SOME PHYSIOLOGICAL STUDIES ON THE DIGESTION OF CELLULOSE

BY RUMEN MICROORGANISMS

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the
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By

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INTRODUCTION

The decomposition of cellulose in the rumen is a fermentation that has intrigued and attracted scientists of many fields and the results of the process are not only essential to the host animal but also have a direct bearing on the well being of man. Microorganisms in the rumen are solely responsible for this breakdown of cellulose and without these microorganisms the ruminant could not consume and digest roughages in its diet. The dependence of man on the meats, dairy products, hides, and other by-products of these same ruminants is indeed great.

The very nature of the rumen environment makes a study of the organisms involved in the process of cellulose degradation a challenging one. One must consider the anaerobic nature of the organ and bear in mind the techniques necessary to culture organisms from such an environment. The total count of true rumen organisms that are anaerobes probably exceeds the total count of aerobes by approximately a hundred fold. As nearly as can be determined by slide counting methods now available; the total count of bacteria, regardless of oxygen requirements, ranges from 56 million to 96 billion per milliliter. In addition to this vast bacterial population there exists a protozoan population of considerable size. The possibilities for synergistic effects among microorganisms in such a population are immeasurable.
When one considers so large and complex a bacterial population it soon becomes obvious that a great majority of the rumen organisms have no direct bearing on the process of cellulose degradation. In addition to this, many of the microorganisms found in the rumen are only transient contaminants and serve but to confuse a study of the true residents. Because of these facts it seems essential that a more or less constant population type be maintained in any study of the organism or organisms responsible for the breakdown of cellulose. In order to accomplish this purpose in this investigation a mixed culture capable of digesting cellulose was transferred at regular intervals.

The organism or organisms responsible for the breakdown of cellulose in the rumen and the exact mechanism by which this breakdown occurs is still somewhat obscure. A study of the factors and organism(s) involved in this process should lead to more efficient utilization of roughage diets by the host animal and be of direct benefit to man.
STATEMENT OF THE PROBLEM

Attempts on the part of most investigators to isolate pure cultures of cellulolytic bacteria from the rumen have met with failure. This failure has been due largely to the lack of knowledge of the nutritional requirements of such organisms and to their anaerobic nature. No technique exists which enables an investigator to isolate pure cellulose-digesting organisms from the rumen with any consistent amount of success. The goal of the present study was the accomplishment of this end and to investigate the nutritional requirements of these organisms. In the study the techniques used by other workers in the field as well as modifications of these and other techniques were employed. Few cellulolytic cultures have been isolated from the rumen and the purity of these cultures has been doubted.

As the study progressed it became increasingly apparent that additional information about factors stimulatory to cellulose digestion was needed before pure cultures could be isolated and studied. No such study has been made with cultures maintained on laboratory media and digesting soluble cellulose and it was felt that factors found to be stimulatory to such a mixed culture, when added to a medium, should enhance the isolation of pure cellulose-digesting cultures. Such a study would also add to the existing knowledge of cellulose breakdown by rumen bacteria.
LITERATURE REVIEW

Studies of cellulose-digesting organisms from the rumen have been directed in the main at the nutritional requirements of such organisms either as pure cultures or in mixtures. A vast amount of knowledge is accumulating and it is only recently that many segments of these studies are being fitted into a whole.

Studies to determine the best type of medium for growing rumen organisms have been numerous. Gall, Stark, and Loosli (1947) made an extensive study of the techniques and media which were necessary to isolate the predominating rumen flora of cattle and sheep. These workers felt that a rich organic medium was the most desirable for the isolation of most rumen organisms. Huhtanen, Rogers, and Gall (1952) used a similarly rich organic medium for the isolation of rumen bacteria. Hungate (1950), on the other hand, favors a medium containing only inorganic salts, rumen fluid, and a carbohydrate. McNeill, Doetsch, and Shaw (1953) found no material which could replace rumen fluid for the growth stimulation of all types of organisms. They noted that many of the rich nitrogenous materials were inhibitory to many rumen organisms. King and Smith (1955) have compared the two types of media commonly employed and concluded that the type of medium employed by Hungate is superior to that of Gall. Their conclusions were based on total counts and cellulolytic counts as well as other criteria.
Vitamins play an important role in the metabolism of rumen microorganisms. McElroy and Jukes (1940) demonstrated the synthesis of biotin in the rumen of the cow. That other members of the Vitamin B complex are synthesized in the rumen was demonstrated by Wegner et al. (1940). These authors were able to show a significant synthesis of thiamin, riboflavin, nicotinic acid, pyridoxine, and pantothenic acid as well as biotin. A relationship between diet and synthesis of B-vitamins was made by Hunt and co-workers (1954). When a steer was fed alfalfa hay there was a more effective synthesis of Vitamin B_{12} than was the case when the steer was on a diet of poor hay. It was also shown by these authors that the organisms which synthesize riboflavin, niacin, and Vitamin B_{12} have a sulfur requirement.

The significance of amino acids in the rumen has been emphasized by a number of studies. MacLeod and Brumwell (1954) made an in vitro study of cellulose degradation by mixed cultures and determined that a mixture of 18 amino acids was even more active in stimulating cellulose breakdown than were whole soluble supplements. Tsuda (1953) investigated the products of four different bacteria from the bovine rumen. He found all of the cultures produced all of the familiar amino acids, with the doubtful exceptions of histidine and tryptophane. Experiments by Loosli et al. (1949) bear
out this significance of amino acids. They fed urea as the only dietary source of nitrogen and found ten essential amino acids to be synthesized in ruminants. The role of the amino acids in forming branched-chain fatty acids was postulated by Al-Shazly (1952). He felt these fatty acids were formed from valine, leucine, and isoleucine by Stickland reactions. A more direct role of an amino acid in the formation of a fatty acid was shown by Sirotnak et al. (1954). These investigators studied the fatty acids produced from aspartic acid by mixed bacterial suspensions. They present data to show the probable pathway from aspartate to acetate and butyrate.

Lactic acid has also been shown by Waldo and Schultz (1956) to be a precursor of the fatty acids -- acetic, propionic, and butyric. It is interesting to note that the type of salt used has an effect on the amount of acid produced. Huetter et al. (1956) in an in vivo study noted that when sodium lactate was fed to ruminants a much smaller amount of propionate and butyrate was formed than when the calcium salt of lactic acid was fed.

