Hydrolytic Products of Cellulose And the Cellulolytic Enzymes

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The chemistry of cellulose digestion by rumen microorganisms was investigated with special reference to identification of the carbohydrates formed and the cellulolytic enzymes. In order to identify the carbohydrate intermediates, a number of organic and inorganic compounds were tested for their ability to arrest fermentation by rumen microorganisms at the reducing sugar stage. Among those found effective were toluene, thymol, and sodium fluoride. Inhibited cellulose-digesting cultures were prepared and the media analyzed at hourly intervals for the carbohydrate intermediates formed. Filter paper chromatography revealed the principal intermediate compound of cellulose degradation by rumen organisms to be glucose. The cellulolytic enzymes of rumen microorganisms are not present as such in rumen fluid, but are associated with the bacterial cells, as centrifuged and filtered rumen fluid failed to digest cellulose. Cell-free enzyme extracts of mixed rumen culture and of an isolated rumen organism were prepared which degraded cellulosic substrates. An optimum temperature of 40° C. and an optimum pH of 5.5 were found for the maximum activity of the cell-free preparations. The cell-free preparations are stable at low temperature, but are partially inactivated when allowed to stand at room temperature for 144 hours.

THE CHEMISTRY OF RUMINANT DIGESTION is unique in that rumen bacteria elaborate various enzymes which play an essential part in the well-being of ruminant animals. The Mammalia do not have the capacity to secrete cellulolytic enzymes, but in the rumen of the herbivore large numbers of microorganisms are present which digest cellulosic plant materials, through the agency of their enzyme systems, and produce end products which may be utilized by the host. A symbiotic relationship therefore exists between the rumen microorganisms and the animal in question.

Although cellulose is by far the most important carbohydrate in ruminant nutrition, there is little knowledge as to the method of its actual breakdown in the rumen. During the past 70 years various attempts have been made to show that microorganisms elaborate enzymes that have the capacity to hydrolyze cellulose. Tappeiner (*20*), in 1884, appeared to be the first to show experimentally that the disappearance of cellulose in the digestive tract is the result of a fermentation brought about by microorganisms inhabiting the alimentary canal. In 1912, Pringsheim (*15*) demonstrated the production of cellobiose from the hydrolysis of cellulose. He postulated the existence of two hydrolytic enzyme systems, cellulase and cellobiase, the former producing cellobiose from cellulose and the latter hydrolyzing the disaccharide to glucose. By the use of various antiseptics such as toluene and iodoform, he was able to inhibit the growth of the microorganisms in a vigorous thermophilic fermentation. This resulted in an accumulation of reducing substances identified as cellobiose and glucose.

Norman and Fuller (*14*) stated that the primary attack on cellulose during anaerobic fermentation is as follows:

$$\text{Cellulose} \xrightarrow{\text{HOH}} \text{cellobiose} \xrightarrow{\text{HOH}} \text{glucose}$$

These workers were able to isolate the degradation products of cellulose fermentation—namely, cellobiose and glucose. Hungate (*8*) while studying the organism *Clostridium cellobioparus*, which he isolated from the rumen, observed that cellobiose did not accumulate in the medium until after the fermentation ceased. The rate of formation of cellulose digestion products was apparently the factor limiting the rate of growth of the microorganisms. It was found by Hungate that liquid media containing cellulose showed a somewhat slower development of the bacteria than the same media with glucose or cellobiose.

Woodman and Evans (*26*) showed that antiseptics, such as toluene, favored the production of reducing sugars in rumen liquid. A few years later Meites, Burrell, and Sutton (*12*) stated that toluene was the most effective antiseptic to prevent further metabolism of reducing substances that were produced during the fermentation of cellulose by rumen bacteria.

To date cellulolytic enzymes have not been isolated in pure form. According to Siu (*19*), a number of investigators have demonstrated cellulolytic activity of crude enzyme preparations from various sources such as the higher plants (wheat, barley, etc.), animals (shipworm, snail, termite, etc.), protozoa, fungi, and bacteria.

In 1948, Saunders, Siu, and Genest (*17*) obtained a cellulolytic enzyme preparation from the mold *Myrothecium verrucaria* and showed that the main breakdown product from cellulose was glucose. A few years later, Whitaker (*24, 25*) outlined a method of purifying the cellulase of this mold and, with a col-

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league (*1*), performed various inhibition and stimulation studies on the purified enzyme preparation. Recently Whistler and Smart (*23*) noted that a crude cellulolytic enzyme preparation from *Aspergillus niger* hydrolyzed swollen cellulose to glucose as the only degradative product. However, when the preparation was purified by adsorption on and elution from powdered cellulose, both cellobiose and glucose were isolated from the enzymic hydrolyzate of cellulose.

It is well known that bacteria of the rumen are markedly cellulolytic, degrading cellulose to end products which are utilized by the ruminant animal. The closeness with which the rumen cellulolytic bacteria adhere to the cellulose particles is explained (*17*) upon the hypothesis that an extracellular cellulase is formed. The closer the bacterium approaches the substrate, the greater benefit it derives from its extracellular enzymes. It is evident that the farther the bacterium is from the cellulose fiber, the less is the chance the hydrolytic enzymes will be able to react on that material. The extracellular nature of the cellulase formed by *Clostridium cellobioparus* was clearly demonstrated by Hungate (*8*). Around each colony on cellulose agar tubes a clear area developed in which the cellulose had been digested.

