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Effect of Soluble Carbohydrates on Digestion of Cellulose by Pure Cultures of Rumen Bacteria†

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The rate of cellulose digestion in the presence of either glucose or cellobiose was studied for the three predominant species of cellulolytic rumen bacteria: *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Bacteroides succinogenes*. When a soluble carbohydrate was added to cellulose broth, the lag phase of cellulose digestion was shortened. Presumably, this was due to greater numbers of bacteria, because increasing the size of the inoculum had a similar effect. Cellulose digestion occurred simultaneously with utilization of the soluble carbohydrate. The rate of cellulose digestion slowed markedly for *B. succinogenes* and *R. flavefaciens* and slowed less for *R. albus* after the cellobiose or glucose had been utilized, and was accompanied by a decrease in pH. Both the rate and the extent of cellulose digestion were partially inhibited when the initial pH of the medium was 6.3 or below. *R. albus* appeared to be less affected by a low-pH medium than were *B. succinogenes* and *R. flavefaciens*. When a soluble carbohydrate was added to the fermentation during the maximum-rate phase of cellulose digestion, the rate of cellulose digestion was not affected until after the soluble carbohydrate had been depleted and the pH had decreased markedly. Prolonged exposure of the bacteria to a low pH had little if any effect on their subsequent ability to digest cellulose. Cellulase activity of intact bacterial cells appeared to be constitutive in nature for these three species of rumen bacteria.

When cereal grains are included in the diet of ruminants, fiber or cellulose digestibility of the roughage component is generally reduced (11). The addition of 20 to 30% glucose to a 2:2:1 timothy hay-corn-cottonseed meal ration was found to decrease fiber digestion by about 25% in both calves and sheep (10, 20). This reduction in fiber digestion could be caused by a depletion of noncarbohydrate nutrients by the saccharolclastic bacteria, by an excess production of acid, or by an inhibition of cellulase by soluble sugars (15).

Using mixed cultures of rumen bacteria in vitro and nylon bag techniques in vivo, el-Shazly et al. (7) were able to show that the depression of purified-cellulose digestion caused by the addition of starch was the result of nitrogen limitation to the cellulolytic bacteria. Stewart (28) investigated the effect of pH on digestion of cotton yarn by rumen contents in vitro. When barley was added as a substrate to the fermentation, cellulose digestion was depressed; however, when the pH of the medium was maintained at 6.6, no depression occurred. Cellulolytic activity of rumen contents was found to be maximum at pH 7.0, decreasing to near zero at pH 6.0. The number of filter paper-degrading bacteria decreased from 10^6/ml at pH 6.9 to 10^3/ml at pH 6.0. These studies suggest that depletion of noncarbohydrate nutrients, excess production of acid, or both could be responsible for the depression of cellulose digestion observed when feeding grain or starch to ruminants. However, neither of these factors should be of major importance in pure culture studies with a well-buffered medium containing a relatively low concentration of soluble carbohydrate.

Fusee and Leatherwood (8) studied the effects of a soluble sugar on cellulose digestion by growing *Ruminococcus albus* in cellulose-agar roll tubes with various concentrations of added cellobiose. They estimated the extent of cellulose digestion by observing the size of clear zones that were formed. When they increased the concentrations of cellobiose, they noted that the relative size of the clear zones was diminished. Similar results had been obtained earlier by Hungate (15), using a cellulolytic strain of *Butyrivibrio fibrisolvens*. On the basis of these reports and studies of their own, Hitchner and Leatherwood (12) and Leatherwood (18) proposed that cellulase is a repressible enzyme and

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that all microorganisms, including the cellulolytic species in the rumen, are governed by catabolite repression of cellulase. In contrast, studies by Smith et al. (27) suggest that cellobiose inhibits the action of the cellulase rather than its production. Without specific information on the mechanism involved, the inhibition observed with cellobiose in cellulose-agar roll tubes should probably be described as a catabolite regulatory mechanism of cellobiose upon cellulase activity (24).

The present study was undertaken to investigate the ability of pure cultures of the three predominant species of cellulolytic rumen bacteria to digest cellulose in the presence of a readily available carbohydrate.