Clark (1953) states that rumen gases consist of 67 per cent carbon dioxide, 26 per cent methane, 6 per cent nitrogen, and 0.1 per cent hydrogen sulfide. Oxygen is present in only trace amounts. Koistinen (1946) presents data that suggests that most acetic acid is formed from
a reaction between CO$_2$ and CH$_4$ and points out the importance of feeding carbonates since their carbon can be converted to organic acids in the rumen. Using isotopes Huhtanen, Carleton, and Roberts (1954) in a study of a pure culture isolate of the rumen showed the incorporation of CO$_2$ into fatty acids. Contrary to this finding Otagaki et al. (1955) in another isotopic study demonstrated that carbon from CO$_2$ fixation was found mostly in the cellular material and very little was detected in the volatile fatty acids. Perlin and Michaelis (1946) reported an absolute requirement for CO$_2$ in the decomposition of cellulose by _Vibrio perimastrix_ and another unidentified bacterium when the two decomposed cellulose as a mixed culture.

The complex nature of the factors involved in ruminal cellulose digestion is indicated from a study by Burroughs and his associates (1950). In this investigation a continuous _in vitro_ fermentation was maintained and it was determined that additions to the fermentation of a complex salt solution, ash of alfalfa extract, autoclaved rumen liquor, or an autoclaved water extract of manure proved to be beneficial to the process of cellulose degradation. Bentley and his co-workers (1954a) showed that while compounds such as biotin, vitamin B$_{12}$, p-ABA, and certain purine or pyrimidine bases improved cellulose digestion, these compounds were not as stimulatory as
rumen juice or water extracts of alfalfa, yeast, or molasses.

That the type of ration fed an animal has an influence on the kinds and numbers of bacteria isolated from the rumen was shown by Bryant and Burkley (1953a). They noted that the bacterial flora was more complex when alfalfa hay or alfalfa hay concentrate rations were fed to animals than when concentrate or wheat straw was the ration. Some differences were noted in the groups of bacteria isolated from different animals fed the same ration. Pounden and Hibbs (1948) have also defined the relationship between the type of ration fed an animal and the kinds of organisms isolated. In their study of hay and grain rations they delineated the predominant types of organisms found into two groups for each ration. Group I of the hay flora was described as quite large gram positive coccoids existing in closely knit pairs. Group II of the hay flora consisted of large gram positive, thick, fairly square-ended rods; very large gram negative cigar-shaped rods; and smaller gram negative short rods in fours or multiples of fours. The two groups of the grain flora consisted of medium-sized, comparatively thin, gram positive rods which were sometimes granular and of variable length and gram negative rods which resembled coliforms. Later, Pounden, Ferguson, and Hibbs (1950) described these types of organisms as they existed in the various parts of the
digestive tract of cattle. A study was made by Pounden and Hibbs (1950) of calves raised without these characteristic organisms and protozoa. In their investigation one group of calves was inoculated with cud material while another group was uninoculated. While both groups of animals gained weight at about the same rate, the uninoculated animals developed rougher coats of hair and exhibited "pot-bellied" abdomens. These animals were lacking in the usual protozoa found in the rumen and showed some variations in the characteristic rumen microflora described above.

In an attempt to more or less standardize the description of types of ruminal isolates, Moir and Masson (1952) have presented an illustrated scheme of rumen microorganisms from sheep. These authors described some 33 different types of bacteria and presented photo micrographs for comparison to organisms isolated by other investigators. Huhtanen and Gall (1953a, 1953b) have described groups of rumen organisms according to their reactions on carbohydrates, the end products formed, and the morphology of the organisms. These authors describe an organism designated as RO-C8 which closely resembles Veillonella gazogenes isolated by Johns (1951). Both organisms form similar end products and are gram positive in young cultures but quickly revert to gram negative in 12 to 18 hours. The description of such a gram variability
in organisms is not uncommon in the literature of rumen bacteria.

A number of investigators have reported the isolation of pure cultures of anaerobic, cellulose-digesting bacteria. Yet today the validity of many of these reports is doubted by numerous investigators in the field. These doubts have been raised for the most part by the difficulty in repeating the isolations when the same techniques were employed.

Omeliansky (1902) was probably the first to isolate anaerobic cellulose-digesting bacteria. The source of his isolations was river mud and horse feces from which he isolated two different organisms. Khouvine (1923) obtained pure cultures from the soil, herbivora, and the intestinal tract of man. Werner (1926) reported isolating a cellulose-digesting bacterium from the larvae of a rose beetle. His isolate, like that of Khouvine, would not grow without cellulose. Cowles and Rettger (1931) also used horse feces as a source of material and isolated a cellulolytic rod which closely resembled that studied by Omeliansky.

Pochon (1934) was the first investigator to isolate a cellulolytic bacterium from the rumen. He isolated an organism, *Plectridium cellulolyticum*, from an ox. On initial isolation the organism was only slightly cellulolytic and grew best at a pH of 8. After 15 months of
passage on artificial media the organism became tolerant of a wide pH range, grew aerobically as well as anaerobically, and was cellulolytically active. Hungate (1942a) isolated an organism from cattle which he briefly described but did not name. This organism could be passed from cellulose to glucose and back again to cellulose without losing its cellulolytic ability. The nutritional requirements of the organism were met by supplying biotin in addition to ammonium salts and cellulose. Hungate (1942b) has also reported on a pure culture of cellulolytic protozoan from cattle and on two pure cultures of bacteria isolated from the termite (1944a). Both of these organisms were obligate anaerobes, one being an actinomycete which was named *Micromonospora propionici*. This organism was later isolated by Hungate (1946) from the rumen of cattle.

No other worker has enjoyed so great a degree of success in isolating pure cultures of cellulolytic bacteria from the rumen than has Hungate (1944a, b, 1947). Since his efforts have been so rewarding it is noteworthy to briefly describe his technique for obtaining pure cultures. A complete description of the technique is given in his review article (1950). A rumen juice sample is diluted in a cellulose-agar medium. The dilutions are made in rubber stoppered tubes which are kept anaerobic by continual flushing with carbon dioxide as the dilutions
are prepared. The tubes containing the agar dilutions are incubated for a period of one week. From the highest dilutions are selected colonies which have digested the finely ground up cellulose which is suspended in the agar. The digestion of cellulose is evidenced by a clear zone surrounding the colony. The colony is again diluted in a solid medium containing finely ground cellulose and is transferred until only one kind of colony appears to be present. The colony is also subcultured in glucose or cellobiose in a dilution series. If growth occurs in high dilutions, a colony is transferred back to cellulose. If rapid and typical cellulose digestion occurs and there is no indication of contaminants it is concluded that a pure culture of the cellulose digesting bacterium has been isolated.

