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Fermentation of Cellodextrins by Cellulolytic and Noncellulolytic Rumen Bacteria

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Water-soluble cellodextrins were prepared from microcrystalline cellulose by using fuming hydrochloric acid and acetone precipitation. This cellodextrin preparation contained only trace amounts of glucose and cellobiose and was primarily composed of cellotetraose and cellopentaose. When various species of cellulolytic and noncellulolytic bacteria were cultured with cellodextrins, their growth rates and maximal optical densities were in most cases similar to those observed with cellobiose. Time course samplings and analyses of cellodextrins by high-pressure liquid chromatography indicated that longer-chain cellodextrins were hydrolyzed extracellularly to cellobiose and cellotriose. Cellodextrin utilization by noncellulolytic rumen bacteria and extracellular hydrolysis of cellodextrins increase the possibility that cross-feeding occurs in the rumen and help to explain the high numbers of noncellulolytic bacteria in ruminants fed fibrous diets.

Most animals are herbivores, and they provide most of the animal products consumed by humans. All heterotrophs are either directly or indirectly dependent upon photosynthetic organisms, and plants in turn have evolved cell walls that are resistant to digestion. The digestive secretions of animals are unable to hydrolyze major plant cell wall components (i.e., cellulose and hemicellulose), but certain rumen and intestinal bacteria have this capacity (8, 10, 15, 16).

Cellulose is resistant to rumen digestion, and few of the predominant rumen bacteria exhibit high levels of cellulase activity. *Bacteroides succinogenes* and *Ruminococcus flavefaciens* synthesize very active cellulases which can even hydrolyze crystalline cellulose (1, 4, 6, 12). *Ruminococcus albus* and some strains of *Butyrivibrio fibrisolvens* are also cellulolytic, but these organisms can only degrade the more amorphous types of cellulose rapidly (1, 3, 7).

When Scheifinger and Wolin cocultured *Selenomonas ruminantium* with *Bacteroides succinogenes* in cellulose broth, *Selenomonas ruminantium* persisted even though it is unable to grow on intact cellulose (14). These investigators hypothesized that *Selenomonas ruminantium* was living on "cellulose fragments" that were produced by *Bacteroides succinogenes*. Direct utilization of cellodextrins by *Selenomonas ruminantium* was not demonstrated.

The magnitude of cellodextrin cross-feeding in vivo has not been examined, but it is interesting to note that a number of noncellulolytic rumen bacteria are able to utilize cellobiose (8). Cellodextrin cross-feeding may help to explain the high numbers of noncellulolytic bacteria in cows fed poor-quality forage (2). The following experiments indicated that noncellulolytic strains of *Selenomonas ruminantium*, *Bacteroides ruminicola*, and *Streptococcus bovis* were able to utilize purified, water-soluble cellodextrins.

MATERIALS AND METHODS

Organisms. *Bacteroides ruminicola* B_14, *Butyrivibrio fibrisolvens* A38 and 49, *Selenomonas ruminantium* HD_4, *Eubacterium ruminantium* GA195, *Lachnospira multiparus* 40, *R. albus* 7, and *R. flavefaciens* C94 and FD1 were obtained from M. P. Bryant, University of Illinois, Urbana. *Bacteroides succinogenes* S85 and A3C and *R. albus* B199 were obtained from T. L. Miller, New York State Department of Health, Albany. *Bacteroides ruminicola* 23 and *Selenomonas ruminantium* D were obtained from K. A. Dawson, University of Kentucky, Lexington, and C. S. Stewart, Rowett Research Institute, Aberdeen, Scotland, supplied *Streptococcus bovis* 26. The JB1 strain of *Streptococcus bovis* was isolated at the University of California, Davis, Calif (13).

