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Effects of Condensed Tannins on Endoglucanase Activity and Filter Paper Digestion by *Fibrobacter succinogenes* S85†

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The effect of condensed tannins from birdsfoot trefoil (*Lotus corniculatus* L.) on the cellulolytic rumen bacterium *Fibrobacter succinogenes* S85 was examined. Condensed tannins inhibited endoglucanase activity in the extracellular culture fluid, at concentrations as low as 25 μg ml^{-1}. In contrast, cell-associated endoglucanase activity increased in concentrations of condensed tannins between 100 and 300 μg ml^{-1}. Inhibition of endoglucanase activity in both the extracellular and the cell-associated fractions was virtually complete at 400 μg of condensed tannins ml^{-1}. Despite the sharp decline in extracellular endoglucanase activity with increasing concentrations of condensed tannins, filter paper digestion declined only moderately between 0 and 200 μg of condensed tannins ml^{-1}. However, at 300 μg ml^{-1}, filter paper digestion was dramatically reduced and at 400 μg ml^{-1}, almost no filter paper was digested. *F. succinogenes* S85 was seen to form digestive grooves on the surface of cellulose, and at 200 μg ml^{-1}, digestive pits were formed which penetrated into the interior of cellulose fibers. Cells grown with condensed tannins (100 to 300 μg ml^{-1}) possessed large amounts of surface material, and although this material may have been capsular carbohydrate, its osmiophilic nature suggested that it had arisen from the formation of tannin-protein complexes on the cell surface. The presence of electron-dense extracellular material suggested that similar complexes were formed with extracellular protein.

Condensed tannins are hydroxyflavanols formed from the polymerization of leucoanthocyanidin and catechin (18, 33). These compounds readily form hydrophobic and hydrogen bonds with proteins to form tannin-protein complexes. The formation of these complexes with both feed proteins and digestive enzymes reduces the nutritional value of forages for ruminants (1).

Condensed tannins in feeds decrease voluntary feed intake and digestion of forages by ruminants (2, 18). Reduction in forage intake has been attributed to the effects of condensed tannins on palatability (23); however, the mechanisms responsible for the depression in forage digestion are less defined. It is likely that condensed tannins suppress forage digestion by inhibiting both microbial enzymes in the rumen and mammalian enzymes in the small intestine. The bacteriostatic and bactericidal effects of condensed tannins on pathogenic microorganisms are well known (14). Inhibition of cellulose digestion (19), ruminal microbial enzymes (3, 30), and cell wall synthesis in *Cellvibrio fulvus* (14) suggests that condensed tannins retain their antimicrobial properties in the rumen.

Although alterations in enzymatic activity and bacterial growth may contribute to the negative effect of condensed tannins on forage digestion, little is known about the specific effects of these compounds on rumen bacteria. *Fibrobacter succinogenes* is recognized as the most active of the rumen bacteria in the digestion of cellulosic substrates in the rumen (10, 13, 32), and consequently, many of its fibrolytic enzymes have been characterized in detail (21, 22). Thus, the objective of this study was to determine the specific effects of condensed tannins isolated from birdsfoot trefoil (*Lotus corniculatus* L.) on the digestion of cellulose by *F. succinogenes* S85.

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MATERIALS AND METHODS

Organisms and growth medium. *F. succinogenes* subsp. *succinogenes* S85, formerly *Bacteroides succinogenes* (ATCC 19169) (24), was obtained from the Lethbridge Research Station Culture Centre. It was grown in a modified liquid medium (10 ml) of Scott and Dehority (29). Modifications included (i) removal of casein hydrolysate, (ii) addition of autoclaved, clarified rumen fluid (10%, vol vol^{-1}), and (iii) addition of 1.0 cm^2 of Whatman no. 1 filter paper as the sole source of carbohydrate. Cultures were grown at 39°C and were transferred every 3 days for a 2-week period prior to experimentation with condensed tannins. The anaerobic technique of Hungate (15), as modified by Bryant and Burkey (6), was used throughout the experiment.