As reported in another place (*9*), a number of bacterial isolates have been cultivated from the rumen contents of the bovine and their fermentation characteristics studied. It was shown that all of the 32 isolates obtained in these laboratories fermented starch, dextrin, maltose, cellobiose, and glucose readily. However, only two isolates (Nos. 31 and 32) fermented cellulose and carboxymethylcellulose (CMC-70-L) at an active rate. The morphology, staining characteristics, and oxygen requirements of cultures 31 and 32 were also investigated and reported (*9*).

During investigation of the digestion of cellulose by rumen microorganisms, cell-free enzyme preparations were obtained from the mixed rumen culture and from a cellulolytic isolate. These enzyme preparations were able to convert cellulose to reducing sugars, and some information as to the properties of the cellulolytic enzyme has been secured.

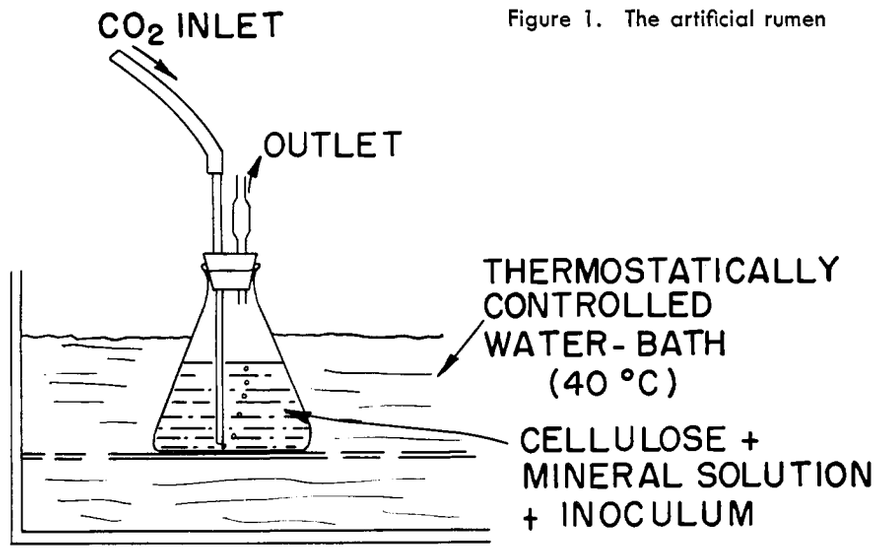


Figure 1. The artificial rumen

Hydrolytic Products

Fermentation Apparatus and Procedure

The metabolism of cellulose by the rumen bacteria can be studied adequately in the laboratory. This in vitro study, used to simulate the in vivo processes, has been made possible by the use of the laboratory apparatus which is now commonly termed the artificial rumen.

The artificial rumens employed in the work reported consisted of a number of Erlenmeyer flasks or test tubes of various sizes, depending on the type of experiment performed and the amount of cellulose-digesting culture required. The culture vessels were placed in a thermostatically controlled water bath at 40° C. for a prescribed length of time. The physical arrangement, similar to that described by Burroughs, Frank, Gerlaugh, and Bethke (*2*) and by Ruf (*16*), is shown in Figure 1.

In most of the experiments that were conducted during the investigation of cellulose digestion by the microorganisms of the rumen, the fermentation cultures were prepared in 500-ml. wide-mouthed Erlenmeyer flasks. The materials placed initially in the flasks of this size were: 4 grams of cellulose or a derivative of cellulose; 32.1 ml. of the mineral solution recommended by Burroughs, Headley, Bethke, and Gerlaugh (*3*) containing, per liter, 26.25 grams of monobasic sodium phosphate, 26.25 grams of sodium bicarbonate, 3.75 grams of potassium chloride, 3.75 grams of sodium chloride, 1.125 grams of magnesium sulfate, 0.375 grams of calcium chloride, 0.075 gram of ferrous sulfate, 0.04 gram of manganous sulfate, 0.04 gram of zinc sulfate, 0.02 gram of copper sulfate, and 0.01 gram of cobalt chloride; 4.5 ml. of urea solution containing 84 grams of urea per liter (*16*); and enough warm distilled water to make the total volume 200 ml. To this, 200 ml. of inoculum was added. The inocula were obtained from a fistulated bovine, the fresh rumen contents being strained through cheesecloth and used immediately. The total volume of the flask cultures was 400 ml., and the cellulose concentration was 1%. The medium at this stage was usually at the desired pH of 6.5 to 7.0 and thus did not require any alteration. Immediately following the preparation of the experimental cultures, an aliquot was withdrawn from each flask for analyses. The flasks were then placed in the thermostatically controlled water bath. The rubber stopper assembly for each flask was inserted and the stream of carbon dioxide directed through the media. The pH of each

Table I. Fermentation of Cellulose and Soluble Cellulose Derivatives by Rumen Microorganisms

Cellulose Added	Toluene Added	Reducing Substances (as Glucose) Formed, G./100 Ml.			
		0 hr.	12 hr.	24 hr.	48 hr.
None	+	0.000	0.037	0.046	0.053
Filter paper	+	0.000	0.157	0.163	0.192
Alphacel	−	0.000	0.001	0.000	0.000
Alphacel	+	0.000	0.168	0.179	0.180
CMC-120-M	−	0.000	0.000	0.000	0.000
CMC-120-M	+	0.000	0.188	0.216	0.227
CMC-70-L	−	0.000	0.021	0.028	0.029
CMC-70-L	+	0.000	0.255	0.268	0.318
CMC-70-M	−	0.000	0.023	0.033	0.040
CMC-70-M	+	0.000	0.253	0.274	0.296
CMC-70-H	−	0.000	0.014	0.020	0.025
CMC-70-H	+	0.000	0.256	0.279	0.282