Media. The basal medium contained 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 1,000 mg of Na_2CO_3, 600 mg of cysteine hydrochloride, 1 mg of hemin, 0.25 mmol each of isobutyrate, isovalerate, 2-methylbutyrate, and valerate, 0.05 g of yeast extract, and 0.05 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.) per liter and was prepared anaerobically as described by Hungate (9). The ruminococci grew poorly in basal medium; therefore, 0.25 mmol of phenyl acetate, 0.25 mmol of 3-phenylpropionate (11), and 10% (vol/vol) clarified rumen fluid were added. *Bacteroides ruminicola* 23 and *Butyrivibrio fibrisolvens* 49 also grew poorly in basal medium and were given 10% rumen fluid and additional yeast extract (1.0 g/liter), respectively. Cellobiose or cellodextrins were prepared as separate solutions and added to basal medium after being autoclaved. All incubations were done in Butyl-rubber-stoppered serum bottles that each contained 50 ml of medium. The temperature was 39°C and the pH was 6.7.

Cellodextrins. Cellodextrins were prepared by a modification of the method of Freer and Detroy (5). Avicel (10 g; FMC Corp., Philadelphia, Pa.) was dissolved in ice-cold fuming HCl and incubated at 25°C for 2 h. The HCl was then partially removed by vacuum, and the cellodextrins were precipitated by the addition of 10 volumes of ice-cold acetone. The cellodextrins were reprecipitated five more times with 10 volumes of acetone and collected by centrifugation (1,300 × *g*, 0°C, 30 min). The pellet was dissolved in 100 ml of H_2O, neutralized with basic Dowex 2-8×-100 (Sigma Chemical Co., St. Louis, Mo.), and clarified on a polypropylene filter (pore size, 50 μm). Water-soluble cellodextrins were concentrated on a rotary evaporator (45°C) and reprecipitated with 10 volumes of acetone. Residual acetone was removed by vacuum. The final yield of cello-

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TABLE 1. Utilization of cellobiose and cellodextrins by pure cultures of rumen bacteria

Organism	Cellobiose		Cellodextrins	
	OD_{600}[a]	μ[b] (h^{-1})	OD_{600}	μ (h^{-1})
Ruminococcus albus 7	1.26	0.42	1.43	0.67
Ruminococcus albus B199	0.85	0.34	0.79	0.31
Ruminococcus flavefaciens FD1	0.81	0.44	1.13	0.48
Ruminococcus flavefaciens C94	0.92	0.56	0.82	0.40
Bacteroides succinogenes S85	0.95	0.48	1.09	0.44
Bacteroides succinogenes A3C	0.97	0.34	0.88	0.45
Butyrivibrio fibrisolvens A38	0.95	0.45	1.27	0.41
Butyrivibrio fibrisolvens 49	0.71	0.34	0.53	0.31
Selenomonas ruminantium HD_4	0.94	0.73	1.10	0.53
Selenomonas ruminantium D	0.78	0.71	0.93	0.55
Bacteroides ruminicola B_14	1.14	0.31	1.60	0.46
Bacteroides ruminicola 23	1.19	0.20	1.16	0.24
Streptococcus bovis 26	0.85	0.76	0.50	0.40
Streptococcus bovis JB1	0.83	0.91	0.15	<0.05
Eubacterium ruminantium GA195	0.82	0.49	0.10	<0.05

[a] OD_{600}, Maximum change in optical density at 600 nm (Gilford spectrophotometer model 260, 1-cm light path). Readings were taken at 8, 24, and 48 h.
[b] μ, Maximum specific growth rate.

dextrins from Avicel was approximately 10%. Water-insoluble cellulose accounted for most of the remaining Avicel.

Analyses. Samples (3.2 ml) were withdrawn from the incubation bottles with a hypodermic syringe, and the optical density at 600 nm (Gilford spectrophotometer model 260, with cuvettes with a 1-cm light path) was recorded. Cells were removed from the medium by centrifugation (0°C, 10,000 × *g*, 10 min), and the supernatant was acidified by the addition of 0.1 ml of 3.6 N H_2SO_4. The acidified sample was then mixed with 1 g of acidic Dowex 50-×4-100 (Sigma). After 10 min the Dowex was allowed to settle, and 2 ml of supernatant was transferred to another tube. This supernatant was then neutralized with 0.4 g of basic Dowex 2-8×-100. When the pH increased to 5.7 to 6.0, the salt-free supernatant was removed and frozen (−15°C) until used for analysis.

