bunsen valves and incubated at 39°C. Leaf sections were sampled for digestion at 4, 10 to 12, 24, and 48 h.

Electron microscopy. For scanning electron microscopy (SEM), sections digested for 4 or 10 h were prepared as described (1). For transmission electron microscopy (TEM), blades incubated for up to 48 h were prepared as described (5). Ultrathin sections were made from digested areas of the blades with the highest number of protozoa. These ultrathin sections were stained with uranyl acetate and lead citrate and observed at 50 kV, and the negatives were processed under identical conditions to ensure valid comparisons of electron density.

RESULTS AND DISCUSSION

Leaf blades of orchardgrass incubated with washed cell suspensions of rumen bacteria (i.e., without protozoa) plus 1.6 mg of streptomycin per ml indicated the lack of generalized cell wall digestion as shown by intact, contiguous cells having electron-dense cell walls (Fig. 1) similar to those in undigested leaf blades similarly pre-

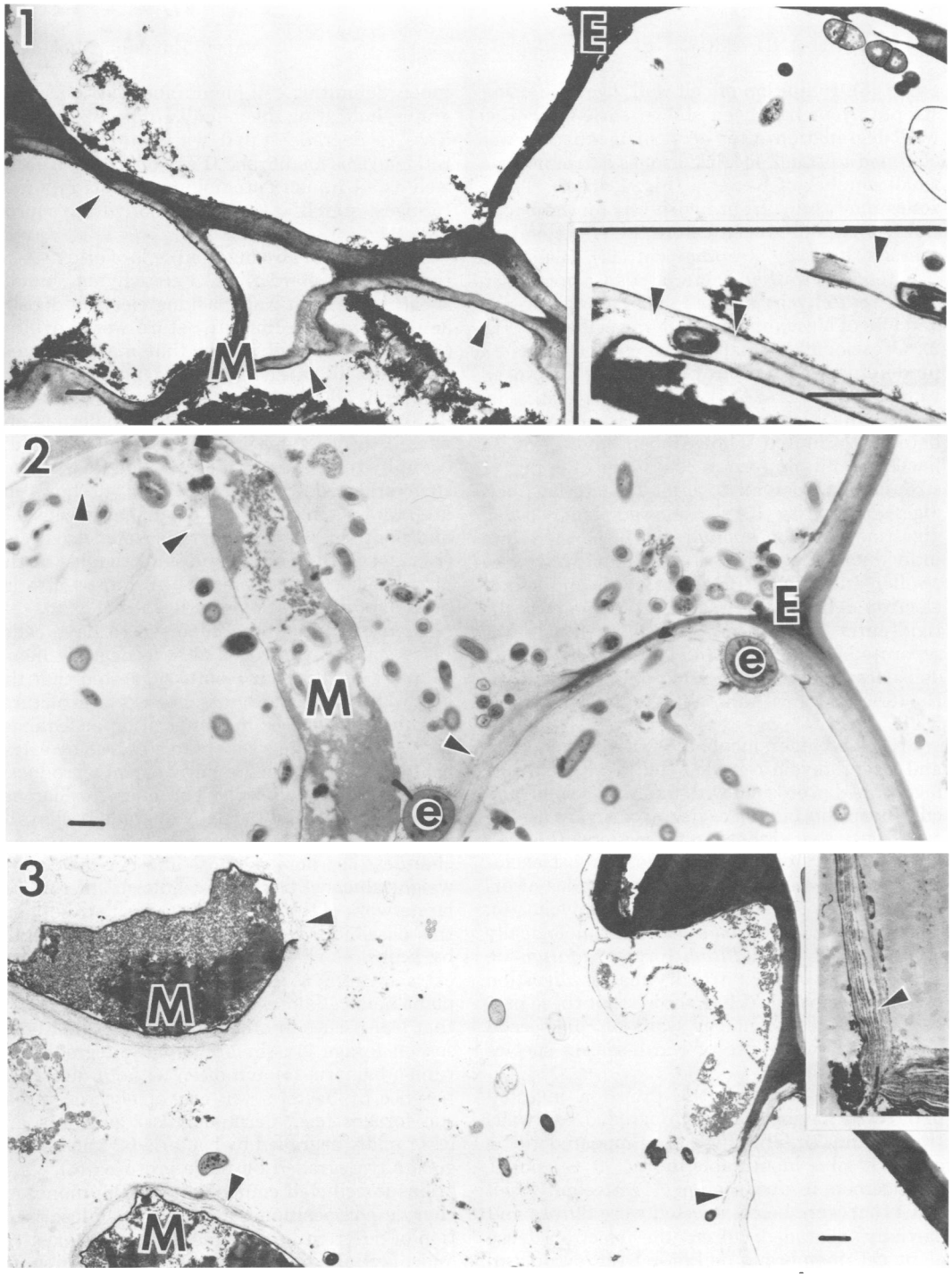


FIG. 1. *TEM of blade incubated for 48 h in a washed cell suspension of rumen bacteria with streptomycin, showing intact nature of epidermis (E) and mesophyll (M) cells and electron density of cell walls (arrows). Digestion was limited to small, localized zones (arrows) surrounding few bacteria (inset). Bars, 1 μm each.*

FIG. 2. *TEM of blade incubated for 12 h in a washed bacterial suspension without streptomycin, showing generalized erosion and loss of electron density (arrows) of mesophyll (M) and epidermal (E) cell walls and attachment to walls by encapsulated cocci (e). Bar, 1 μm.*

FIG. 3. *TEM of blade incubated for 24 h in rumen fluid/streptomycin, showing separation of mesophyll tissue into cells (M) with walls having lost electron density and swollen to reveal microfibrils (arrows). Bacteria are not attached to plant walls. Bar, 1 μm. Enlarged section of the inner part of the epidermal cell wall showing a distinctly frayed fibrillar nature (arrow).*

pared (5). Inhibition of cell wall digestion using streptomycin had been shown earlier (6). Cell wall degradation after 48 h of incubation was confined to small, localized zones surrounding a small number of bacteria (Fig. 1, inset). These zones may result from hydrolysis by the bacterial enzymes produced before the effect on protein synthesis by streptomycin (12). Blades incubated with washed bacterial suspensions without streptomycin showed