MATERIALS AND METHODS

Bacteria and media. *R. albus* 7 (3), *Ruminococcus flavefaciens* C94 (3), and *Bacteroides succinogenes* A3c (4) were used in this study. All strains were maintained on rumen fluid-glucose-cellobiose-agar slants (2). Inoculum cultures were grown for about 24 h in 0.5% cellobiose broth, which was similar in composition to rumen fluid-glucose-cellobiose-agar medium except that glucose and agar were omitted. The complete medium of Scott and Dehority (26) was used for all experiments. Cellobiose was omitted, and a 3% solution of 16 to 18-h ball-milled cellulose (Sigmacell 20, a microcrystalline cellulose obtained from Sigma Chemical, Co., St. Louis, Mo.) was added to give a final concentration of 0.2% cellulose. When glucose or cellobiose was added, previously sterilized solutions of the appropriate concentration were added to the tubed cellulose medium.

The inoculum was prepared by diluting the 24-h rumen fluid-cellobiose broth culture to an optical density of 0.2 with substrate-free complete medium. A 0.2-ml portion of this dilution was used to inoculate all media. Optical density was measured in culture tubes (16 by 150 mm) at 600 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. The anaerobic culture technique of Hungate (14), including the modifications of Dehority (6), was used throughout.

Analysis of substrates. Cellulose was analyzed by transferring the entire contents of the culture tube to a tared test tube, centrifuging at ca. 1,800 rpm for 10 min, and decanting the supernatant and adding 5 ml of acid-detergent-fiber solution to it (30). The tubes were then mixed and heated in an autoclave for 1 h at 100°C. The insoluble residue was separated by centrifugation, the supernatant was discarded, and the sediment was washed twice with boiling distilled water. The tubes were dried overnight in an oven at 100°C, placed in a desiccator until cool, and weighed. In a preliminary experiment, various levels of bacterial cultures were added to the cellulose medium, and cellulose levels were determined in the unincubated tubes. The added bacteria did not contribute to the weight of the cellulose recovered. In other experiments with uninoculated tubes, the acid-detergent-fiber-treated cellulose residue was compared with the residue left without acid-detergent-fiber treatment or autoclaving. Recovery in replicate trials was 102 and 94%. Cellobiose and glucose levels were determined by the orcinol procedure (1, 21).

All fermentations were performed in duplicate. A set of the appropriate number of tubes was inoculated at the beginning of each experiment and then incubated at 38°C. At specified intervals, duplicate tubes were removed and assayed for residual soluble carbohydrate, cellulose, or both. The mean value for each pair was calculated. All experiments were repeated in two, and in some cases three, replicates. Unless otherwise designated, all data points in the figures represent the mean of at least four separate fermentation tubes.

RESULTS

Effect of soluble carbohydrate and inoculum size on cellulose digestion. With the addition of a soluble carbohydrate to the cellulose fermentation, it was anticipated that bacterial numbers should increase more rapidly than with cellulose alone. To test the effect of greater numbers of organisms on the rate of cellulose digestion, we compared the standard inoculum of 0.2 ml with a larger inoculum of 1.0 ml. Figure 1 shows the rate of cellulose digestion for *R. albus* 7, using 0.2 ml of inoculum (control), 1.0 ml of inoculum, and 0.2 ml of inoculum plus 0.15% cellobiose. This low concentration of cellobiose was chosen so that the fermentation would proceed without