Isolation of condensed tannins. Condensed tannins were obtained from a high-tannin variety of birdsfoot trefoil (*Lotus corniculatus* L.) which contained 3% condensed tannins on a dry matter basis. Fresh, whole plants were homogenized with 70% acetone containing 0.1% (wt vol^{-1}) ascorbic acid in a Waring blender. Acetone extracts were reduced to the aqueous phase by evaporation under reduced pressure at 35°C. The resulting aqueous phase was extracted with petroleum ether followed by ethyl acetate until both organic solvents became clear. Traces of ethyl acetate were removed from the remaining aqueous fraction by rotary evaporation. The resulting fraction was diluted 1:1 with methanol and was applied to a Sephadex LH-20 column equilibrated in 50% aqueous methanol. Columns were then washed with 50% aqueous methanol until the elutant was clear. Condensed tannins were eluted from the columns by using 70% acetone and were evaporated to the aqueous fraction. The aqueous phase was lyophilized, and the extracted condensed tannins were stored in the dark in a desiccator at 5°C.

Culture techniques with condensed tannins. After 3 days of growth on filter paper, cultures of *F. succinogenes* S85 were mixed and centrifuged (10,000 × *g*, 20 min, 4°C) and the

supernatant was discarded. Cells were suspended in 1 ml of fresh medium, and 100 μl of the bacterial suspension was used to inoculate test tubes containing 25 mg of Whatman no. 1 filter paper and 4 ml of medium. Condensed tannins were dissolved in Bryant's anaerobic salt solution (6) and were filter sterilized. Stock solutions of condensed tannins were prepared so that addition of 100 μl to cultures of *F. succinogenes* S85 resulted in the desired final concentration of condensed tannins. The effect of condensed tannins on cellulolytic activity and the digestion of filter paper was examined at concentrations ranging from 25 to 300 μg ml^{-1} (25-μg increments) and from 300 to 800 μg ml^{-1} (100-μg increments). Quadruplicate cultures were prepared for each concentration of condensed tannins and were incubated for 3 days at 39°C. Three of the cultures were used to estimate cellulolytic activity and filter paper disappearance, while the fourth culture was prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Filter paper digestion. Cultures were vacuum filtered to collect remaining filter paper residue onto preweighed Whatman no. 54 filter paper. The resulting filtrate was placed on ice. The filter paper was then washed three times with 50 mM sodium phosphate buffer (1 ml) and was washed once with methanol (1 ml). The filter paper residue was dried at 80°C for 24 h and was weighed to estimate the extent of filter paper digestion.

Cell fractionation and endoglucanase activity. Collected filtrate (1.0 ml) was centrifuged (15,600 × *g*, 5 min, 4°C) to separate supernatant and cell-associated fractions. Pelleted cells were washed twice with 1.0 ml of 50 mM sodium phosphate buffer (pH 6.5) and were centrifuged (15,600 × *g*, 5 min, 4°C). Cells were suspended in 0.5 ml of 50 mM sodium phosphate buffer (pH 6.5) maintained at 4°C and disrupted by pulsed sonication for 30 s on a Sonic 300 Dismembrator (Artek Systems Corporation, Farmingdale, N.Y.) at 60% output. Crude cell extract was obtained by centrifuging disrupted bacteria (15,600 × *g*, 5 min, 4°C) to remove cell debris.

Endoglucanase activity of the extracellular fluid and crude cell extract was determined with the soluble chromogenic substrate ostazin brilliant red-hydroxyethyl cellulose (4). Extracellular fluid or crude cell extract (0.1 ml) was mixed with 0.1 ml of substrate solution containing 8 mg of ostazin brilliant red-hydroxyethyl cellulose ml^{-1} (Sigma Chemical Co., St. Louis, Mo.) in 50 mM sodium phosphate buffer (pH 6.5). Mixtures were incubated at 39°C for 2 h; reactions were stopped by the addition of 0.8 ml of an ethanol-acetone solution (2:1, vol vol^{-1}). The mixture was allowed to stand at room temperature for 30 min, and the precipitated substrate was removed by centrifugation (15,600 × *g*, 5 min, 4°C). A_{550} of the clear supernatant was measured spectrophotometrically (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Activity was expressed against a commercial endoglucanase from *Penicillium funiculosum* (EC 3.2.1.4; Sigma Chemical Co.) with 1 U of activity defined as the absorbance given by 1 U of commercial endoglucanase ml^{-1} h^{-1}.

Protein determination. The amount of protein in the crude cell extract was determined by the Coomassie blue dye-binding method of Bradford (5). Bovine serum albumin (Sigma) was used as a standard, and all reagents were obtained in a protein assay kit (Bio-Rad Laboratories Ltd., Chemical Division, Richmond, Calif.). Protein in the extracellular fluid fraction was not measured because of interference of free condensed tannins with the Bradford assay.