generalized erosion and loss of electron density of the cell walls (Fig. 2). Occasionally, encapsulated cocci (Fig. 2e), a predominant cellulolytic bacterium in the mixed rumen microflora (1–3), adhered to and degraded the plant cell walls. Leaf blades incubated with diluted, whole rumen fluid (i.e., with bacteria and protozoa) containing 1.6 mg of streptomycin per ml to inhibit bacterial fiber-digesting activity (13) also showed plant cell wall digestion. The mesophyll was separated into individual cells (Fig. 3), and plant walls were swollen and diffuse, revealing fibrils and loss of electron density (Fig. 3, arrows). Often even the rigid outer part of the epidermal cell wall was separated from the cuticle, lacked electron denseness, and revealed a microfibrillar network. Bacteria did not adhere to and degrade cell walls.

SEM of blades incubated with rumen fluid and streptomycin revealed that a tapered, surface-ribbed protozoan with a terminal spine and cilia near the buccal cavity exclusively associated with the orchardgrass sections (Fig. 4). High numbers of these protozoa almost exclusively had been found within leaf blades of orchardgrass incubated with streptomycin (6). This type of protozoan was morphologically identical to *E. ecaudatum* form *caudatum* (9, 10). Tests using in vitro dry-matter digestion procedures and SEM had shown that these protozoa degraded plant cell walls of cool-season grasses, but not those of warm-season species (6).

TEM revealed that the protozoa ingested plant cells having partially degraded cell walls (Fig. 5 and 7). This ingestion appeared to be aided by cilia, identifiable by the "9 + 2 fibril" arrangement in cross section (11). Mesophyll cell walls that were being ingested were diffuse and partially degraded; often the electron-dense plant cell membrane delimiting the cytoplasm was observed (Fig. 5). The microfibrillar network of the cell wall was often exposed during ingestion by protozoa (Fig. 5, inset). Chloroplasts with voids resulting from starch grain degradation and lacking the plant cell wall and membrane were present within protozoa (Fig. 6a). Other chloroplasts within protozoa retained the inner, delimiting cell membrane and the starch grains, indicating the inability of protozoal amylases to degrade starch within cell walls that retained this membrane (Fig. 6b). *Epidinium*, as well as all rumen entodiniomorphs, is reported to ingest starch as a chief carbohydrate source (10).