Bryant and Burkey (1953b) in a study of 896 strains of organisms isolated from the rumen found 8 of the strains to be cellulolytic. These authors used the method of Hungate in their isolations. Bryant and Doetsch (1954) were also successful in obtaining pure cellulolytic organisms by this method. The organisms isolated by these investigators were similar to Bacteroides succinogenes, a gram negative rod first isolated by Hungate (1950).

Bryant and Small (1956) have recently reported on a group of organisms which they feel is important in the digestion of fiber in the rumen. Of the 48 strains
tested 3 were able to digest cellulose after an incubation period of a year. Their results suggested that the ability of these organisms to digest cellulose is a variable characteristic and when the organisms are transferred in a medium containing glucose and cellobiose the ability to digest cellulose, as determined visually, is lost.

Numerous reports exist in the literature of the beneficial effect of one or more organisms on the digestion of cellulose by a cellulose utilizing bacterium. Sijpesteijn (1951) noted that growth on cellulose was favored by the addition of Clostridium sporogenes, or a certain amount of sterilized medium in which this organism had previously grown, to a culture of Ruminococcus flavefaciens, a cellulose-decomposing anaerobic coccus of the rumen. A similar phenomenon was noted earlier by Sanborn (1926). He found the rate of cellulose digestion by Cellulomonas folia to be increased when extracts of Bacillus mycoides, B. cereus, or B. subtilis were added to the medium. Treccani (1953) made several unsuccessful attempts at isolating a pure culture of cellulose-decomposing organisms of the goat rumen. He felt his work strengthened the hypothesis that anaerobic cellulose digestion in the rumen is the result of a symbiotic activity of coccal and rod forms. Stanier (1942) expressed his opinion on this "symbiotic" effect among
anaerobic cellulose-decomposers. He pointed out that in the ultimate analysis of such an effect; physical and chemical grounds or both would explain the failure to obtain pure cultures. He states "... with sufficient persistence and ingenuity a separation of the two forms should be possible". No evidence; however, is cited to rule out such a possibility.

Soluble cellulose has been widely used in the study of cellulose-decomposing organisms. Reese, Siu, and Levinson (1950) discussed the chemical nature of this substance and, from data collected from studies of filtrates of molds and bacteria, postulated an enzymatic pathway for cellulose degradation. Reese (1955) discusses the views on the question as to whether there is only one type or several cellulases.

Underkofler, Kitts, and Smith (1953) found carboxymethylcellulose (CMC) to be superior to insoluble cellulose for studies with ruminal organisms. After 48 hours incubation, 0.143, 0.41, and 0.61 grams of volatile acid (as acetic acid) were obtained from 100 milliliters of culture medium containing 1 per cent of ground filter paper, alphacel, and CMC respectively. By using CMC, Kitts and Underkofler (1954) demonstrated that cellulolytic enzymes of the rumen are not present as such in rumen fluid, but are associated with the bacterial cells. This was shown by centrifuging out the cells, filtering
the supernatant liquid and incubating it with CMC to test for cellulolytic activity. Their findings are not in agreement with those of King (1956) who found a considerable portion of rumen bacterial cellulases to be truly extracellular.

Freeman, Baillie, and Macinnes (1948) were presented with the problem of loss of viscosity of suspensions of CMC which had been stored for long periods of time. Upon investigating they found that the suspensions had become contaminated with several types of bacteria. The contaminants were transferred as a mixed culture from which several pure cultures were obtained. Of these pure cultures, only one organism was capable of digesting CMC. This organism, probably a Cellulomonas, was capable of growing on comminuted wood in a liquid sulfate medium. In this investigation the authors determined the activity of the organism by using a viscometer and measuring the change in viscosity of CMC suspensions. Levinson and Reese (1950) used a similar technique as did Holden and Tracy (1950) in measuring the cellulose activities of organisms in their studies. More recently King (1956) has employed this method in a study of rumen enzymes which are capable of digesting CMC.
MATERIALS AND METHODS

Source of Samples

Most of the experiments were made on samples obtained from two fistulated sheep and one fistulated steer which were made available through the cooperation of Dr. William Tyznik of the Department of Animal Science, The Ohio State University. The samples were removed via the permanent fistula and strained through cheese cloth into a bottle. The larger particles were thusly removed, and either discarded or placed back in the rumen. The bottle containing the sample was securely stoppered and transported directly to the laboratory where it was either used in dilutions or stored in the refrigerator. Samples stored in the refrigerator were used in the preparation of media. The samples were stored under an atmosphere of pure carbon dioxide in tightly stoppered bottles.

The sample used for preparing CMC media for determining factors stimulatory to cellulose digestion was obtained from David Davies Meat Packing Company in Columbus, Ohio. This sample was obtained from a freshly slaughtered steer and was transported, filtered, and stored as were the above samples.

Dilution Fluid

The fluid used to dilute rumen juice samples was that described by Lepovetsky (1954) with the exception that methylene blue in a final concentration of 0.0001
per cent was used as an indicator of anaerobiosis in place of resazurin. The dilution fluid was autoclaved in milk sample dilution bottles and 20 x 150 mm culture tubes and then securely stoppered with rubber stoppers. The dilution blanks so prepared were adjusted under aseptic conditions to pH 7 before use by bubbling carbon dioxide through the fluid.

Medium for Mixed Cultures

The medium used for transferring mixed cultures or for experiments with samples taken directly from the rumen was a modification of Hungate's Medium (1950) as proposed by Lepovetsky (1954) and was composed of the following:

<table>
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<tr>
<th>Component</th>
<th>Concentration</th>
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<tr>
<td>KH₂PO₄</td>
<td>0.06 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.09 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.02 g</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>0.09 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.09 g</td>
</tr>
<tr>
<td>NaThioglycollate</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.15 g</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>0.2 g</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.5 mg</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>n-Valeric Acid</td>
<td>32 mg</td>
</tr>
<tr>
<td>n-Butyric Acid</td>
<td>28 mg</td>
</tr>
<tr>
<td>iso-Butyric Acid</td>
<td>14 mg</td>
</tr>
<tr>
<td>p-ABA</td>
<td>50 μg</td>
</tr>
<tr>
<td>Biotin</td>
<td>20 μg</td>
</tr>
<tr>
<td>Trace Elements Solution</td>
<td>1 ml</td>
</tr>
<tr>
<td>Rumen Fluid</td>
<td>28 ml</td>
</tr>
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</table>