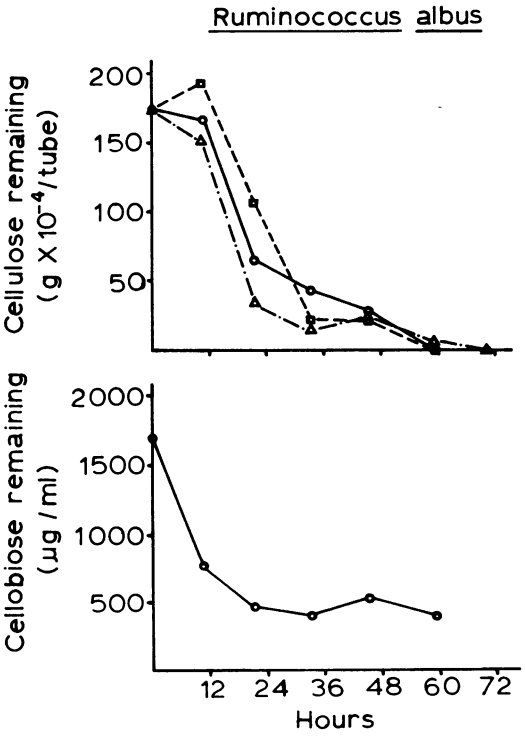

FIG. 1. Effect of added cellobiose on cellulose digestion by *R. albus*. Dashed line (□), 0.2-ml inoculum (control); broken line (△), 1.0-ml inoculum; solid line (○), 0.2-ml inoculum plus 0.15% cellobiose.

being inhibited by the accumulation of end products. Rates of cellulose digestion were similar between 10 and 21 h in the fermentations with the larger inoculum and those with added cellobiose, both being slightly faster and initiating sooner than the control. Cellobiose was utilized to the maximum extent by about 21 h, and it was after this time that the rate of cellulose digestion slowed. With *B. succinogenes* (Fig. 2), initiation of cellulose digestion appeared to lag somewhat in the control, but the apparent rate between 38 and 54 h was similar to that of the other fermentations between 18 and 24 h. Both the control and the large inoculum fermentations continued at nearly their same rates until the cellulose was almost depleted. However, after 24 h, the rate of cellulose digestion decreased markedly in the fermentation containing cellobiose and by 38 to 54 h had essentially ceased. Cellobiose was digested simultaneously with cellulose, and by 24 h, cellobiose had been utilized to the fullest extent. Thus, it was only after utilization of the soluble carbohydrate that a marked decline was noted in the rate of cellulose digestion. Similar results were obtained for these two strains when glucose was the added soluble carbohydrate. Cellulose digestion by *R. flavefaciens*, which does not utilize glucose, was affected in the same manner in fermentations with cellobiose and in those with a larger inoculum. For all species, the extent of cellulose digestion in the control and the large inoculum tubes was similar. These results suggested that an initial increase in the rate of cellulose digestion, or more specifically, a decrease in lag time, occurring with a soluble carbohydrate added to the medium could result from a rapid increase in bacterial numbers. Cellulose and soluble carbohydrate appeared to be utilized simultaneously. For *B. succinogenes* and *R. flavefaciens*, the rate of cellulose digestion decreased markedly after about 36 h in those fermentations with added glucose or cellobiose. The soluble carbohydrate had been completely utilized by this time, which suggested that a large drop in pH could have been responsible for this change.

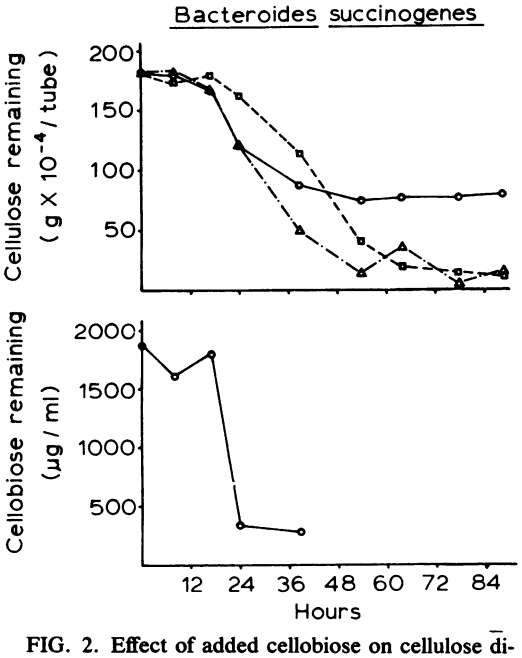

FIG. 2. Effect of added cellobiose on cellulose digestion by *B. succinogenes*. Dashed line (□), 0.2-ml inoculum (control); broken line (△), 1.0-ml inoculum; solid line (○), 0.2-ml inoculum plus 0.15% cellobiose.