Cellodextrins were assayed by high-pressure liquid chromatography with a Beckman model 334 liquid chromatograph, a model 156 refractive-index detector, a 421 CRT data controller, a CR1A integrator, and a Bio-Rad 42-A carbohydrate column. The column temperature was set at 85°C with a column heater, high-pressure liquid chromatography and grade water (J. T. Baker Chemical Co., Phillipsburg, N.J.) was pumped through the column at 0.25 ml/min. When a steady base line was obtained, 50 μl of salt-free sample was injected into the column. The samples had to be salt free so chloride ions would not precipitate with silver groups in the column. A decrease in integrated area was used as an indicator of cellodextrin utilization.

RESULTS AND DISCUSSION

Previous reports indicated that *R. albus* 7, *R. flavefaciens* FD1 and C94, and *Bacteroides succinogenes* S85 and A3C are cellulolytic (7), and preliminary experiments with acid-treated Avicel (the cellulose left after cellodextrin preparation; see above) verified that these strains were still cellulolytic. The fastest rates of cellulose disappearance were observed with *Bacteroides succinogenes* S85. This correlated with the ability of this organism to degrade even the most crystalline forms of cellulose (1). *Butyrivibrio fibrisolvens* strains A38 and 49 were also reported to be cellulolytic, although the rates of cellulose digestion were generally slow (3). In our experiments, the visual disappearance of cellulose occurred with strain A38 after 7 days, but little disappearance occurred with strain 49 even after 14 days. All of the cellulolytic strains and noncellulolytic (*Selenomonas ruminantium* HD_4 and D, *Bacteroides ruminicola* B_14 and 23, *Streptococcus bovis* 26 and JB1, and *Eubacterium ruminantium* GA195) strains grew rapidly on cellobiose (Table 1).

The cellodextrin preparation contained only trace amounts of glucose and cellobiose; it was primarily composed of cellotetraose and cellopentaose (Fig. 1). When the various strains of rumen bacteria were cultured with the cellodextrin mixture, growth rates and maximal optical densities were in most cases similar to those observed with cellobiose (Table 1). *Streptococcus bovis* JB1 and *E. ruminantium* GA195 grew rapidly on cellobiose, but little growth was observed with the cellodextrin preparation. *Streptococcus bovis* 26 was able to grow on cellodextrins, but the final optical density and growth rate were lower on cellodextrins than on cellobiose.

To ascertain whether the bacteria preferred particular cellodextrins and to determine if cellodextrins were hydrolyzed extracellularly, separate time course incubations were performed (Fig. 2). When *Bacteroides succinogenes* S85 was incubated with the cellodextrin mixture, the amount of cellohexaose and cellopentaose decreased during the first 4 h of incubation, and this decrease was associated with a large increase in cellotriose and a smaller increase in cellobiose (Fig. 2a). The increases in cellotriose and cellobiose indicated that most of the longer-chain cellodextrins had been hydrolyzed extracellularly prior to transport and fermentation. Similar patterns of cellodextrin utilization were observed with *R. albus* 7 (Fig. 2b) and *R. flavefaciens* (Fig. 2c). With *Butyrivibrio fibrisolvens* A38, longer-chain cellodextrins were also rapidly hydrolyzed, but there was no increase in cellotriose (Fig. 2d). There was a small increase in cellobiose, and this would indicate that at least some of the cellodextrins were hydrolyzed extracellularly.

Noncellulolytic strains of *Bacteroides ruminicola* (Fig. 2e) and *Selenomonas ruminantium* (Fig. 2f) also degraded cellodextrins with a chain length of up to six and even seven glucose units. Celloheptaose data are not shown because the