Distilled Water to make a total volume of 100 ml
The medium, containing all the compounds except glucose, fatty acids, and sodium carbonate, was boiled in a 500 milliliter round bottom flask until the methylene blue became decolorized. Carbon dioxide was continuously passed into the gaseous phase of the flask in order to effect a quicker reduction of the oxidation-reduction indicator and to maintain anaerobic conditions. Rumen fluid was added and the boiling was continued until the indicator again became reduced. A rubber stopper was tightly fitted into the neck of the flask and wired into place. The flask was then autoclaved for 20 minutes at 121 C. When the medium had cooled, sterile glucose, fatty acids, and sodium carbonate solutions were added and the pH adjusted to 7 with sodium hydroxide or hydrochloric acid. During this time sterile carbon dioxide was passed into the flask to displace the air and maintain anaerobic conditions. The medium was dispensed as described in a later section.

Glucose and sodium carbonate were prepared as 45 and 6 per cent solutions respectively and autoclaved for 15 minutes at 121 C. The sodium carbonate was stored under carbon dioxide in a rubber-stoppered flask.

The volatile fatty acids were sterilized by Seitz filtration and added to the medium in the amounts indicated.

The solution of trace elements consisted of 100
milliliters of distilled water containing 20 mg of CoCl$_2$·6H$_2$O, 280 mg of H$_3$BO$_3$, 210 mg of MnSO$_4$·2H$_2$O, and 75 mg of Na$_2$MoO$_4$.

All of the other compounds used in the medium were prepared as concentrated stock solutions and stored in the refrigerator.

In subsequent sections the above described medium will be referred to as the transfer medium. The same medium containing two per cent agar is referred to as rumen juice agar.

**Cellulose Substrate**

Cellulose was added to the medium as Whatman filter paper, soluble cellulose, or purified wood cellulose. Hercules CMC (70 premium low viscosity), a soluble cellulose obtained from the Hercules Powder Company, Wilmington, Delaware, served as the soluble cellulose substrate. The purified wood cellulose, Solka Floc 40, was obtained from Brown Company, Berlin, New Hampshire. A one per cent concentration of soluble cellulose or Solka Floc was used in the medium. Filter paper was cut into strips so that the medium in the tubes contained about 0.2 g of filter paper per 10 milliliters of medium.

For the nutritional studies CMC was used in the media at a one per cent concentration. In order that all the media for one experiment would have about the same viscosity, the CMC was prepared and added to the medium.
as a concentrated stock solution. In preparing the stock solutions the CMC was allowed to soak overnight in order to bring about complete solution.

Dispensing of Media

Hungate (1950) strongly stresses the necessity of strictly anaerobic conditions during the preparation of media and gives detailed instructions for maintenance of such conditions. This technique involves filling a sterile pipette with carbon dioxide before pipetting to tubes a sterilized medium from the flask in which it was prepared. The technique was attempted but proved to be too cumbersome and time consuming. A method was developed whereby media could be dispensed anaerobically in much less time and which eliminated some of the chances of contamination which were seemingly inherent in the method of Hungate. Figure 1 shows the apparatus used to dispense sterile media. Ring stands at H, I, and J supported the apparatus. The glass and rubber tubing sections labeled A, B, C, and D were individually wrapped in brown paper and sterilized as a complete unit in the autoclave just prior to use. The tubing was then connected at A to a tank of pure carbon dioxide and the gas turned on. Clamps were placed at E and F so that all gas would pass through the tubing at D. The rubber-stoppered, sterilized flask of medium was then opened and the tubing D inserted loosely into the medium. Glucose, volatile fatty acids, and sodium
carbonate were added and the medium adjusted to pH 7. The stopper containing the glass tubing (D) was then firmly seated in the neck of the flask as the clamp was removed from F. If Solka Floc was present in the medium the flask was frequently swirled in order to keep the particles suspended. The wrapping paper was removed from the capillary pipette (B) which was then placed in one of the tubes which had been previously sterilized and stoppered with sterile rubber stoppers. By pinching the rubber tubing at G, the tube was flushed with carbon dioxide before the medium was dispensed into it. Next the pinchcock E was opened and approximately 10 milliliters of medium was allowed to enter the tube. The stopper was then removed from a second tube, tubing C was placed in the second tube, the stopper from the second tube was placed in the first tube as the capillary pipette was removed and placed in the second tube. Tube two was flushed with carbon dioxide in the same manner as was tube one and the operation was continued until the flask of medium was dispensed.

**Culture Transfers**

Mixed cultures growing on filter paper as a substrate were transferred each time the filter paper began to disintegrate. Two to five days were usually necessary for the cultures to digest the filter paper to the point of disintegration. When soluble cellulose was the substrate
Figure 1. Apparatus for Anaerobic Dispensing of Media.
the cultures were transferred at two or three day intervals. Hungate's (1950) procedure was followed for transferring the cultures under an atmosphere of carbon dioxide.

**Inocula for CMC Media**

Cultures maintained on CMC served as the inoculum source for experiments dealing with nutritional requirements of the cellulolytic mixed culture. The organisms were grown anaerobically in Erlenmeyer flasks in the medium described. After an incubation period of 30 hours at 40°C the organisms were harvested in a Sorval angle head centrifuge. The cells were washed three times using eight milliliters of sterile 0.85 per cent saline per washing and were suspended in saline to a volume equivalent to one half the volume of the growth medium. The inoculum used for the experiments was 2.5 per cent by volume.

**Media for Nutritional Studies**

To determine the nutritional requirements of the mixed culture a basal medium was prepared containing the following in a total volume of 100 milliliters of distilled water: 0.06 g $\text{KH}_2\text{PO}_4$, 0.09 g $\text{K}_2\text{HPO}_4$, 0.02 g $\text{MgSO}_4\cdot7\text{H}_2\text{O}$, 0.09 g $(\text{NH}_4)_2\text{SO}_4$, 0.09 g NaCl, 2.5 mg CaCl$_2$, 0.01 mg FeSO$_4$, 0.2 mg methylene blue, 0.05 g sodium thioglycollate, 0.2 g Na$_2$CO$_3$, 1 g CMC, and 28 milliliters of rumen fluid. If a compound was found to be stimulatory to the digestion of CMC that compound was included in a
basal medium which was used to determine the effect of another added compound. By this method it was determined which of the compounds used in the transfer medium were stimulatory to the digestion of CMC.