Bacterial counts. The growth and viability of *F. succinogenes* S85 in various concentrations of condensed tannins were determined with an anaerobic dilution series as described by Bryant and Burkey (6). Cultures of *F. succinogenes* were grown for 3 days in medium containing condensed tannins at 100 to 800 μg ml^{-1} (100-μg increments). Cultures (1 ml) were inoculated into a series (10^{-1} to 10^{-9}) of triplicate dilution tubes containing the modified Scott and Dehority medium containing 1 cm^2 of filter paper and no condensed tannins. Tubes were incubated at 39°C for 5 days and examined for filter paper digestion, and the most probable number was estimated (8).

SEM. Residual filter paper from the fourth replicate culture was fixed for 2 h in 5% glutaraldehyde (J. B. EM Services Inc., Dorval, Quebec, Canada) in 0.1 M sodium cacodylate buffer (pH 7.2). Specimens were then washed five times for 10 min each in 0.1 M cacodylate buffer, dehydrated in a graduated ethanol series, and critical point dried with CO_2. Specimens were mounted on aluminum stubs with silver paste, sputter coated with gold, and viewed with a Hitachi S-570 SEM. Samples were viewed with an accelerating voltage of 7 to 10 kV and were photographed with Ilford FP4 panchromatic film.

TEM. Cultures of *F. succinogenes* S85 were grown at 39°C on modified Scott and Dehority's medium containing 0.2% glucose (wt vol^{-1}) instead of filter paper and 300 μg condensed tannins ml^{-1}. After 24 h, cells were collected by centrifugation, prefixed in 0.5% glutaraldehyde for 30 min, enrobed in 4% agar, and fixed for 2 h in 5% glutaraldehyde at 5°C. Samples were washed five times with 0.1 M sodium cacodylate buffer, postfixed for 2 h in 3% osmium tetroxide, and dehydrated in graded ethanol. Specimens were infiltrated and embedded in Spurr's resin (J. B. EM Services Inc.) (31). Ultrathin sections were cut with a Reichert model OM U3 ultramicrotome and were stained with 2% uranyl acetate and lead citrate (28). Specimens were viewed with a Hitachi H-500 TEM at an accelerating voltage of 75 kV and were photographed with Kodak electron microscope film.

RESULTS

Condensed tannins and endoglucanase activity. The effect of increasing concentrations of condensed tannins extracted from birdsfoot trefoil on the endoglucanase activity of *F. succinogenes* S85 is illustrated in Fig. 1. The majority of endoglucanase activity was associated with the culture supernatant. Extracellular and cell-associated endoglucanase activities were almost completely inhibited by 400 μg ml of condensed $tannins^{-1}$. Exposure to condensed tannins caused a continuous decrease in extracellular endoglucanase activity up to 400 μg of condensed tannins ml^{-1} (Fig. 1A). This decline in extracellular endoglucanase activity was apparent at concentrations of condensed tannins as low as 25 μg ml^{-1} (data not shown). In contrast, cell-associated endoglucanase activity was initially increased by exposure to concentrations of condensed tannins between 100 and 300 μg ml^{-1} (Fig. 1B). This increase in endoglucanase activity was accompanied by a decline in the amount of cell-associated protein (Fig. 1B) and an increase in the specific activity of cell-associated endoglucanases (Fig. 2).

Condensed tannins and filter paper digestion. The effects of condensed tannins on filter paper digestion correlated closely with their effects on endoglucanase activity (Fig. 3). However, the decline in filter paper digestion was not as abrupt as the inhibition of extracellular endoglucanase activity. Digestion of filter paper by *F. succinogenes* S85 was reduced by 8, 10, 45, and 92% when condensed tannins were