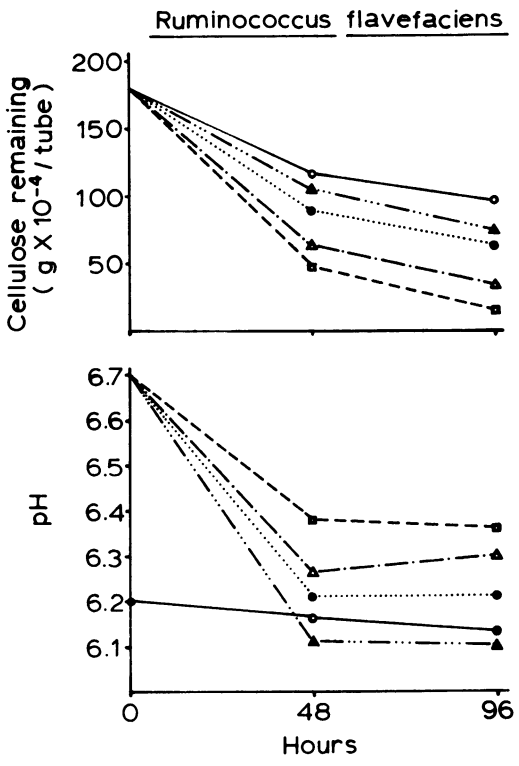

FIG. 3. Effect of three levels of cellobiose and initial medium pH on cellulose digestion by *R. flavefaciens*. Symbols: (○), low-pH medium, no cellobiose; (□), regular-pH medium, no cellobiose (control); (△), regular-pH medium plus 0.05% cellobiose; (●), regular-pH medium plus 0.10% cellobiose; (▲), regular-pH medium plus 0.15% cellobiose.

Level of soluble carbohydrate and pH. Either cellobiose or glucose was added at three levels (0.05, 0.10, or 0.15%) to the regular cellulose medium (pH 6.7 to 6.8). Two media without added soluble carbohydrate were also used, one at pH 6.7 to 6.8 and the other adjusted to pH 6.2 to 6.3. Data for *R. flavefaciens* and cellobiose are shown in Fig. 3. After 48 h, cellulose diges-

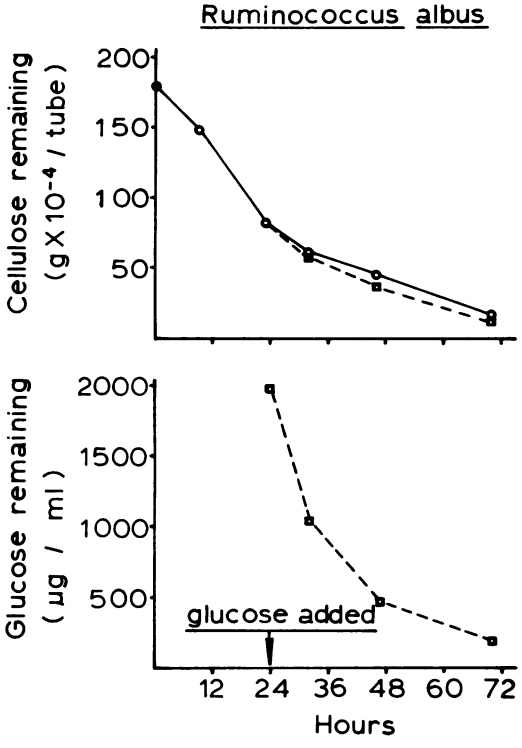

FIG. 4. Effect of adding glucose after initiation of cellulose digestion by *R. albus*. Solid line (○), no glucose (control); dashed line (□), 0.15% glucose added.

tion was lowest in the fermentation with the low initial pH, whereas cellulose digestion in the control with no cellobiose added was highest. Cellulose digestion in the fermentations with cellobiose varied inversely with the concentration of cellobiose; i.e., the higher the concentration, the less cellulose digested. As expected, pH levels also followed this trend: the higher the cellobiose concentration, the lower the pH dropped. From 48 to 96 h, some additional cellulose digestion occurred, but little additional decrease was noted in pH. A similar pattern of results was obtained with all three organisms. In general, the extent of cellulose digestion was only slightly affected by pH for *R. albus*, moderately affected for *B. succinogenes*, and markedly reduced for *R. flavefaciens*.