For carboxymethylcelluloses numerals refer to degree of substitution (70 means that each 100 anhydroglucose units have 70 carboxymethyl groups) and letters refer to viscosity (L, M, H = low, medium, high).

culture was checked periodically throughout the experiment by inserting into the medium electrodes from a McBeth pH meter. When the pH was found to be below 6.2 it was adjusted to 6.8, using a saturated solution of sodium carbonate. During various time intervals aliquots were withdrawn from the fermentation cultures for analyses.

Analytical Procedures Samples of the fermentation media were deproteinized by a slight modification of the procedure of Doak (*5*) using cadmium hydroxide. The solution was not filtered during the clarification procedure, but instead the deproteinized sample was centrifuged and the clear supernatant decanted from the residue for further use.

The method of Underkofler, Guymon, Rayman, and Fulmer (*21*) was followed for the quantitative determination of reducing sugars. The total reducing substances in each sample were calculated as glucose per 100 ml. of fermentation medium.

The distillation of the volatile acids was conducted as outlined by Neish (*13*). The distilled acids were titrated with standard sodium hydroxide solution to the phenol red end point. The total volatile acids were calculated as acetic acid per 100 ml. of fermentation medium.

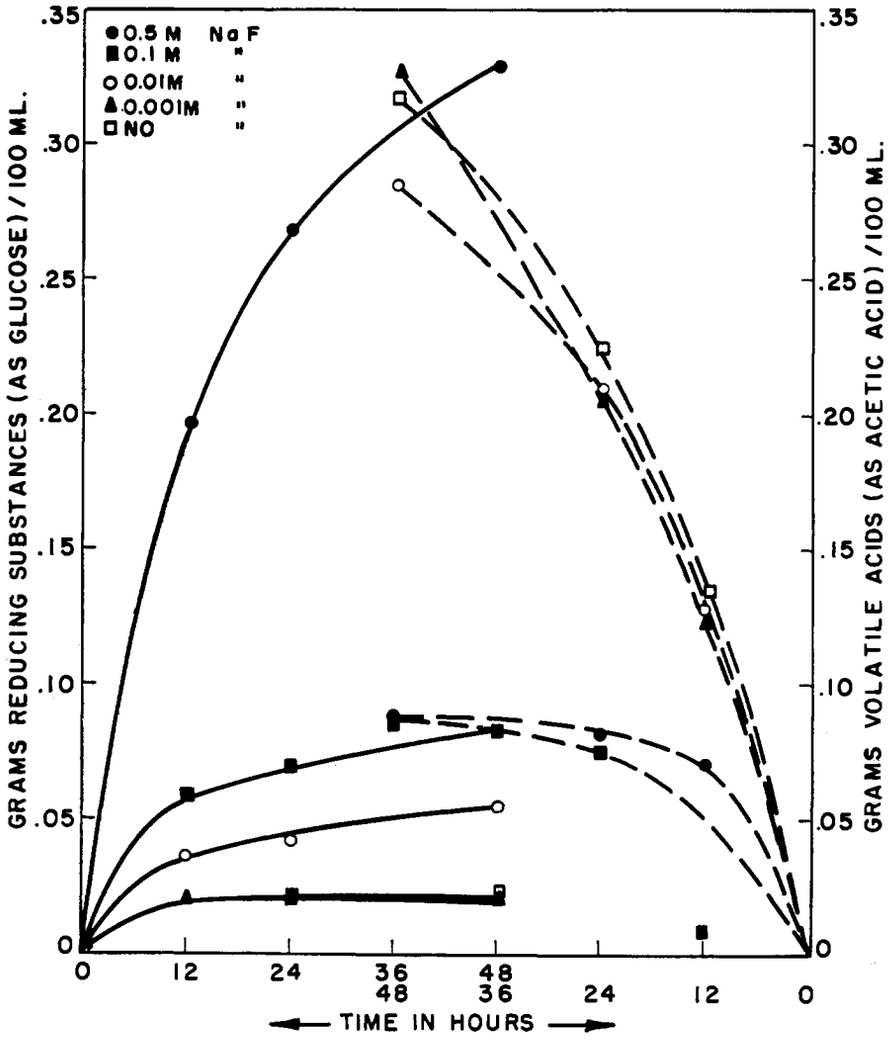
The method of detecting reducing sugars by filter paper chromatography was essentially the same as that used by French, Knapp, and Pazur (*7*). The solutions were spotted on a line near the bottom of 8 × 8⅝ inch sheets of Eaton and Dikeman 613 filter paper. After the spots had dried, the sheet was formed into a cylinder and ascending chromatograms were made; the developing solvent was *n*-butyl alcohol–pyridine–water, 6:4:3 parts by volume. The chromatograms were dried at room temperature, sprayed with an alkaline copper reagent, heated for 5 minutes in an oven at 110° C., and then sprayed with a phosphomolybdic acid solution which brought out as blue spots the areas where reduction of the copper by sugar had occurred.