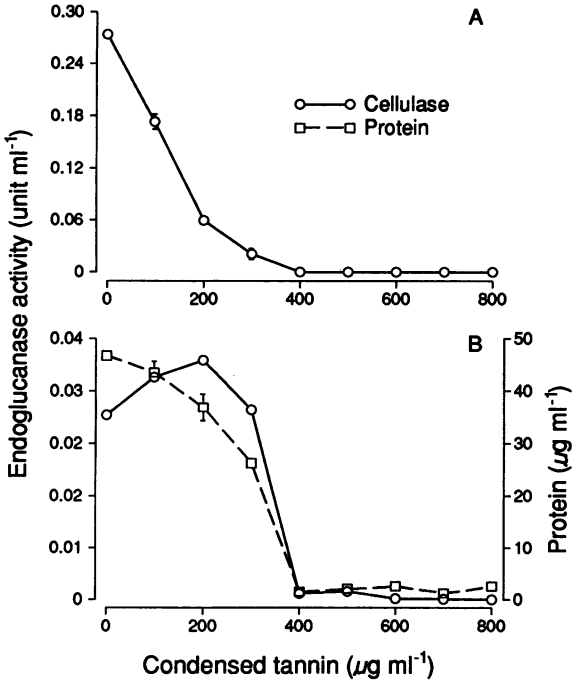

FIG. 1. Effect of birdsfoot trefoil condensed tannins on endoglucanase activity of the culture supernatant (A) and cell-associated endoglucanase activity and protein levels (B). Values are the means of triplicate cultures. Where not shown, standard error bars are within symbols.

100, 200, 300, and 400 μg ml^{-1}, respectively. *F. succinogenes* S85 was virtually unable to digest filter paper at condensed tannin concentrations greater than 400 μg ml^{-1}.

Most probable number of viable bacteria. Exposure of *F. succinogenes* S85 to 400 μg of condensed tannins ml^{-1} inhibited the growth of *F. succinogenes* S85 (Table 1). At concentrations below 400 μg ml^{-1}, growth was not seriously inhibited and numbers of viable bacteria were approximately 10^3 greater than the original inoculant. In contrast, in cultures with >600 μg of condensed tannins ml^{-1}, the numbers of viable bacteria were lower than those in the original inoculant. Although growth of cells at these higher concentrations was severely inhibited, at 800 μg of condensed tannins ml^{-1} some cells remained viable.

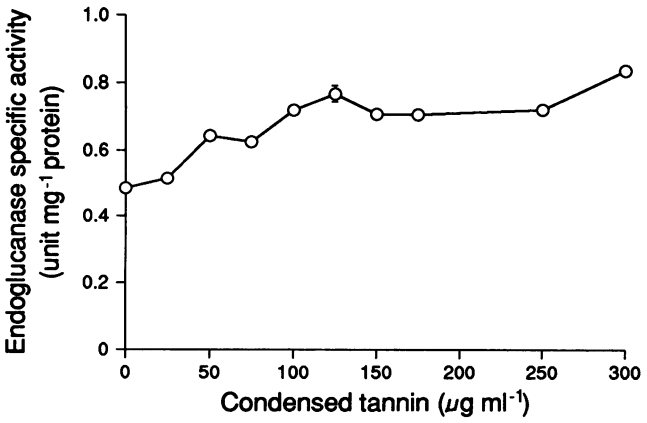

FIG. 2. Effect of birdsfoot trefoil condensed tannins on cell-associated specific endoglucanase activity of *F. succinogenes* S85. Values are the means of triplicate cultures. Where not shown, standard error bars are within symbols.

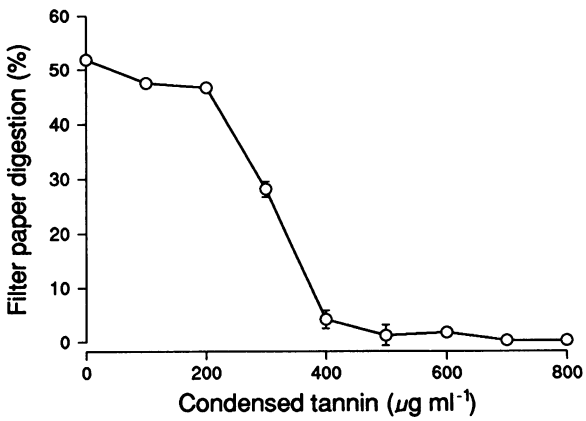

FIG. 3. Effect of birdsfoot trefoil condensed tannins on the digestion of filter paper by *F. succinogenes* S85. Values are the means of triplicate cultures. Where not shown, standard error bars are within symbols.