Addition of soluble carbohydrate during period of active cellulose digestion. After cellulose fermentation with *R. albus* had proceeded for 24 h, glucose was added to give a concentration of ca. 0.15%. Cellulose digestion was not adversely affected (Fig. 4); in fact, the rate of cellulose digestion with glucose was nearly identical to that in the control fermentation. Glucose was utilized quickly, and cellulose and glucose digestion occurred concurrently. Results from similar experiments with *B. succinogenes* are shown in Fig. 5. For the first 8 h after glucose was added, cellulose and glucose were utilized simultaneously with no change in the rate of cellulose digestion. After this period of rapid glucose fermentation, the rates of both glucose and cellulose digestion slowed. The rate of cellulose digestion in the fermentation with added glucose was noticeably slower than that in the control between 46 and 72 h, and glucose utilization essentially ceased.

In additional experiments, a level of 0.5% cellobiose was added in addition to the 0.15% level to determine the effects of overloading the system with a normal catabolite. For *B. succinogenes* and *R. flavefaciens*, the rate of cellulose digestion in the fermentations with 0.15% cellobiose was only slightly slower than that in the control; however, the rate of cellulose digestion decreased to a greater extent in the presence of 0.5% cellobiose. The extent of cellulose digestion was slightly less with 0.15% cellobiose and markedly reduced when 0.5% cellobiose was added. As before, these effects were related to the magnitude of decrease in pH resulting from fermentation of the soluble carbohydrate. Results from similar experiments with *R. albus* differed slightly in that the rates of cellulose

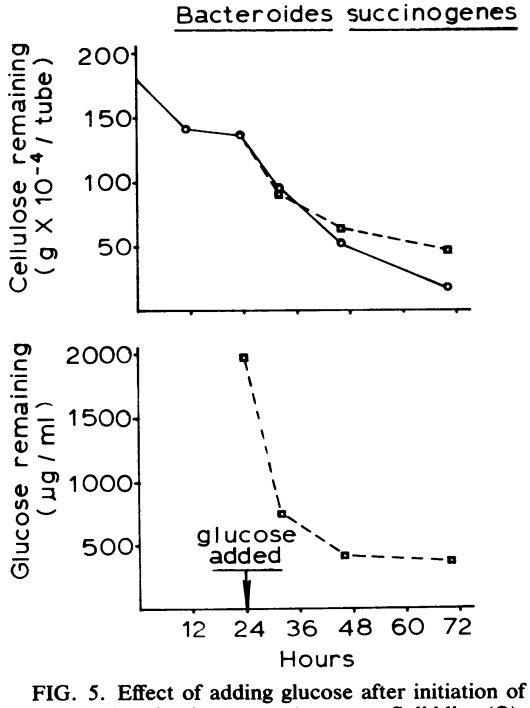

FIG. 5. Effect of adding glucose after initiation of cellulose digestion by *B. succinogenes*. Solid line (○), no glucose (control); dashed line (□), 0.15% glucose added.

digestion in the fermentation with both levels of added cellobiose were very close to that of the control, and the decrease in pH was less.

Adjustment of pH. Cellulose digestion by *B. succinogenes* was compared in the fermentations with cellulose, cellulose plus 0.5% cellobiose, and cellulose plus 0.5% cellobiose with pH adjustment. The pH was adjusted back to ca. 6.9 at 9, 22, and 33 h. Cellulose digestion in the pH-adjusted fermentations was somewhat less than that in the control, but it was 12.5 and 25 percentage units higher than that in the unadjusted fermentations at 22 and 71 h, respectively.