Table II. Fermentation of 1% CMC-70-L by Rumen Microorganisms in Presence and Absence of Sodium Fluoride

Molar Concn. of Sodium Fluoride	Reducing Substances (as Glucose) Formed, G./100 Ml.				Total Volatile Acids (as Acetic Acid) Formed, G./100 Ml.			
	0 hr.	12 hr.	24 hr.	48 hr.	0 hr.	12 hr.	24 hr.	48 hr.
0.000	0.000	0.021	0.021	0.021	0.000	0.132	0.224	0.316
0.001	0.000	0.021	0.020	0.020	0.000	0.124	0.207	0.326
0.01	0.000	0.037	0.042	0.055	0.000	0.128	0.210	0.285
0.1	0.000	0.059	0.069	0.084	0.000	0.008	0.076	0.087
0.5	0.000	0.197	0.268	0.328	0.000	0.071	0.082	0.088
0.5[a]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

[a] Control, no inoculum added.

Figure 2. Fermentation of CMC-70-L by rumen microorganisms in presence and absence of sodium fluoride



Experimental Results Underkofler, Kitts, and Smith (*22*) recently reported that rumen microorganisms are able to digest cellulose in the form of finely ground filter paper or as soluble cellulose derivatives, the carboxymethylcelluloses (CMC), to produce the metabolic end products. A number of carboxymethylcellulose products were obtained from the Hercules Powder Co. and an experiment was designed to determine which of these compounds was most susceptible to the action of the rumen organisms. The results of this study (see Table I) showed that all of the carboxymethylcellulose samples used were digested rapidly by the microbial action. Finely ground filter paper and Alphacel (a powdered cellulose obtained from the Nutritional Biochemicals Corp.) were degraded at a much slower rate. In all cases toluene was found to prevent the conversion of the reducing substances to volatile fatty acids.

A number of chemical compounds were tested in order to find other inhibitory substances besides toluene which would prevent the conversion of reducing sugars to short-chain fatty acids during the digestion of cellulose by rumen microorganisms. These compounds included: sodium cyanide, iodoform, sodium fluoride, malonic acid, thymol, iodoacetic acid, toluene, formaldehyde, phenol, chloroform, *m*-xylene, and benzoic acid. During the fermentation of CMC-70-L by the rumen microorganisms in the presence and absence of the various chemical compounds, aliquots were removed at intervals from the culture media and analyzed for reducing substances and volatile acids.

Table III. Accumulation of Reducing Substances and Production of Volatile Acids in Inhibited and Noninhibited Fermentation Media

Inhibitor	Reducing Substances (as Glucose) Formed, G./100 Ml.		Total Volatile Acids (as Acetic Acid) Formed, G./100 Ml.	
	With inhibitor	Without inhibitor	With inhibitor	Without inhibitor
Sodium cyanide, 0.1*M*	0.062	0.015	0.092	0.616
Iodoform, 0.1*M*	0.069	0.023	0.088	0.347
Sodium fluoride, 0.5*M*	0.328	0.021	0.088	0.316
Malonic acid, 0.001*M*	0.060	0.018	0.267	0.404
Thymol, 0.1*M*	0.474	0.018	0.000	0.404
Iodoacetic acid, 0.5*M*	0.103	0.016	0.062	0.225
Toluene, 50 ml.	0.222	0.024	0.012	0.203
Formaldehyde, 15 ml.	0.025	0.024	0.050	0.203
Phenol, 19 g.	0.026	0.024	0.014	0.203
Chloroform, 50 ml.	0.372	0.024	0.014	0.203
m-Xylene, 50 ml.	0.167	0.024	0.031	0.203
Benzoic acid, 5 g.	0.054	0.024	0.278	0.203

Typical experimental results are shown in Table II and Figure 2.

In order to compare the effectiveness of the several chemical compounds tested for their inhibitory effect during the fermentation of CMC-70-L by rumen bacteria, the data are tabulated in Table III. The values given for the accumulation of reducing substances and volatile fatty acids produced in the various fermentation experiments are the amounts that were formed after 48 hours' fermentation. In the table only the results for the level of each inhibitor producing maximum accumulation of reducing sugars are shown. The data indicate that thymol, chloroform, sodium fluoride, toluene, *m*-xylene, and iodoacetic acid, in decreasing order, are the most effective in preventing the conversion of simple soluble sugars to short-chain fatty acids. As a result, reducing substances accumulated in the cellulose fermentation media.

In most of the subsequent experiments thymol or sodium fluoride was used to stop the fermentation of cellulose by rumen microorganisms at the glucose stage. By this means the pathway of cellulose degradation by rumen bacteria with special reference to the carbohydrate intermediates formed could be studied.

Filter Paper Chromatographic Studies A number of preliminary experiments were conducted in order to obtain information as to the identity of the reducing sugars which accumulated in a cellulose-digesting culture having an inhibitor added. The fermentation media containing ground filter paper or CMC-70-L as the cellulosic substrates, and toluene as inhibitor, were inoculated with fresh strained rumen liquid and incubated under carbon dioxide at 40° C. for 48 hours. After this period of time the media were tested qualitatively for reducing sugars, by the filter paper chromatographic technique.