SEM. Examination of cultures by SEM showed that when grown with 300 μg of condensed tannins ml^{-1}, *F. succinogenes* S85 colonized and formed digestive grooves on the surface of cellulose fibers (Fig. 4A). However, at 400 μg of condensed tannins ml^{-1}, large areas of the surface of filter paper showed no evidence of colonization or digestion (Fig. 4B). *F. succinogenes* S85 grown with 300 μg of condensed tannins ml^{-1} possessed large amounts of surface material (Fig. 4C), a phenomenon that was not observed when condensed tannins were absent during growth (Fig. 4D). At 200 μg of condensed tannins ml^{-1}, cells formed deep digestive grooves, and in some areas complete penetration of cellulose fibers was observed (Fig. 5).

TEM. Condensed tannins are osmiophilic and appear as electron dense areas in transmission electron micrographs of plant cells (27). TEM revealed electron-dense material on the cell surface of *F. succinogenes* S85 grown with condensed tannins (300 μg ml^{-1}) (Fig. 6A), but cells grown

TABLE 1. Effect of increasing concentrations of condensed tannins on retention of viability of *F. succinogenes* S85[a]

Condensed tannins[b] (μg ml^{-1})	Viable count (most probable no. ml^{-1})
0	4.3×10^8
100	2.3×10^8
200	9.3×10^7
300	2.3×10^8
400	2.3×10^6
500	4.3×10^5
600	2.3×10^3
700	9.3×10^2
800	2.4×10^3

[a] Triplicate tubes were initially inoculated with a total of 1.4×10^5 viable cells. Cultures were incubated for 3 days in modified Scott and Dehority medium with Whatman no. 1 filter paper and condensed tannins. Estimation of viable cells ml^{-1} was done with medium which contained no condensed tannins.

[b] Condensed tannins were obtained via an acetone extraction of birdsfoot trefoil (*Lotus corniculatus* L.).