The protozoa also ingested cells of other tissue types in orchardgrass. Parenchyma bundle sheath cells with walls lacking electron density and showing the fibrillar nature were engulfed (Fig. 7). SEM had shown that mesophyll, parenchyma bundle sheath, and portions of the epidermis all were degraded by the protozoa (6). The walls of guard cells in the epidermis appeared to resist enzymatic breakdown, as indicated by their retention of electron density and structural rigidity. However, Fig. 8 shows an attempt by a protozoan to engulf guard cells (G) and another partially degraded epidermal cell (E), and the inset shows loss of rigidity of the guard cell wall near cilia (C). Protozoa also ingested remnants of plant cell walls (Fig. 9).

Epidinium has been reported to have cellobiase activity and to be able to degrade hemicellulose (8, 10). Our results suggested that the cell walls of orchardgrass possess a structural organization (either in composition or arrangement) that was degradable to a degree by extracellular, cell wall-degrading enzymes produced by this entodiniomorph. The microfibrillar nature of swollen and partially degraded cell walls could be due to the removal of matrix polyaccharides (i.e., hemicellulose and low-molecular-weight glucans) from the cellulosic, microfibrillar network of the plant wall (7). Data to support this possibility come from an exhaustive study by Bailey et al. (8) in which they showed that extracts from *E. ecaudatum* degraded various plant hemicelluloses. Previous work has shown that leaf tissues in orchardgrass and other cool-season forage grasses are rapidly degraded by rumen bacteria (4) and often without direct adherence by bacteria (2); conversely, warm-season forages (e.g., bermudagrass) generally are less rapidly degraded by bacteria (4) and are not generally degraded by these protozoa (6). Variations in cell wall components and in monosaccharide composition of the hemicellulose fraction of different forage grass species do exist (7) and may influence plant cell wall digestion by the rumen protozoa as well as by the rumen bacteria (4).

Our results indicated that a protozoan resembling *E. ecaudatum* form *caudatum* can partially hydrolyze cell walls of orchardgrass tissues apparently by extracellular enzymes. Consequently, these tissues separate into individual