In all cases only glucose and a trace of xylose were present in the cellulose digestion cultures. There was no trace of the disaccharide cellobiose in any of the samples tested. It is likely that after 48 hours' incubation the height of the cellulose fermentation had passed, and if cellobiose was formed during the degradation of the cellulosic substrate by the rumen microorganisms it could have been hydrolyzed to glucose. With this probability in mind, a more comprehensive investigation was conducted to study the carbohydrate intermediates formed during the rumen microbial breakdown of cellulose.

In order to follow the fermentation of CMC-70-L by the rumen bacteria, a 2% concentration of the cellulosic material was used in the fermentation media instead of the usual 1%. This gave larger yields of cellulose degradation products and thus a greater concentration of the sugars for chromatographic detection.

A number of cellulose fermentation cultures having CMC-70-L or Alphacel as the cellulosic substrates and sodium fluoride or thymol as inhibitors were prepared. At hourly intervals for 16 hours an aliquot was withdrawn from each culture and prepared for qualitative analysis for reducing sugars. It was found that the usual method for clarifying the media was inadequate and thus the samples to be analyzed chromatographically were deproteinized by using methanol. The sample was first brought to a boil and hot methanol was added until its concentration reached 75% in the mixture. The sample was then heated for a few minutes, cooled, and centrifuged. The clear supernatant was decanted carefully and evaporated to one third its original volume. After the sample was cooled, it was qualitatively analyzed for reducing sugars by means of filter paper chromatography.

A tracing from a typical chromatogram, that for the sample taken after 2 hours of incubation, is shown in Figure 3. The principal intermediate compound of the degradation of Alphacel and CMC-70-L was glucose. Slight traces of cellobiose were evident up to 6 hours of incubation, but no evidence of this sugar appeared on subsequent chromatograms. Faint spots indicating xylose were evident on all the chromatograms over the 16-hour period of investigation. No evidence was ever found of any reducing oligosaccharides larger than cellobiose on the chromatograms.

Cellulolytic Enzymes

Methods The experimental methods used in this investigation, involving the artificial rumen technique, analysis for products, and paper chromatography, were essentially the same as described above.

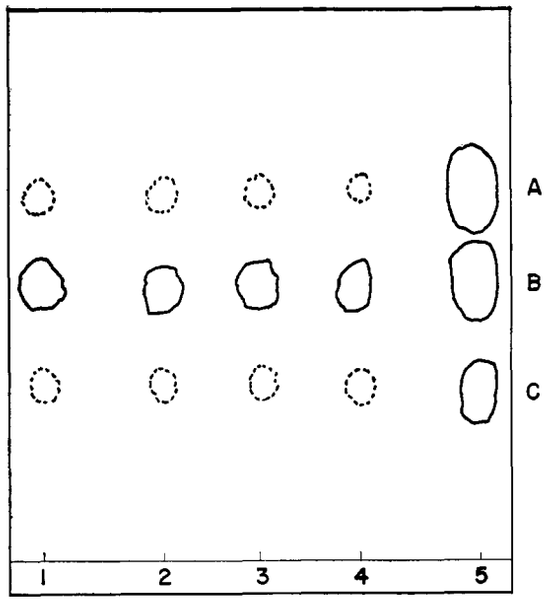


Figure 3. Tracing of paper chromatogram showing sugars formed by degradation of cellulosic substrates by rumen microorganisms after 2-hour incubation

A. Xylose
B. Glucose
C. Cellobiose
1. CMC-70-L + sodium fluoride
2. Alphacel + sodium fluoride
3. CMC-70-L + thymol
4. Alphacel + thymol
5. Control

Control spots resulted from 0.01 mg. of each sugar applied

Experimental spots for xylose and cellobiose are shown by broken lines to indicate that the spots were very faint and represented only traces of these sugars present

For the determination of the cellulolytic activity of different preparations, the amount of reducing substances formed from CMC-70-L or Alphacel when incubated at 40° C. anaerobically under carbon dioxide was estimated. The anaerobic incubation should not be necessary for the enzyme preparations, but was employed so that comparison could be made between the enzymic activities of living organisms and the preparations. In each test, 5 ml. of the preparation was mixed with 5 ml. of a phosphate-citrate buffer (*10*) of pH 5.5, and 100 mg. of cellulosic material. The buffer was prepared by mixing 5.46 volumes of 0.2*M* secondary sodium phosphate solution with 4.5 volumes of 0.1*M* citric acid solution. Sodium fluoride (0.21 gram) or thymol (0.20 gram) was usually added to the mixture in the reaction vessel as an antiseptic to prevent bacterial contamination. At various times during the period of incubation, aliquots were withdrawn, filtered, and analyzed for reducing substances by reducing sugar determination and by paper chromatography.