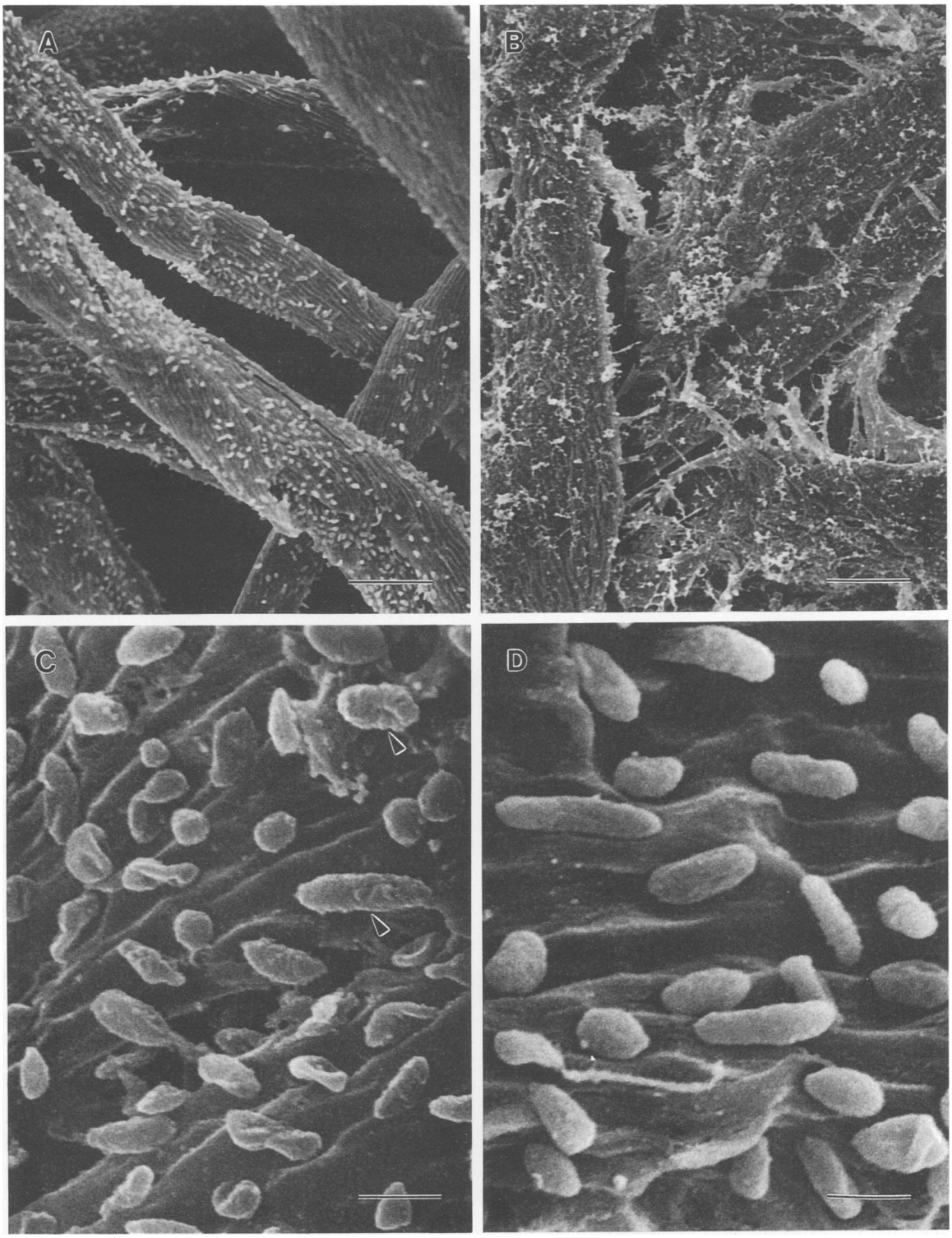

FIG. 4. SEM of Whatman no. 1 filter paper incubated with *F. succinogenes* S85 at 39°C for 72 h in the presence or absence of condensed tannins. (A) Condensed tannins present at 300 μg ml^{-1}. Colonization has occurred, and digestive grooves have been formed on the surface of cellulose fibers. Bar, 10 μm. (B) Condensed tannins present at 400 μg ml^{-1}. There is no evidence of bacterial adhesion or digestion on the surface of cellulose fibers. Bar, 10 μm. (C) Condensed tannins present at 300 μg ml^{-1}. Large amounts of surface material are present on these cells (arrowheads). Bar, 1 μm. (D) No condensed tannins present during growth. Typical digestion grooves are evident, but cells do not possess the surface material seen in panel C. Bar, 1 μm.

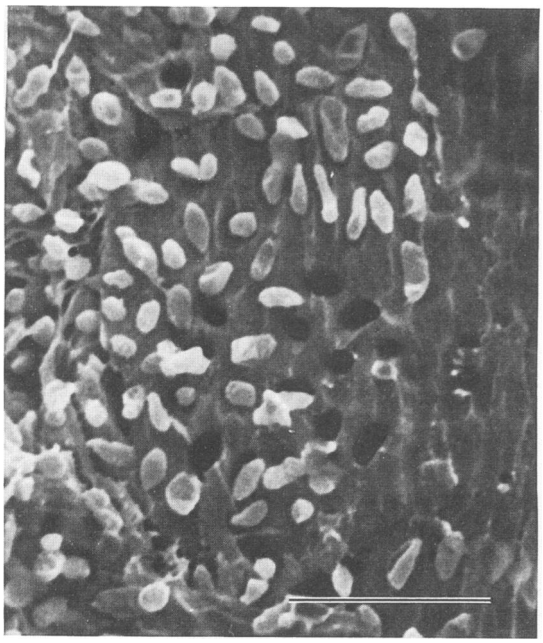

FIG. 5. SEM of *F. succinogenes* S85 grown in the presence of 200 μg of condensed tannins ml^{-1}. Note that cells have formed digestion pits that penetrate into the interior of the cellulose fiber. These pits were not produced by cells grown in the absence of condensed tannins (Fig. 4D). Bar, 5 μm.

without condensed tannins showed no comparable material (Fig. 6B). In the presence of condensed tannins (300 μg ml^{-1}), cells were surrounded by large amounts of extracellular material (Fig. 6C). In contrast, cells grown without condensed tannins exhibited normal cell morphology, and extracellular material was not seen (Fig. 6D).