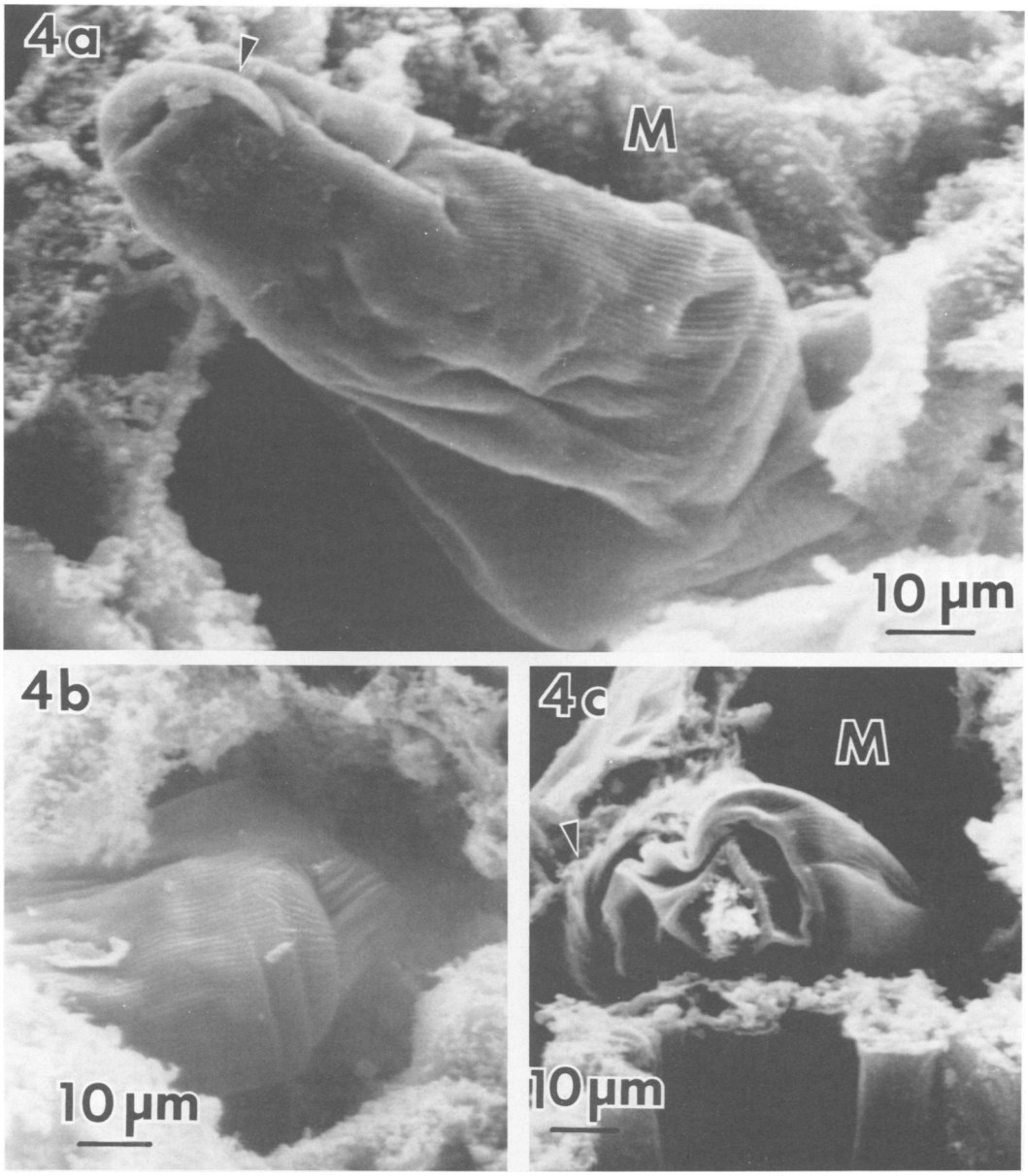


FIG. 4. *SEM of blade incubated for 4 to 10 h in rumen fluid/streptomycin, showing morphological characteristics of the protozoa that degrade plant tissues. (a) A protozoan degrading mesophyll tissue (M) has a ribbed surface, a tapered body, and a single, terminal spine (arrow). (b) A similar protozoan within degraded mesophyll. (c) Protozoan showing cilia (arrow) near head within mesophyll (M) that is totally degraded.*