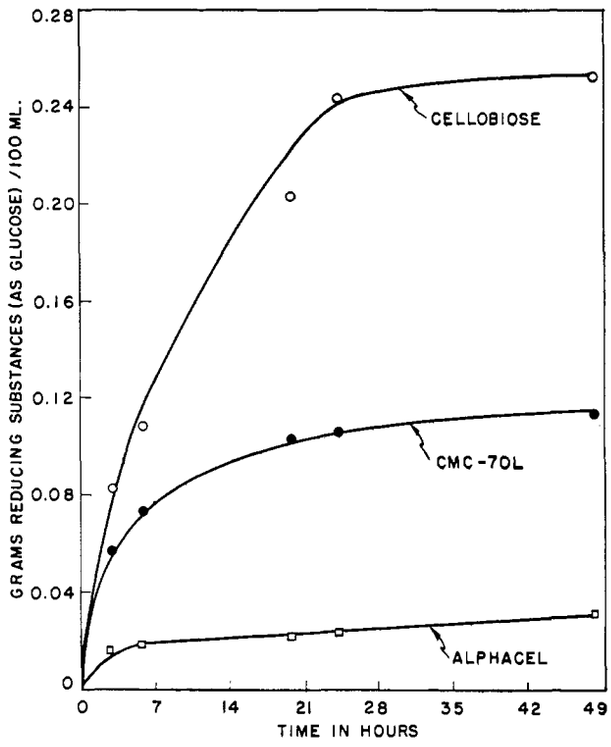


Figure 4. Reducing sugar formation from CMC-70-L, Alphacel, and cellobiose by cellulolytic cell-free extract

Experimental Results

In the microbiological degradation of cellulose, specific enzymes are elaborated which are able to split the larger polysaccharide molecule into smaller water-soluble compounds. These latter compounds can then diffuse into the interior of the organism for further metabolism. Many workers have observed that the cellulolytic microorganisms from the rumen act upon insoluble cellulose fibers only by direct contact with the fibers.

Various attempts were made in this study to determine whether the cellulose-splitting enzymes were excellular and present in the rumen liquid. In one experiment fresh rumen contents taken from a fistulated bovine were first strained through four thicknesses of cheesecloth and then centrifuged at 10,000 r.p.m. for 20 minutes. Part of the supernatant was tested for its cellulose-digesting activity. The remaining portion was filtered through a Berkefeld filter to remove bacteria that might still have been present in the liquid. The filtered sample was also tested for its cellulose-digesting activity.

The results of this experiment, as shown in Table IV, indicated that the filtered rumen liquid had no cellulolytic activity, while the centrifuged, nonfiltered sample possessed slight activity. This slight activity undoubtedly was the result of a small number of cellulolytic microorganisms present in the nonfiltered sample. It appears that the specific enzymes in question are not liberated as such in the external medium but are associated with the bacterial cells. Perhaps they are tightly adsorbed on the outer surface of the cell membrane.

Attempts were made to prepare cell-free enzyme extracts from the rumen organisms by supersonic vibration. The extracts obtained were of very low cellulolytic activity. Success was achieved, however, by use of the method outlined by McIlwain (*11*) for the preparation of cell-free enzyme extracts from microorganisms.

Four quarts of fresh sample of rumen liquid was obtained from the fistulated animal and strained through four thicknesses of cheesecloth. The resulting liquid was centrifuged at 1000 r.p.m. for 2 minutes to remove plant material and protozoa (*18*). The bacterial cells in the supernatant liquid were then harvested by high speed centrifugation, employing the Sharples centrifuge. After the cells had been washed three times with the 0.05*M* phosphate buffer of Meites, Burrell, and Sutton (*12*) (an equal mixture of 0.067*M* secondary sodium phosphate and 0.033*M* primary potassium phosphate) of pH 6.98, they were repacked in the centrifuge, weighed, and placed in a previously cooled mortar. Polishing Alumina A-303 (obtained from the Aluminum Ore Co.) was added in the amount of 2.5 to 3 times the weight of the cells. The mixture was then thoroughly ground with a cold pestle for 5 to 10 minutes. After this time cold, sterile physiological saline solution was added, the amount being two times by volume of the weight of the cells. The mixture was thoroughly stirred until a uniform suspension was obtained. This suspension was then centrifuged under refrigeration for 1 hour at 12,000 to 15,000 r.p.m. The resulting clear liquid was decanted carefully and used as the cell-free cellulolytic bacterial extract.

A number of experiments were conducted to determine the activity of the cell-free extract. The extract was tested in media containing 1% CMC-70-L in the presence and absence of sodium fluoride and thymol. It was observed, as indicated in Table V, that this extract possessed good cellulolytic activity. The addition of sodium fluoride or thymol prevented the reaction mixture from becoming contaminated. In the absence of such inhibitor, the reducing

Table IV. Fermentation of 1% CMC-70-L by Supernatant and Filtrate from Rumen Liquid

0.5M Sodium Fluoride Added	Inoculum Added	Reducing Substances (as Glucose) Formed, G./100 Ml. 0 hr.	24 hr.	48 hr.
−	Supernatant	0.000	0.012	0.013
+	Supernatant	0.000	0.055	0.055
−	Filtrate	0.000	0.000	0.000
+	Filtrate	0.000	0.000	0.000

Table V. Enzymic Breakdown of 1% CMC-70-L by a Cell-Free Extract of Rumen Microorganisms in Presence and Absence of Inhibitors

CMC Added	Inhibitor Added	Extract Added	Reducing Substances (as Glucose) Formed, G./100 Ml. 0 hr.	6 hr.	20 hr.	24 hr.
+	Thymol	−	0.000	0.003	0.003	0.003
−	Thymol	+	0.000	0.006	0.006	0.006
+	Thymol	+	0.000	0.102	0.104	0.112
+	NaF	+	0.000	0.106	0.131	0.140
+	None	+	0.000	0.113	0.057	0.062

sugars first formed were partially utilized by contaminants on longer incubation. Each mixture was analyzed at 24 hours for total volatile acids. It was found that no short-chain fatty acids were produced.