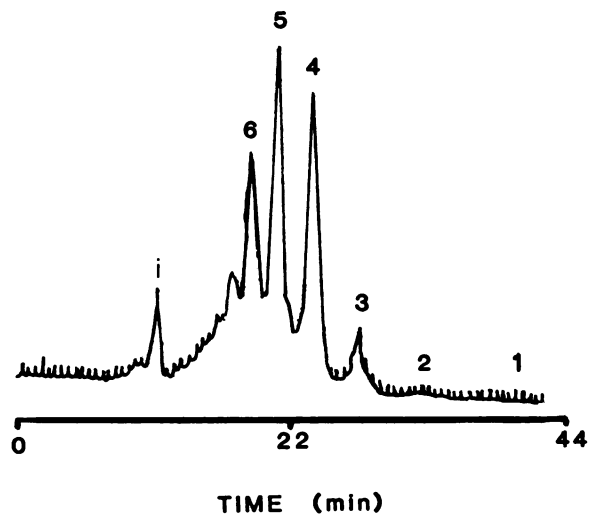


FIG. 1. High-pressure liquid chromatogram of cellodextrins showing the injection peak (i), glucose (peak 1), cellobiose (peak 2), cellotriose (peak 3), cellotetraose (peak 4), cellopentaose (peak 5), and cellohexaose (peak 6).