DISCUSSION

In agreement with results from previous studies (9, 12), the majority of endoglucanase activity produced by *F. succinogenes* S85 was associated with the culture supernatant. Extracellular endoglucanases of *F. succinogenes* S85 were rapidly inhibited by condensed tannins from birdsfoot trefoil and were completely inactivated at concentrations of >400 μg ml^{-1}. Inhibition of extracellular endoglucanases was evident at concentrations of condensed tannins as low as 25 μg ml^{-1}, a concentration similar to that which inhibited the growth of *C. fulvus* and *Clostridium cellulosolvens* (14). The activity of cell-associated endoglucanases, however, increased with initial increases in the concentration of condensed tannins. In fact, the increase in specific activity of cell-associated endoglucanases increased between 0 and 300 μg of condensed tannins ml^{-1} at a rate that was almost linear. Consequently, the decline in the digestion of filter paper with increasing concentration of condensed tannin was not as pronounced as the decline in extracellular endoglucanase activity. As with extracellular endoglucanases, cell-associated endoglucanases were virtually inactivated by 400 μg of condensed tannins ml^{-1}. Thus, the net effect of exposure of *F. succinogenes* S85 to condensed tannins (0 to 300 μg ml^{-1}) was a transient increase in the activity of cell-associated endoglucanases, accompanied by a decline in the activity of extracellular endoglucanases, with cell-associated endoglucanases accounting for an increasing proportion of total endoglucanase activity. As the concentration of condensed tannins approached 400 μg ml^{-1}, all endoglucanases were inhibited and cellulose digestion ceased.

Cells of *F. succinogenes* S85 were seen to adhere intimately to cellulose fibers. Detachment of *F. succinogenes* S85 from cellulose fibers by methylcellulose revealed the presence of parallel digestive grooves on the surface of cellulose fibers (17). Similar grooves were formed on the surface of cellulose fibers when *F. succinogenes* S85 was grown in the absence or presence of up to 300 μg of condensed tannins ml^{-1} but were not evident when cultures were grown with 400 μg ml^{-1}. At 200 μg of condensed tannins ml^{-1}, digestive grooves were present and were accompanied by individual pits that penetrated directly into the interior of cellulose fibers, a phenomenon which was not observed in the absence of condensed tannins. These pits may result from enhanced cell-associated endoglucanase activity at the site of cell attachment, a conclusion that seems reasonable considering the increase in the activity of cell-associated endoglucanases in the presence of 100 to 300 μg of condensed tannins ml^{-1}.

F. succinogenes S85 cells grown in the presence of condensed tannins (100 to 300 μg ml^{-1}) were seen to possess large amounts of electron-dense surface-associated material. Although it is possible that this surface material is capsular carbohydrate, electron-dense material was still evident in TEM preparations that were not post-stained with lead citrate and uranyl acetate. Considering that condensed tannins are highly osmiophilic (27) and bind to rumen bacteria (16), it is feasible that this electron-dense material is a result of formation of complexes between condensed tannins and the cell surface. Formation of tannin-protein and/or tannin-carbohydrate complexes may also account for the electron-dense extracellular material observed in cultures grown with condensed tannins.

The present study confirms the bacteriostatic properties of condensed tannins reported by others (14, 20). *C. fulvus*, *Sporocytophaga myxococcoides*, and *Bacillus subtilis* differ in their sensitivity to condensed tannins (14), and it is likely that similar differences in sensitivity exist among rumen microorganisms. Although condensed tannins were undeniably bacteriostatic to *F. succinogenes* S85, the recovery of living cells from cultures containing 800 μg of condensed tannins ml^{-1} demonstrates that these compounds are not completely bactericidal.

The inhibitory effects of condensed tannins on cellulose digestion may not be solely related to their inactivation of extracellular enzymes through the formation of tannin-enzyme complexes. Adhesion is thought to be essential for the digestion of cellulose (7, 25). The mechanisms of adhesion are not fully characterized, but treatment of *F. succinogenes* S85 cells with trypsin, pronase, or glutaraldehyde reduces the adhesion of cells to cellulose, implicating the involvement of proteins on the cell surface in the adhesion process (11). Apparent formation of protein-tannin complexes on the cell surface of *F. succinogenes* S85 makes it plausible that condensed tannins may interfere with the adhesion process.

This report clearly demonstrates the inhibitory effects of condensed tannins on cellulose digestion. Further work is required to assess the effects that condensed tannins have on microbial adhesion, penetration, colonization, and consortium formation, processes which are essential for the ruminal digestion of feed. Although microorganisms capable of degrading protein-tannin complexes have been isolated from ruminants (26), there are no reports of rumen microorgan-