Paper chromatographic analysis of the degradation of CMC-70-L by the cellulolytic extract was also performed at various time intervals during the above experiment. In the early hours of the investigation only glucose was detected in the reaction mixtures; however, after 20 and 24 hours of incubation, glucose and traces of xylose appeared on the chromatograms. The presence of xylose suggested that a little xylan was present in the cellulosic substrate, and that the cellulolytic preparation contained an enzyme capable of hydrolyzing the xylan to xylose.

Cellobiose was not detected in any of the tests as a degradation product of CMC-70-L by the extract. It was possible that cellobiose was not formed, or that if it was it could have been rapidly hydrolyzed to glucose during the reaction period. In view of the latter possibility, the action of the cellulolytic preparation on CMC-70-L, Alphacel, and cellobiose was studied. The reaction mixtures were prepared as described above.

From Figure 4, it may be observed that the extract had the ability to hydrolyze Alphacel as well as CMC-70-L, but at a slower rate. Cellobiose was likewise hydrolyzed to glucose. Its rate of hydrolysis was much greater than that of CMC-70-L or Alphacel. It is probably true that the detection of cellobiose in the reaction mixtures during the degradation of CMC-70-L or Alphacel would be difficult because of the rapid rate at which the cellobiose would be hydrolyzed to glucose.

In order to be sure that the action of the cellulolytic preparation was not due to contaminating microorganisms, samples of the enzyme solution were tested after sterilization by bacteriological filtration through a sintered-glass filter. The results clearly showed that the conversion of CMC-70-L to reducing sugars was not brought about by contaminating organisms. The unfiltered extract gave 0.090 and 0.094 gram, and the filtered extract gave 0.094 and 0.099 gram of reducing substances (as glucose) per 100 ml. after 6 and 20 hours' incubation, respectively.

A number of experiments were conducted to study the properties of the cellulolytic bacterial extract. Phosphate buffer solutions (*10*) were employed in this and subsequent experiments. A series of enzyme-buffer-carboxymethylcellulose mixtures of pH 4.0 to pH 8.0 was prepared and incubated anaerobically at 40° C. The results, shown in Figure 5, indicated that 5.5 was the optimum pH for the cellulolytic activity of the extract.

Table VI. Enzymic Breakdown of 1% CMC-70-L and 1% Alphacel by a Cell-Free Extract of Culture 32

Cellulose Added	Reducing Substances (as Glucose) Formed, G./100 Ml. 0 hr.	16 hr.
CMC-70-L	0.000	0.026
Alphacel	0.000	0.017
None	0.000	0.007

The optimum temperature for enzymic action was determined by preparing a number of enzyme-buffer-carboxymethylcellulose mixtures at pH 5.5 and incubating them at temperatures from 10° to 80° C. under carbon dioxide. The results of this study, as given in Figure 6, showed that the optimum temperature for maximum cellulolytic activity of the extract was between 40° and 50° C. It was noticed that the activity was greater at 50° C. for the 6-hour reaction period, but on longer periods of incubation the extract showed an optimum temperature of 40° C.

In testing the stability of the extract, portions of the same preparations were kept at −15°, 10°, and 25° C. for 144 hours. During this period cellulolytic activity of the samples was tested periodically using CMC-70-L. After 144 hours of storage the frozen and chilled samples showed no loss of activity, while the sample held at room temperature lost 34% of its enzymic activity.

Since McIlwain's procedure (*11*) resulted in the successful preparation of a cell-free cellulolytic bacterial extract from mixed rumen culture, a similar extract was prepared from the isolated rumen culture 32. The extract obtained was tested for its cellulolytic activity (Table VI) and it was shown to have the ability to hydrolyze CMC-70-L and Alphacel. Glucose was detected by filter paper chromatography, as the only degradative product of the cellulosic substrates by this enzyme preparation.

Discussion and Conclusions

Preliminary experiments conducted in order to elucidate a part of the pathway of cellulose digestion by rumen microorganisms suggested that glucose was the only product formed. However, these investigations were made with inhibited cultures that had been incubated for 48 hours. It is reasonable to assume that the height of the cellulose fermentation had passed, and if cellobiose, cellotriose, or higher oligosaccharides were formed during the degradation of the cellulosic substrate they could have been hydrolyzed to glucose by microbial action during that period of time.

Cellulose-digestion cultures, inhibited with sodium fluoride or thymol, and analyzed qualitatively at hourly intervals for carbohydrate intermediates showed three reducing sugars as compounds of cellulose degradation. Of these glucose was the principal compound. Xylose and cellobiose were detected only in traces. The presence of xylose may be explained by the fact that the cellulosic substrates are contaminated with traces of xylan. This polysaccharide is very susceptible to enzymic attack and thus would be hydrolyzed to xylose. Cellobiose could be the primary degradation product of cellulose from which glucose is formed by hydrolysis, or glucose might itself be a primary product of cellulose digestion by rumen organisms. This question is not settled.

Whitaker (*25*) with his purified cellulase from the mold *Myrothecium verrucaria* found that both glucose and cellobiose were formed by the action of his enzyme, and that cellobiose was hydrolyzed by the enzyme. He concluded, however, that the formation of glucose by this cellulase is not dependent on formation via cellobiose.