Effect of prolonged low pH. The possible effects of low pH, which normally occurs in the rumen after feeding, on the subsequent ability of organisms to digest cellulose were investigated. With two different cellulose media, one at pH 6.7 and the second at pH 5.8 to 6.0, fermentation proceeded for 48 h, followed by adjustment of the pH back to about 6.8 in half of the low-pH-medium tubes. In general, after pH adjustment, both the rate and the extent of cellulose digestion were similar to those of the controls. Results of typical experiments for *B. succinogenes* and *R. flavefaciens* are shown in Fig. 6. For *R. albus*, more cellulose was digested in the low-pH medium, with a less-marked increase in rate after adjustment.

Nature of cellulase activity. If cellulase activity is constitutive, then cellulose digestion should initiate and proceed at similar rates for either soluble-carbohydrate- or cellulose-grown cells. Conversely, a definite lag in cellulose digestion should occur with soluble-carbohydrate-grown cells if cellulase activity is inducible. Sufficient inoculum for the maximum rate of cellulose digestion must be used to compare cellulose digestion rates between cultures grown on different substrates. These levels, 1.0 ml of an inoculum with an optical density of 0.2 for soluble carbohydrates and 1.0 ml of cellulose broth culture, were determined in preliminary studies. For the two ruminococcus strains, no significant differences were found in the amount of cellulose digested at 24, 48, or 72 h, regardless of the inoculum substrate (Table 1). In contrast, the extent of cellulose digestion by *B. succinogenes* at 24 h was significantly lower ($P < 0.05$) for inoculum cultures grown on glucose or cellobiose. Thus, either the cellulase activity of *B. succinogenes* is inducible, a period of time is required to remove a metabolic inhibitor, or a certain amount of time is required for "attachment" of the bacteria to the cellulose fibers. The rates of cellulose digestion were compared for inocula grown on cellobiose, cellulose broth, or the supernatant obtained from cellulose broth after vigorous agitation and low-speed centrifugation. The supernatant obtained from cellulose broth was observed microscopically and found to contain a fairly high concentration of bacterial cells but was essentially free of cellulose particles. At that point, it was treated the same as the cellobiose inoculum, i.e., diluted to an optical density of 0.2. Results of these experiments indicate that when cells grown on cellulose are separated from the cellulose particles, the time required for initiation of cellulose digestion is increased (Table 2). Thus, the lag phase for *B.*

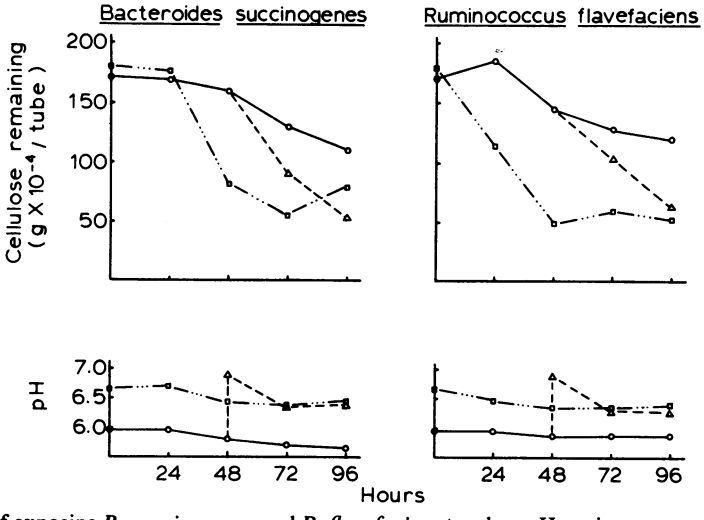

FIG. 6. Effect of exposing *B. succinogenes* and *R. flavefaciens* to a low-pH environment on their subsequent ability to digest cellulose. Solid line (○), low-pH medium; broken line (□), regular medium (control); dashed line (△), low-pH medium adjusted to 6.9.