**Fermentation Flasks**

The nutritional studies were carried out using 500 milliliter round bottom flasks. The media were prepared anaerobically as previously described. After additions to the media were made and the pH adjusted, an aliquot was withdrawn and pipetted into a sterile rubber stoppered test tube. This aliquot was incubated along with the culture flask and served as an uninoculated control for any change in viscosity of the CMC medium during incubation. Sterile 0.85 per cent saline was added to the control tube to compensate for saline used in the inoculum.

Earlier studies had shown that very little change occurred in the viscosity of CMC media during incubation. However, at the time of inoculation of the flasks an aliquot was withdrawn and a viscosity determination was made of the aliquot. If any variation existed between the viscosity of this zero time control aliquot and the uninoculated incubated aliquot, an average of the two was taken and this figure used as the control value for determining viscosity change of the inoculated medium.

After a medium was inoculated and an aliquot
withdrawn, the solid rubber stopper was replaced by a sterile double stopper. The double stopper consisted of a stopper bored to accommodate a smaller serum stopper. A thin film of silicon grease was used to maintain an air tight seal between the tightly fitting stoppers. The double stopper was wired into place in the neck of the flask. By this double stopper arrangement it was possible to withdraw a sample from the flask without opening the flask. When a sample was removed from the flask a sterile syringe and needle was employed and carbon dioxide was injected into the flask to compensate for the volume of medium removed. Carbon dioxide to fill the syringe was obtained by passing the gas through a column of sterile glass wool. Aseptic technique was used throughout the above procedures. Incubation of the flasks was at 40°C.

Viscosity Determinations

Werkman and Stahly (1933) had shown that the liquefaction of gelatin could be quantitated by use of an Ostwald viscometer. A modification of the method used by these authors proved to be very satisfactory in this investigation. The constant temperature water bath employed was essentially the same as that described by those authors and the viscosity change with incubation was expressed as a ratio as described in their publication. The change in viscosity of the medium is
obtained by dividing the outflow time in the viscometer after incubation by the outflow time before incubation.

In the present investigation the determinations were made at 38 C using an Ostwald viscometer which was blown to drain water in 40 seconds.

**Rumen Fluid Used in One Per Cent CMC Media**

It is necessary in viscosity determinations to use a medium free of any debris which would plug up the narrow capillary of a viscometer. Rumen fluid clarified by centrifugation at 26,000 rpm in a Sharples super centrifuge was found to be suitable for such a medium.

**Isolation Technique of Hungate**

Several methods were employed in attempts to obtain a pure culture of a cellulolytic bacterium. The most extensively used method was that of Hungate (1950) which is amply described in his review article. Briefly this method involves making dilutions of a rumen sample in an agar medium containing rumen juice, salts, and acid digested cotton which has been pulverized by grinding in a ball mill for 72 hours. The agar dilution tubes are incubated until colonies appear. The cellulolytic organisms are detectable by the presence of a halo which is present surrounding the colony. The halo results from the finely ground cellulose having been digested.

Capillary pipettes were utilized in one modification
of Hungate's method. After the dilutions were prepared a small amount of agar from each dilution tube was drawn into a sterile capillary pipette. The pipette was then placed in a sterile tube which was flushed with carbon dioxide and a rubber stopper was used to close the tube. After an incubation period of a week to ten days the capillary pipettes were examined for the presence of colonies surrounded by haloes.

Hungate's technique was also extended in the following manner. After duplicate dilutions of the sample were made in the cellulose agar medium in the ordinary manner, the contents of one of the series of dilution tubes were poured into petri plates. These plates were placed in a Smillie Jar which was then flushed with carbon dioxide. The tubes were rotated as the agar was poured so that a thin film of agar remained to coat the interior of the tube. The tube was flushed with carbon dioxide and the rubber stopper was tightly re-inserted. The tubes and plates were incubated at 37 C for two weeks before an examination was made for cellulytic colonies.

An enrichment technique was attempted using cellulose as the only carbohydrate and ammonium sulfate as the only nitrogen source. The medium was that of Hungate (1950) devoid of rumen juice and agar.
Filter Paper Disc Method

A sample was collected and diluted by serial ten fold dilutions in the rumen juice agar. The dilution tubes were kept at 45 C in a water bath. Approximately 10 milliliters of a sterile 2 per cent agar solution containing sodium thioglycollate, pH 7 phosphate buffer, and methylen blue were added to large 22 mm diameter rubber stoppered tubes which were then quickly cooled in an ice bath. One such tube was prepared for each dilution of the sample. Sterile forceps were used to place a sterile filter paper disc on top of the hardened agar in each tube. Medium from each dilution tube was then pipetted onto the filter paper disc in each tube so that the disc was just covered. When this agar had hardened a second 10 milliliter portion of the buffered 2 per cent agar was added and the tubes tightly stoppered. Aseptic technique was followed throughout and the tubes were continually flushed with carbon dioxide while the pipetting operation was in progress.

After the dilution of the sample was pipetted into the tube containing filter paper, the remaining dilution medium in the original tube was poured into a petri dish and allowed to harden. Sterile forceps were used to place a sterile filter paper disc of 70 mm diameter on top of the agar. The filter paper was lightly rubbed with the forceps to assure adhesion to the medium. The lid of the
dish was then replaced with the bottom half of a plate which had just been streaked with a 24 hour broth culture of *Serratia marcescens*. The two petri dish bottoms were then sealed together with parafilm and incubated with the *Serratia*-streaked plate on the bottom. Incubation was at 37°C for a period of two weeks.
RESULTS

Attempts at Isolation of Cellulolytic Bacteria

Hungate's Method. - The method of Hungate for isolating cellulolytic bacteria was attempted several times with different samples from the fistulated sheep and was found to be unsatisfactory. Most of the difficulty encountered in this technique arose from the fact that only a very small portion of the culture in the deep tube was accessible for isolation. Due to the presence of rumen fluid in the medium only a few millimeters at the top of the column of agar was penetrated by light. Most of the agar in the tubes was too opaque to make possible the detection of single well isolated colonies even when a dissecting microscope was employed to examine the tubes.

Roll Tube Method. - The roll tube method was adopted because of the difficulty encountered with Hungate's Method. The plates prepared from the roll tubes were not satisfactory due to the fact that the finely ground cellulose settled to the bottom of the plate before the agar had hardened. The layer of cellulose on the bottom of the plate made the detection of isolated colonies impossible.