In various enzymic starch degradation studies—for example, with *Clostridium acetobutylicum* (*6*)—amyloglucosidases have been found, the principal catalytic activity of which is the hydrolytic removal of individual glucose units from the nonreducing terminus of a starch chain. When a branch point in the starch structure is encountered, the enzymic action stops. From the results of the study of cellulose degradation it appears that the cellulose-splitting enzyme elaborated by rumen micro-

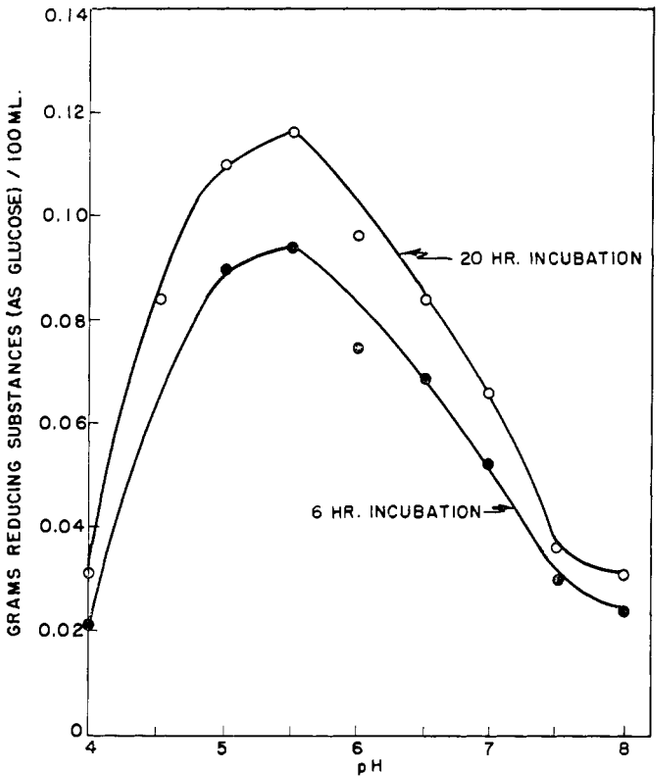


Figure 5. Effect of pH on activity of cellulolytic cell-free preparation

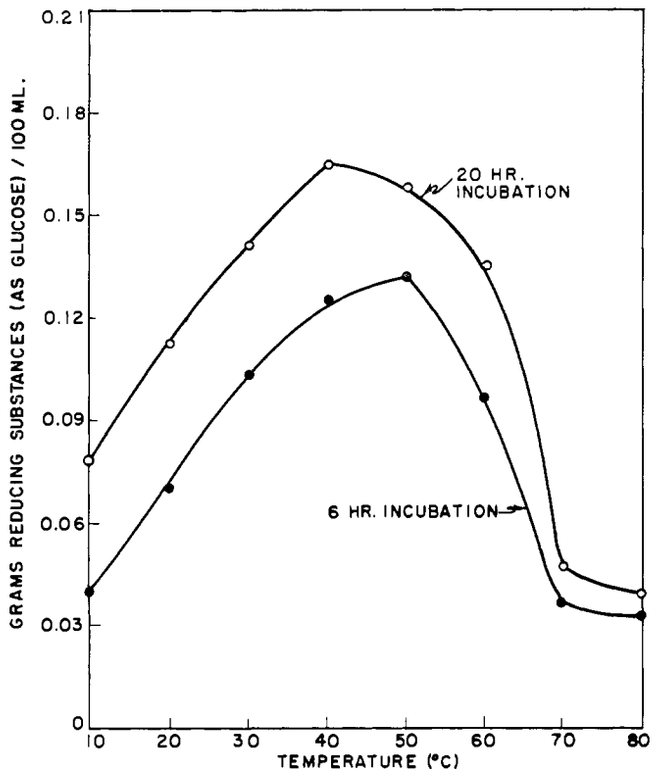


Figure 6. Effect of temperature on activity of cellulolytic cell-free preparation

organisms may be a "celloglucosidase," an enzyme which hydrolyzes individual glucose units from the ends of the cellulose chains. This postulation is similar to the one proposed by Clayson (*4*) in 1943. He believed that the cellulolytic microorganisms begin at the end of the cellulose chain and lopped off one anhydroglucose unit after the other. The failure, under any conditions, to observe formation of oligosaccharides larger than cellobiose as a result of the action of the rumen organisms upon cellulosic substrates tends to confirm the hypothesis that the enzymes specifically split off sugars from the ends of the cellulose chains rather than attack cellulose molecules in a more random fashion similar to that of α-amylase upon starch.

This investigation has shown the possibility of producing cell-free cellulolytic enzyme preparations from rumen microorganisms. The failure to find sugars other than glucose as the result of the action of the cell-free enzyme preparations on cellulosic materials tends to confirm the hypothesis that the cellulose-splitting enzyme of the rumen organisms is a celloglucosidase. However, the rapid rate of hydrolysis of cellobiose by the enzyme preparation, and the possibility that in such a crude enzyme mixture there may be present enzymes which rapidly hydrolyze other possible initial degradation products of cellulose, still leave unsettled whether glucose is a primary degradation product. Further work on the purification of the cellulolytic enzyme is therefore in progress.

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Received for review March 22, 1954. Accepted May 14, 1954. Presented before the Division of Agricultural and Food Chemistry, Fermentation Subdivision, at the 124th Meeting of the AMERICAN CHEMICAL SOCIETY, *Chicago, Ill. Journal Paper J-2367, Iowa Agricultural Experiment Station, Ames, Iowa, Project 1202.*