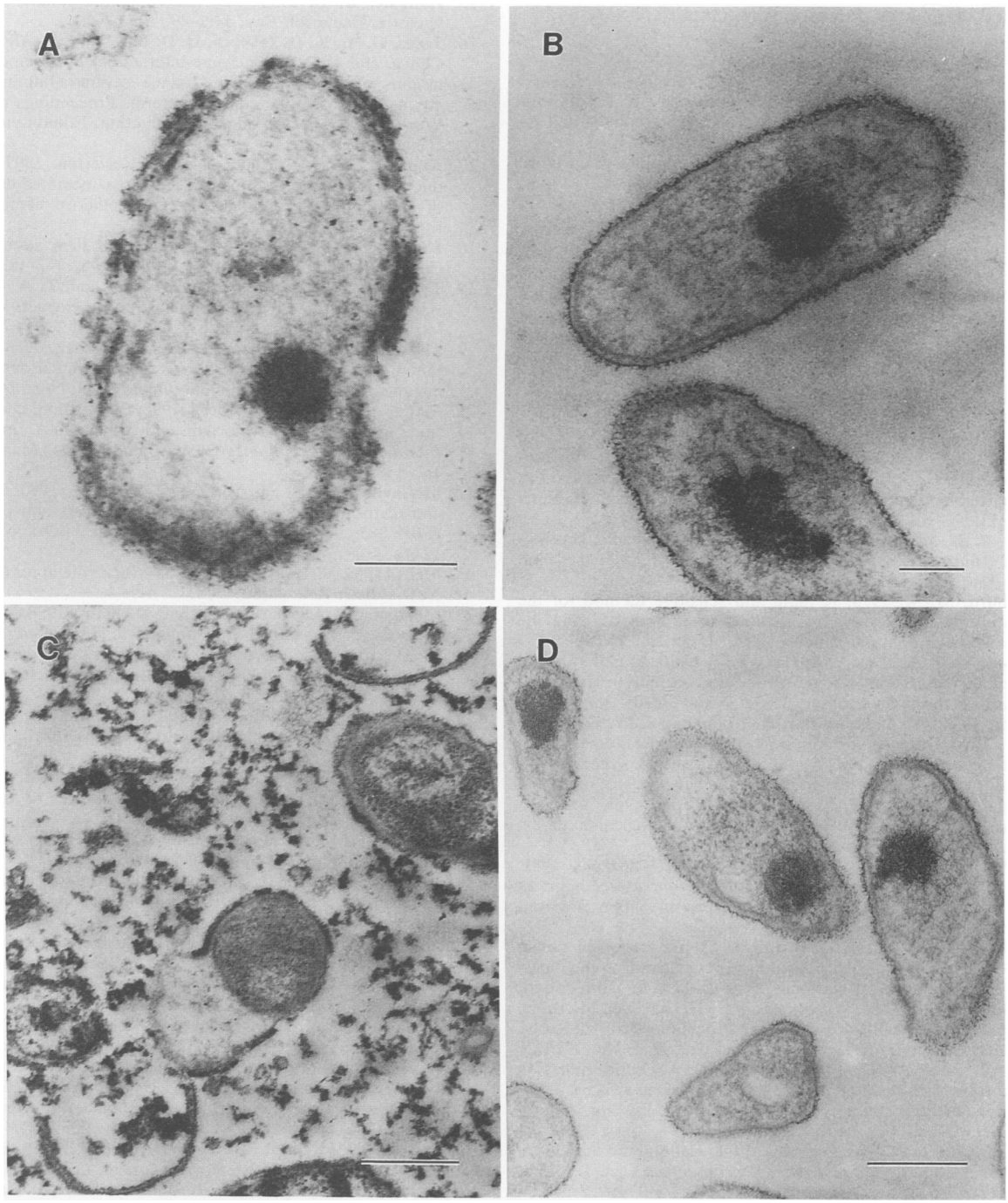

FIG. 6. TEM of *F. succinogenes* S85 grown on 0.2% glucose at 39°C for 24 h with or without condensed tannins. (A) Condensed tannins present at 300 μg ml^{-1}. Note the presence of electron-dense material on the cell surface and apparent damage to the outer membrane. Bar, 0.2 μm. (B) No condensed tannins present during growth. A typical surface carbohydrate layer is apparent, but the thick electron-dense layer seen on the surface of the cell in Fig. 5A is not present. Bar, 0.2 μm. (C) Condensed tannins present at 300 μg ml^{-1}. Note the presence of large amounts of extracellular material. Bar, 0.5 μm. (D) No condensed tannins present during growth. The extracellular material seen in panel C is not present. Bar, 0.5 μm.

isms capable of metabolizing condensed tannins. Isolation of rumen microorganisms with this capability could prove to be the first step in the development of ecological and genetic strategies to improve the utilization of forages containing condensed tannins by domestic ruminants.

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