By the roll tube method and with the aid of a dissecting microscope it was possible to detect the presence of isolated colonies in the thin agar film.
Many of these colonies were of so small a size as to be invisible to the unaided eye. Hungate advises the use of a dissecting microscope for spotting cellulolytic colonies since many very small colonies may be present adjacent to the desired colony. He used sterile capillary pipettes and the halo criterion for isolating pure cellulolytic colonies from his deep dilution tubes.

Due to the smallness of the colonies and their location on the sides of the tubes, picking with a capillary pipette was found to be very difficult. An ordinary inoculating needle, filed to a point and bent into a right angle about three millimeters from the end was used to pick the colonies. Each tube was first examined under the dissecting microscope at a 24X magnification. Single isolated colonies that seemed to be cellulolytically active by the halo criterion were circled with a wax pencil. The colonies were picked while being observed under the dissecting microscope and were transferred to rumen juice agar slants. Approximately 40 single colonies were thus isolated.

a) Testing of isolates for activity. - After the transferred colonies had grown out as anaerobic slant cultures they were tested for cellulolytic activity by transferring to two different media containing cellulose. One of these was the same as that used for transferring mixed cultures and contained ball-milled cellulose. The
other was a medium in which rumen juice was replaced by 130 milligrams of yeast extract per 100 milliliters of medium. This medium also contained ball-milled cellulose. Use of these two media served a dual purpose. First of all, the rumen juice medium was utilized to detect cellulolytic activity. Secondly, by using the clarified yeast extract medium it was possible to determine viability of the organisms since some of the isolates grew very slowly on the slants. The cloudiness of a rumen juice medium makes such viability checks difficult. Also it was desirable to know if cellulose digestion would proceed in a medium where yeast extract replaced rumen juice.

The cultures were examined periodically for indications of cellulose breakdown. To determine whether or not digestion had occurred in the tubes, the level of cellulose was compared to an uninoculated control. The level of cellulose in the bottom of the tubes was marked with a wax pencil since some settling of the cellulose occurred during the long incubation period. Tubes inoculated with actively cellulolytic mixed cultures served as checks on the adequacy of the media.

None of the isolates picked by the halo criterion proved to be capable of the digestion of cellulose after an incubation period of four weeks at 37 C.

Capillary Pipette Method. - The capillary pipette
method for obtaining cellulolytic isolates was no more profitable than was the roll tube method. It was felt that since each colony would be more isolated in a capillary tubing the formation of a halo would be more obvious. The presence of haloes was no more detectable around the discrete colonies formed than was the case with colonies in the roll tubes.

**Enrichment Medium.** - The mixed culture transferred in the enrichment medium, containing cellulose as the only carbohydrate, digested the substrate at a very slow rate. After incubation for two weeks, approximately half of the cellulose had been digested and the culture was transferred to a fresh medium. A six weeks incubation period was required before cellulose digestion was again detectable. No digestion occurred after three months incubation when this culture was transferred for a third time. This approach to obtaining pure cultures of cellulose-digesting organisms was abandoned.

**Filter Paper Disc Methods.** - In the filter paper disc method the digestion of the discs would be more likely to be evident than when finely ground filter paper or Solka Floc was present in the agar deep tubes. The filter paper discs and agar in the tubes could be blown out into a sterile petri dish by using sterile capillary pipettes and carbon dioxide, and the agar separated at the filter paper "joint". The colony in the center of digested areas
on the filter paper could then be transferred to a liquid cellulose medium. It was assumed that this method would provide better anaerobic conditions than the method of sealing plates together whereby the rapidly growing *Serratia* utilizes all free available oxygen in the plates and permits the growth of anaerobic organisms. As it turned out the plate method provided a low enough oxidation-reduction potential to allow the growth of cellulolytic anaerobes and was actually the more convenient method.

In the deep tubes containing discs of filter paper, the paper was almost completely digested in the $10^{-1}$ and $10^{-2}$ dilutions. In dilutions higher than these, no areas of cellulose digestion could be observed on the discs.

On the plates several discrete areas of digestion were evident on the filter paper at the $10^{-1}$ and $10^{-2}$ dilutions. Five such digested areas were transferred with a wire loop to the transfer medium. The organisms from two of these five areas proved to be cellulolytic in the liquid medium. These mixed cultures were transferred each time disintegration of the filter paper became obvious. Several experiments were carried out in which dilutions of these mixed cultures were made in rumen juice agar. These dilutions were plated, sterile filter paper discs added, and the dilution plates were sealed together as before with freshly streaked *Serratia* plates.
Twenty-four, thirty-six, forty-eight, and seventy-two hour cultures were plated in these experiments. At the time of plating no evident digestion of cellulose had occurred in the 24 hour cultures; in the 72 hour cultures the filter paper in the tubes had been attacked to the point of falling apart and had settled to the bottom of the tubes.

In spite of the fact that the mixed cultures were originally isolated by this technique, no digestion of the filter paper occurred in the experiments described above. After an incubation period of three weeks, the growth was scraped from the agar and re-inoculated back into the transfer medium. No digestion of filter paper occurred in the liquid medium.

It seemed likely from these studies and from the literature on the subject that the cellulolytic population of the rumen is relatively low in comparison to other types of organisms. Accordingly, a mixed culture capable of cellulose digestion was passed in the transfer medium containing filter paper in the likelihood that the cellulolytic population would be enlarged. The experiments to be described were made using such a culture.

Heat Susceptibility Method. - Certain species of Clostridium have been shown to digest cellulose (Cowles and Rettger, 1931; Hungate, 1944b). Since the mixed cultures contained some spore-forming rods as determined
by staining, the possibility existed for the separation of cellulolytic organisms on the basis of heat susceptibility. Sub-cultures were made of the original mixed culture and these were heated at 80°C for periods of from one to fifteen minutes. These tubes were then incubated and examined periodically for one month. In those tubes in which organisms grew, no digestion of cellulose occurred.

Dilution Methods. - After the mixed culture had been transferred several times, a dilution method was employed in an attempt to isolate pure cultures from what was hoped to be a predominantly cellulolytic population. One loopful of the mixed culture was transferred to a tube of liquid medium containing only minerals and glucose. Two loopsful were transferred from this tube to a second tube, three loopsful from the second tube were transferred to a third tube, etc. until ten tubes were prepared. After incubation, medium from the last tube which exhibited growth was plated out on rumen juice agar and gram stained as a purity check. This procedure was repeated several times and nine pure cultures were obtained. Six of the isolates were gram negative rods, the others gram positive coco-bacilli. None of the isolates was cellulolytic.