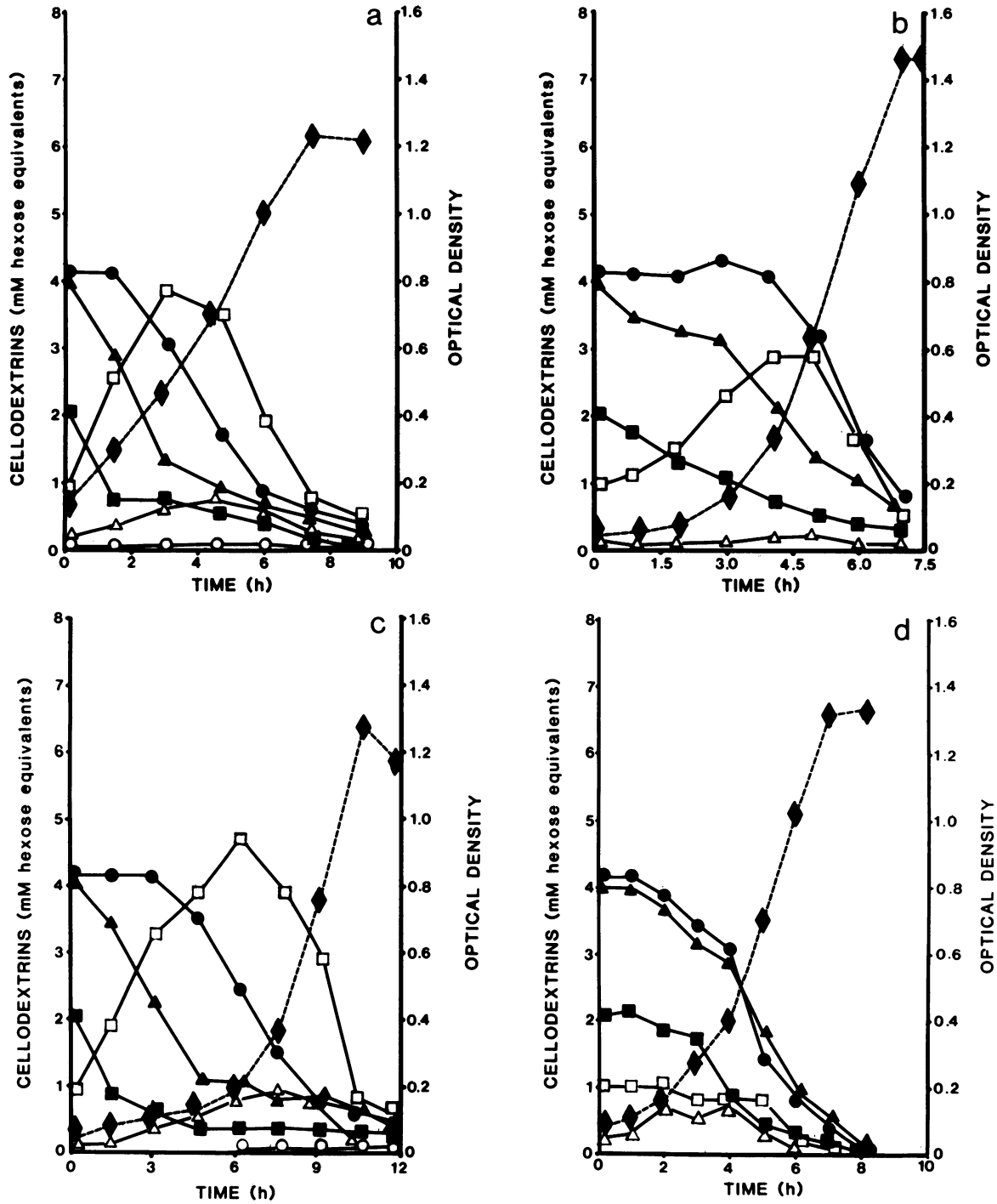
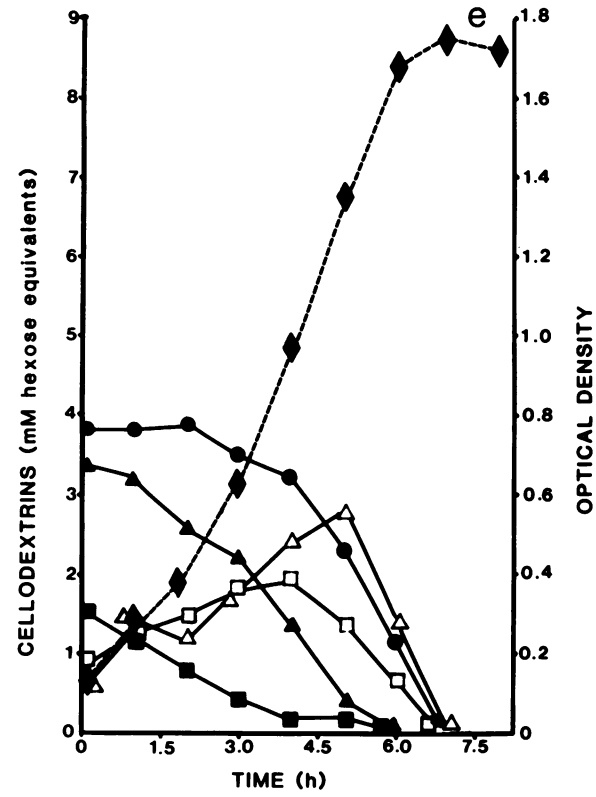
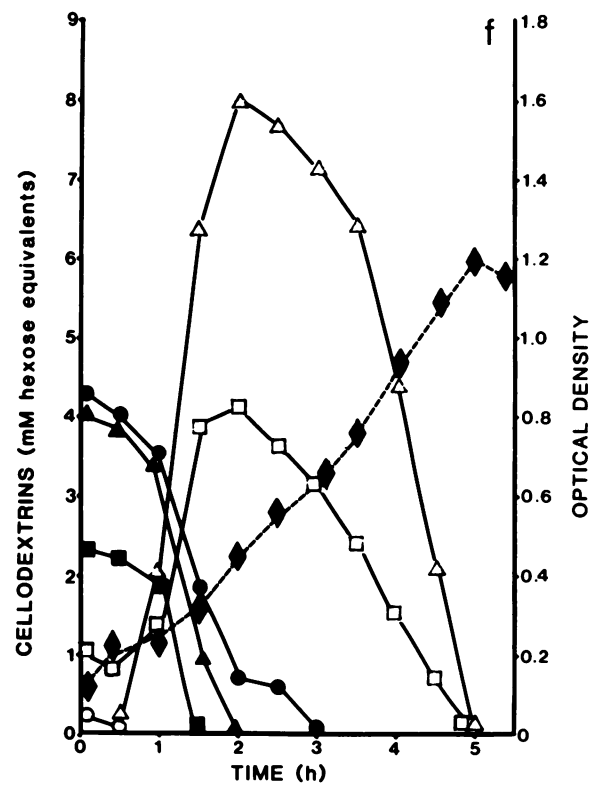