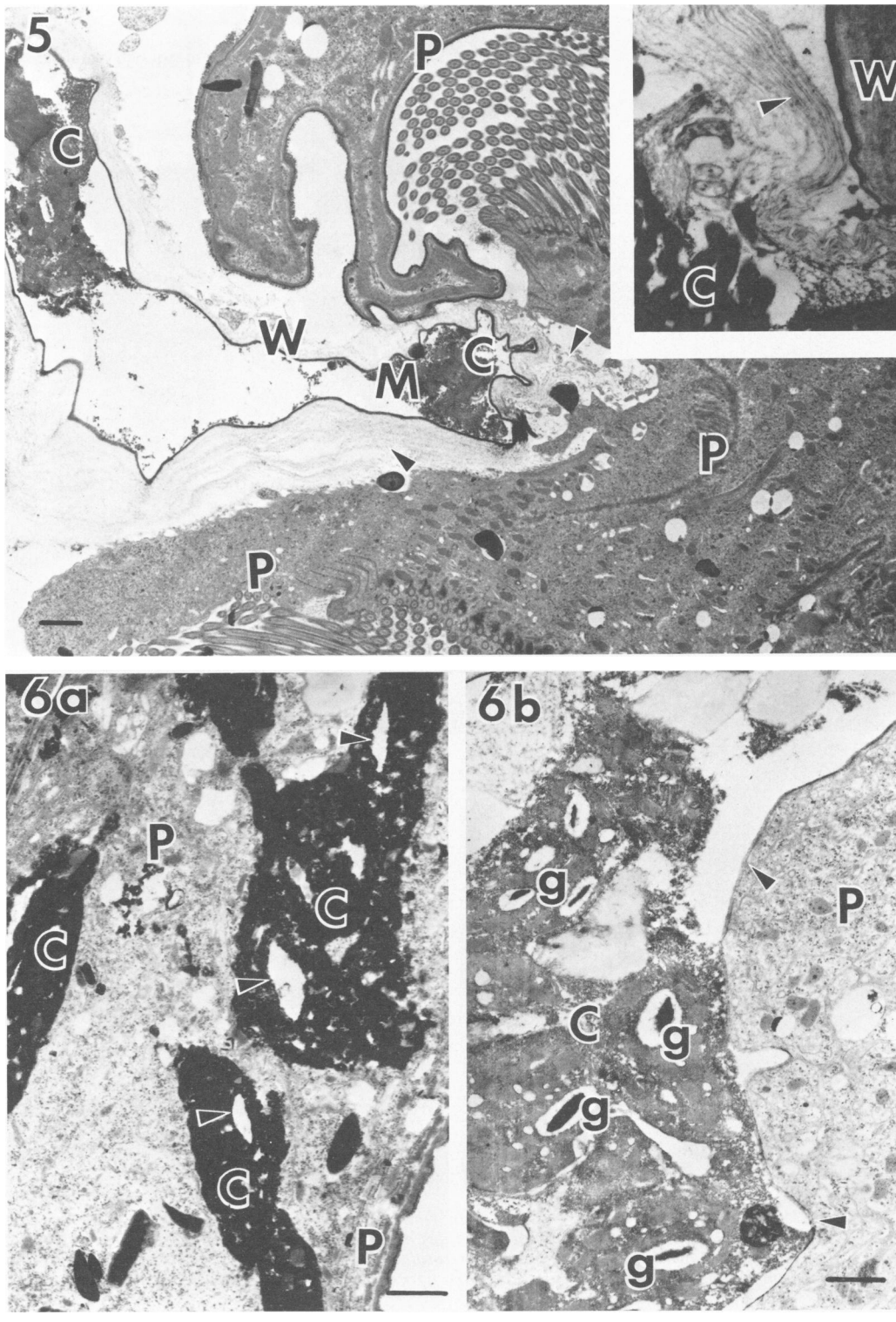
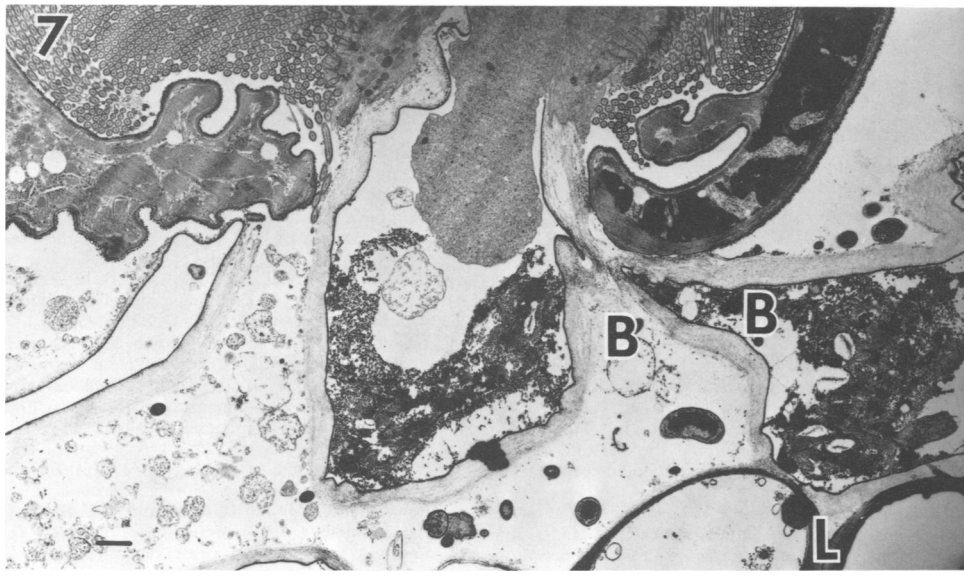
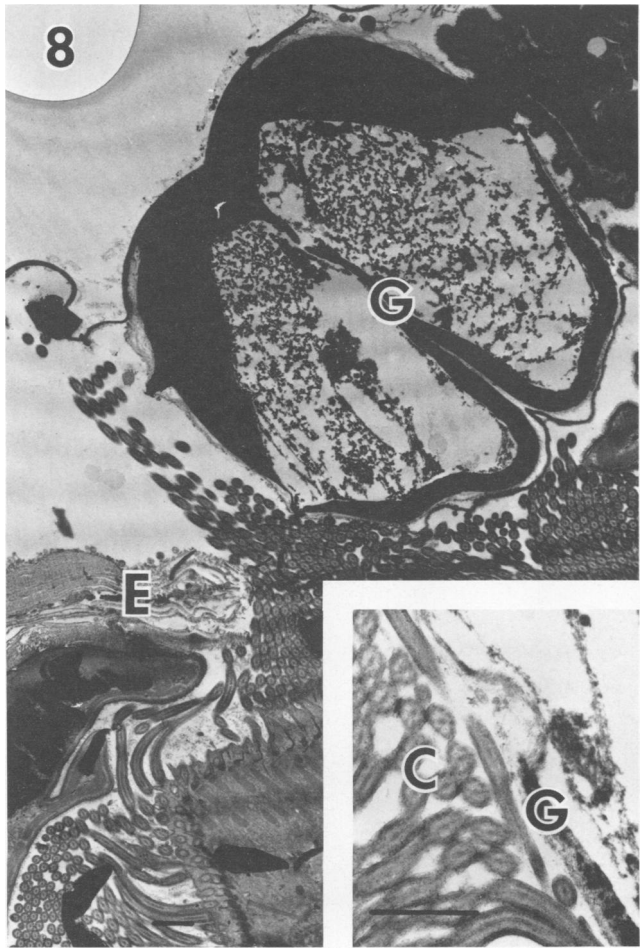