A similar dilution experiment was carried out using the liquid transfer medium containing filter paper
cellulose as the substrate. In this experiment 0.5 ml of the mixed culture was transferred to the first tube of the transfer medium and this amount was used in preparing ten serial dilutions. In addition, from each of the dilution tubes 0.5 ml was also plated out on three different types of media and the plates were sealed together with freshly streaked Serratia plates as described earlier. The three plating media were rumen juice agar medium, the same medium containing 1.5 per cent soluble starch, and rumen juice agar medium containing 3.3 per cent Proteose-Tryptone Agar (Difco) in place of agar-agar.

That the mixed culture was not composed of predominantly cellulolytic organisms was indicated by the fact that growth occurred on all the plates of the dilution series while digestion of the filter paper occurred in only the first seven tubes. The plates prepared from tube seven were used to isolate colonies. All morphologically different colonies from the three media were picked and inoculated into the liquid transfer medium containing filter paper. Of the 18 isolates selected, none was cellulolytic nor was there any digestion of cellulose when all were inoculated together into a single tube of transfer medium.

Millipore Filter. - The possibility of utilizing the Millipore Filter for isolation of cellulolytic organisms was investigated. Since the filtrating membrane is
cellulose acetate it was logical that cellulolytic organisms
might attack this type of cellulose, thereby providing a
rapid and simple technique for obtaining pure cellulolytic
cultures. Hope of this approach was abandoned early when
it was determined that the mixed cultures would not digest
the filtering membrane when it was incorporated as the
cellulose substrate in the transfer medium.

CMC-Agar Plating Method. - The mixed culture which
was capable of digesting filter paper cellulose also
digested the soluble cellulose (CMC). Various levels of
CMC and agar were combined in an attempt to resolve the
minimal amount of agar necessary in combination with CMC
which would provide a solid medium. Three per cent CMC
combined with 0.5 per cent agar suited this purpose and
for plating experiments these concentrations were sub-
sequently used in the transfer medium. When the mixed
culture was stabbed into this medium contained in deep
tubes, liquefaction was produced after an incubation
period of five days.

The solid medium provided by using the above
concentrations of CMC and agar appeared to be an ideal
medium for the isolation of cellulolytic organisms.
Using 0.5 ml as the transfer volume, serial dilutions were
prepared of the mixed culture. The tubes containing
approximately 10 ml of melted medium were kept at 45 C
under carbon dioxide as the dilutions were prepared.
when all the dilutions were completed the contents of the tubes were poured into plates and the glass lid of each plate was replaced with a sterile porous clay lid. The plates were carefully placed in a Smillie Jar which was quickly sealed with plasticine. The Smillie Jar, which is covered by a lid containing an inlet and outlet tube, was then flushed for 15 minutes with carbon dioxide. By working rapidly and allowing the plates to harden while under an atmosphere of carbon dioxide it was possible to maintain anaerobic conditions in the plates.

The plates were incubated for two months at a temperature of 40 °C. Liquefaction was evident in the $10^{-1}$ and $10^{-2}$ dilution plates but no liquefaction was apparent around well isolated colonies present in the high dilution plates.

**Determination of Cellulolytic Activity by Viscosimetric Methods**

The study of Werkman and Stahly (1933) suggested the possibility that some of the well isolated colonies were digesting the CMC at a rate too slow to cause liquefaction of the medium. In their studies of gelatin liquefiers they demonstrated that some of the organisms which did not liquefy gelatin in a stab culture were nevertheless capable of gelatinolysis as indicated by viscosity determinations. Accordingly, another set of dilution plates was prepared and all of the individually isolated colonies
present at the high dilutions were picked and transferred to CMC-agar deep tubes. When growth was evident in these stabs the culture was transferred to the liquid mixed culture medium containing one per cent CMC. These cultures were incubated for one week before viscosity determinations were made on the culture medium. An uninoculated tube of CMC medium which was incubated for the same period of time served as a control.

None of the 11 isolates produced liquefaction of the CMC-agar medium. However, two of these eleven isolates caused a considerable decrease in the viscosity of the liquid medium. As a purity check these two isolates were plated out on CMC-agar. Neither of the isolates was pure and only one of the several colonies picked from the high dilutions of these two cultures digested CMC when viscosity determinations were made. Although plating and gram staining revealed this culture to be pure it was only weakly cellulolytic, reducing the viscosity of the one per cent CMC medium to 0.9 that of the control. This is to be compared to a value of 0.5 obtained from the parent culture from which the organism was isolated. Incubation was for one week in both instances.

**Nutritional Studies**

A convenient tool for the study of cellulose degradation was provided by following the change in viscosity of a one per cent CMC medium which had been
inoculated with cellulolytic organisms. It had become increasingly apparent that a better knowledge of some of the basic nutritional requirements of the cellulolytic organisms present in the mixed culture was of primary importance if these organisms were to be isolated as pure cultures. With this in mind a study was undertaken to determine which of the constituents of the transfer medium were stimulatory to the process of soluble cellulose degradation. A search of the literature revealed that no such study had been made and it was felt that while the investigation might not provide a medium for isolating cellulose digesters, it would supplement the existing knowledge of the factors involved in or necessary for cellulose breakdown by rumen microorganisms.

E__ffect of Yeast Extract. - The medium for nutritional studies listed under "Materials and Methods" served as a basal medium. A 25 per cent yeast extract solution was prepared and sterilized by autoclaving. Three milliliters of this solution was added to one hundred and fifty milliliters of the freshly prepared basal medium giving a final concentration of five tenths of one per cent. A third batch of medium from which rumen juice was omitted contained the same concentration of yeast extract. The flasks of media were inoculated and viscosity determinations were made on withdrawn aliquots as described earlier. The results are given in
Figure 2. In the concentration employed, yeast extract cannot completely replace rumen juice. However, the digestion of CMC in the medium containing yeast extract, but no rumen juice, closely approximated the curve obtained for the basal medium which contained only rumen juice. When yeast extract was added to the basal medium a significant increase in the rate of cellulose digestion was apparent.

Effect of Glucose. - Figure 3 shows the effect of adding glucose to the basal medium containing yeast extract. Although the effect is not great and is not obvious until approximately 20 hours of incubation, stimulation of cellulose digestion is afforded by the presence of glucose in a concentration of 0.15 per cent. This is in agreement with the findings of Hoflund, Quin, and Clark (1948) who determined that the in vitro breakdown of cellulose present as cotton threads was stimulated by the presence of glucose in concentrations of from 0.1 per cent to 0.2 per cent. No attempt was made in the present study to determine that level of glucose which gives optimal digestion of cellulose.