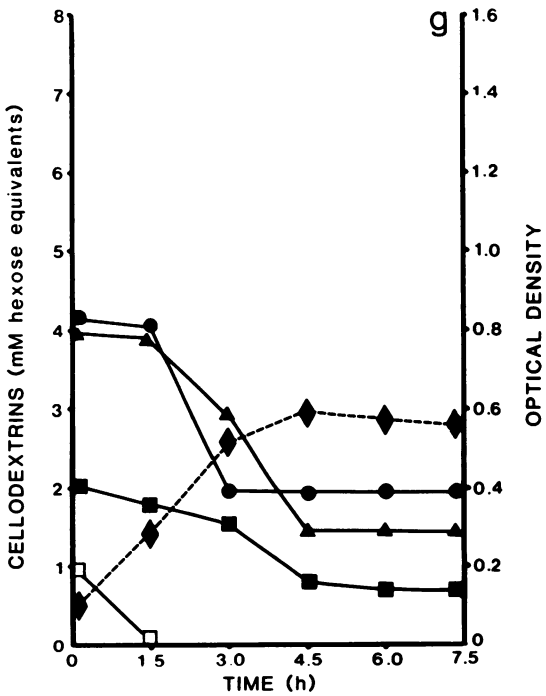
FIG. 2. Utilization of cellodextrins by *Bacteroides succinogenes* S85 (a), *R. albus* 7 (b), *R. flavefaciens* FD1 (c), *Butyrivibrio fibriosolvens* A38 (d), *Bacteroides ruminicola* B_14 (e), *Selenomonas ruminantium* HD_4 (f), and *Streptococcus bovis* 26 (g). Symbols: ◇, Optical density; ○, glucose; △, cellobiose; □, cellotriose; ●, cellotetraose; ▲, cellopentaose; ■, cellohexaose.

abundance of this material in the cellodextrin preparation was low, but qualitative examination of the high-pressure liquid chromatograms indicated a disappearance of this fraction as well. In each case, there was a very large increase in both cellotriose and cellobiose. These data show that cellodextrins with more than four glucose units are hydrolyzed extracellularly and that extracellular hydrolysis is not the rate-limiting step in cellodextrin utilization.

Streptococcus bovis 26 grew on cellodextrins, but the final optical density was less than when a similar amount of cellobiose was provided (Table 1). These data suggested that *Streptococcus bovis* 26 was unable to hydrolyze particular cellodextrins (most likely the high-molecular-weight fractions). Time course incubations, however, did substantiate this tentative hypothesis (Fig. 2g). *Streptococcus bovis* 26 was able to hydrolyze cellodextrins with a chain length as







long as seven glucose units. The lower final optical density resulted from incomplete utilization of the cellohexaose, cellopentaose, and cellotetraose. The medium supported growth to a high optical density with cellobiose, so it is unlikely that a nutritional deficiency was responsible for the incomplete utilization. A possible, although not entirely satisfactory, explanation is that substrate affinity limited growth.

Cellulases from *Bacteroides succinogenes* (6) and *R. albus* (17) are endoglucanases, and this type of activity could yield water-soluble cellodextrins. Wood and Wilson (17) detected cellotriose, cellotetratose, and cellopentaose, but the amounts were not great. Larger cellodextrins could have been hydrolyzed to cellobiose and glucose during the experiment (Fig. 2a and b).

Collectively, these experiments demonstrated that cellulolytic and certain noncellulolytic rumen bacteria produce enzymes that are capable of hydrolyzing water-soluble cellodextrins. In most cases, longer-chain cellodextrin hydrolysis was associated with an increase in either cellobiose or cellotriose in the cell-free medium. Cellodextrin utilization by noncellulolytic rumen bacteria and extracellular hydrolysis increase the possibility that interspecies cross-feeding occurs in the rumen and help to explain the high numbers of noncellulolytic bacteria in ruminants fed fibrous diets. Further work is needed to characterize the enzymes involved and to see if these β-glucosidases are able to hydrolyze even longer chain glucose polymers. Preliminary experiments indicated that cell-free medium from *S. ruminantium* HD4 cultures was able to form reducing sugar from carboxymethyl cellulose.

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