FIG. 5. *TEM of blade incubated for 24 h in rumen fluid/streptomycin, showing ingestion by the protozoan (P) of a mesophyll cell (M) having a partially degraded wall (W) exposing the microfibrils (arrow). Note the electron-dense membrane delimiting the plant cytoplasm (C). The inset shows a cell wall (W) being ingested with the aid of protozoal cilia (C); part of the wall clearly reveals a frayed microfibrillar network (arrow). Bar, 1 μm.*

FIG. 6. *TEM of chloroplasts within protozoa (P) from blades incubated for 24 h in rumen fluid/streptomycin. (a) Chloroplasts (C) lacking cell walls have voids (arrows) indicating starch digestion. (b) Chloroplast (C) with starch grains (g) and the delimiting plant cell membrane (arrows) still present. Bars, 1 μm each.*





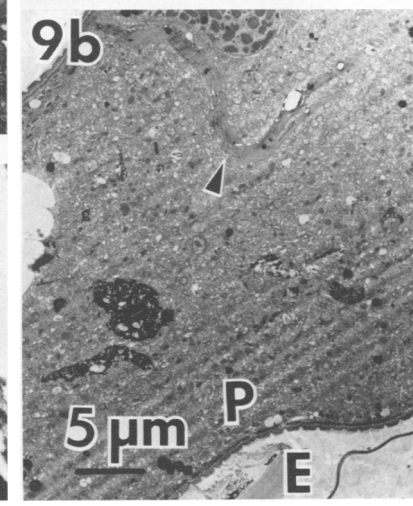


FIG. 7. *TEM of blade incubated for 24 h in rumen fluid/streptomycin, showing protozoal ingestion of parenchyma bundle sheath cells (B). Note electron density of the undigested lignified vascular tissues (L). Bar, 1 µm.*

FIG. 8. *TEM of blade incubated for 24 h in rumen fluid/streptomycin, showing ingestion of guard cells (G) and another partially degraded epidermal cell wall (E). The inset shows destruction of the guard cell wall (G) near cilia (C). Bars, 1 µm each.*

FIG. 9. *TEM of ingested cell wall fragments from blades incubated for 24 h in rumen fluid/streptomycin. (a) Cell wall fragment (arrow) from epidermis (E) being ingested. (b) Cell wall fragment (arrow), possibly from epidermis (E), within a protozoan (P).*

cells and are ingested by the protozoan. Often plant cells delimited by the cell membrane but lacking a cell wall were found within the protozoa, indicating their potential to totally degrade the forage cell wall.

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