TABLE 1. Effect of inoculum substrate on rate of cellulose digestion

Organism	Time (h)	% Cellulose digested with the following inoculum:[a]		
		Glucose	Cellobiose	Cellulose
R. albus	24	43.3 ± 4.9	50.0 ± 3.2	55.3 ± 2.0
	48	54.0 ± 10.6	70.3 ± 4.3	65.7 ± 10.4
	72	51.7 ± 16.6	80.3 ± 4.7	59.0 ± 6.6
B. succinogenes	24	12.0 ± 12.0	16.7 ± 9.8	59.0 ± 6.1[c]
	48	42.7 ± 13.2	69.0 ± 8.1	62.7 ± 9.2
	72	64.7 ± 8.3	71.7 ± 10.9	71.7 ± 5.9
R. flavefaciens	24	ND[b]	51.0 ± 7.0	42.7 ± 0.9
	48	ND	60.7 ± 10.0	60.7 ± 4.1
	72	ND	56.0 ± 9.9	69.7 ± 6.4

[a] Mean and standard error of the mean for three experiments.
[b] ND, Not determined; this strain cannot utilize glucose as an energy source.
[c] Significantly higher ($P < 0.05$) than other means in the same row.

succinogenes is probably related to attachment mechanisms.

DISCUSSION

For all three bacterial species, digestion of cellulose and soluble carbohydrates occurred simultaneously. The only effect of soluble carbohydrates on the rate and extent of cellulose digestion appeared to be an inhibition resulting from a low pH generated by the rapid fermentation of soluble carbohydrates. These results support the concept that cellobiose and glucose do not repress cellulase activity in intact bacteria. Previous studies which have suggested a repression or inhibition of cellulose digestion may have been confounded by the effects of a lowered pH. Hungate (15) and Fusee and Leatherwood (8) observed marked decreases in the size of clear zones in cellulose agar in the presence of a soluble carbohydrate. Because a rapid fermentation of the soluble carbohydrate would be expected in the vicinity of the colony, a slow rate of diffusion of end products in the solid medium might create a microenvironment of extremely low pH. This in turn would affect growth rate and thus the rate and extent of cellulose digestion.

Minimum pH values, or the final pH measured in a poorly buffered medium with an excess of substrate, for the three cellulolytic strains in this study are as follows: *B. succinogenes* A3c, 5.2; *R. albus* 7, 5.4; *R. flavefaciens* C94, 5.6 (3, 4). More recently, Russell and Dombrowski (25) determined for these same species the pH at which growth was inhibited and the culture washed out in a continuous culture system. For *B. succinogenes*, *R. albus*, and *R. flavefaciens*, washout occurred at pH 6.0, 5.9, and 6.15, respectively. These differences in pH tolerance would agree with the results of the present study, i.e., the rate and extent of cellulose digestion were both reduced to a greater extent for *R. flavefaciens* in the presence of a soluble carbohydrate. Although the minimum pH for *R. albus* is higher than that for *B. succinogenes*, the washout pH is lower. This is probably because this strain only produces about half the acid end products per unit of carbohydrate fermented (5). The present study shows that a low level of soluble carbohydrate had minimal effect on the rate and extent of cellulose digestion by *R. albus*. Cellulose digestion by mixed cultures of rumen bacteria has also been found to be markedly reduced at low pH values (28, 29).

On the basis of its growth characteristics in cellulose agar, Hungate (13) suggested that *B. succinogenes* possessed a cell-bound cellulase. More recent studies, using immunofluorescence techniques and electron microscopy, have clearly shown that *B. succinogenes* firmly attaches both to purified cellulose and to plant particles (17, 19). This intimate association is apparently required for cellulolysis (9). Both species of cellulolytic ruminococci have also been shown

TABLE 2. Rate of cellulose digestion by *B. succinogenes*, with cellobiose- and cellulose (attached and unattached)-grown cells as inoculum

Inoculum substrate	% Cellulose digestion[a]			
	24 h		48 h	
	I	II	I	II
Cellobiose	2.3	0	72.5	75.9
Supernatant of cellulose broth	16.6	7.5	76.1	30.1
Cellulose broth	44.8	39.3	74.5	89.0

[a] I and II are separate experiments.

to attach to cellulose and rumen digesta solids (16, 22), and their cellulase activity appears to be cell associated (23). The lag phase in cellulose digestion observed with *B. succinogenes* when the inoculum cells were grown on soluble carbohydrate or freed from particles of cellulose suggests that the time required for attachment or association is longer for this species. This may be related to the markedly different extracellular surface coats of *Ruminococcus* spp. and *B. succinogenes* (16, 17, 22).

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