Effect of Volatile Fatty Acids. The importance of certain fatty acids in the digestion of cellulose has been evidenced by the studies of Bentley and his colleagues (1954b, 1955). The rate of cellulose digestion was first shown by these investigators to be rapidly
Figure 2. The Effect of Yeast Extract Upon the Rate of CMC Degradation as Determined by Viscosity Changes
Figure 3. The Effect of Glucose Upon the Rate of CMC Degradation as Determined by Viscosity Changes

A. Basal rumen juice medium
B. Basal rumen juice medium plus yeast extract
C. B plus glucose
increased by the addition of volatile fatty acids as valeric and caproic. This stimulation by fatty acids was substantiated by the findings of Bryant and Doetsch (1955) who found that C₅ to C₈ straight chain acids in conjunction with a branched-chain acid were required for the growth of Bacteroides succinogenes, a cellulolytic anaerobe of the rumen.

Valeric, butyric, and iso-butyric acids were tested in the basal medium to which glucose and yeast extract had been added in the concentrations mentioned above. Separate batches of media were prepared containing per 100 ml of medium; 32 mg, 28 mg, and 14 mg respectively of valeric, butyric, and iso-butyric acids. These same amounts of the three acids were also added collectively to another batch of medium. Each acid alone was found to be stimulatory to the digestion of CMC. Butyric acid was slightly more stimulatory than were either valeric or iso-butyric and only the curve for butyric acid is shown in Figure 4. No additive effect over that of butyric alone was noted when all three acids were present.

Effect of Biotin and p-Aminobenzoic Acid. - Throughout the nutritional study each experiment was verified at least once by repetition of that experiment. Biotin and p-ABA at concentrations of 20 and 50 micrograms per 100 milliliters of basal medium containing
Figure 4. The Effect of Butyric Acid Upon the Rate of CMC Degradation as Determined by Viscosity Changes
yeast extract, glucose, and butyric acid gave inconsistent results in repeated experiments. In view of this fact it seemed logical that a more nearly synthetic medium would be useful in this and subsequent experiments. Sijpsteijn (1951) had successfully used yeast autolysate as a replacement for rumen juice in his studies with *Ruminococcus flavefaciens*. Since it had been determined that the digestion of CMC was nearly the same when either yeast extract or rumen juice alone was present in the medium, rumen juice was replaced by yeast extract in the basal medium. The two vitamins were again tested separately and together in the concentrations used originally. Figure 5 shows the results of this experiment.

**Urea as a Replacement for Yeast Extract.** - Yeast extract contains the B complex vitamins of which biotin is a member. In order to get a clearer picture of the effect of this vitamin and to determine the effectiveness of urea in substituting for yeast extract as a nitrogen source an experiment was done in which urea at a concentration of 0.17 per cent was employed in the basal medium in place of yeast extract. The urea stock solution was sterilized by Seitz filtration and added to the autoclaved and cooled medium. Very little digestion of CMC occurred in this experiment until after 35 hours of incubation. Essentially the same stimulation with biotin was obtained as when yeast extract was employed as the
Figure 5. The Effects of Biotin and p-ABA Upon the Rate of CMC Degradation as Determined by Viscosity Changes

- A. Basal yeast extract medium plus glucose and butyric acid
- B. A plus biotin
- C. A plus p-ABA
- D. A plus biotin and p-ABA
nitrogen source. However, when p-ABA was present either alone or with biotin the result was less digestion than in the basal medium which contained neither vitamin. The results are given in Table I. Because of the very slow rate of cellulose digestion further experiments with urea did not seem feasible.

**Effect of Trace Elements.** - It was of interest to know if the organisms responsible for the digestion of CMC had a trace element requirement. To determine this 1.5 ml of the trace elements solution was added to 150 ml of the basal medium to which had been added the amounts of glucose, butyric acid, biotin, and p-ABA previously found to be stimulatory. This medium will henceforth be referred to as the basal yeast extract medium.

Very little difference was noted between the basal yeast extract medium and the same medium containing trace elements (Table II). No definite conclusion could be drawn as to the effect of trace elements since it is possible that other components of the medium contained enough trace elements to supply the requirements of the cellulolytic organisms. It can only be stated that the trace elements were of no value in stimulating cellulose digestion.

**Effect of Omission of Sodium Carbonate.** - Throughout these studies sodium carbonate was added routinely to the media since it has been the experience of most
<table>
<thead>
<tr>
<th>Additions to Basal Urea Medium</th>
<th>Hours of Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Glucose / Butyric Acid</td>
<td>1.0*</td>
</tr>
<tr>
<td>Glucose / Butyric Acid</td>
<td>1.0</td>
</tr>
<tr>
<td>/ Biotin</td>
<td></td>
</tr>
<tr>
<td>Glucose / Butyric Acid</td>
<td>1.0</td>
</tr>
<tr>
<td>/ p-ABA</td>
<td></td>
</tr>
<tr>
<td>Glucose / Butyric Acid</td>
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</tr>
<tr>
<td>/ Biotin / p-ABA</td>
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</tr>
</tbody>
</table>

*Viscosity Change.
TABLE II

The Effect of Trace Elements upon the Rate of 
CMC Degradation as Determined by Viscosity Changes

<table>
<thead>
<tr>
<th>Hours of Incubation</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
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</thead>
<tbody>
<tr>
<td>Basal Yeast Extract Medium</td>
<td>1.0*</td>
<td>0.82</td>
<td>0.75</td>
<td>0.67</td>
</tr>
<tr>
<td>Basal Yeast Extract Medium ∆ Trace Elements</td>
<td>1.0</td>
<td>0.85</td>
<td>0.73</td>
<td>0.65</td>
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</tbody>
</table>

*Viscosity Change.
investigators in the field that cellulose digesting organisms from the rumen have a definite carbonate requirement. Basal yeast extract medium was prepared from which sodium carbonate was omitted. The medium was adjusted to pH 7 after autoclaving by adding an appropriate amount of sterile sodium hydroxide. This medium and basal yeast extract medium containing carbonate were inoculated and viscosity determinations were made in the usual manner. Very little digestion of CMC occurred in the medium from which carbonate was omitted indicating a requirement for this compound (Table III).

**Effect of Salts.** - A number of experiments were conducted employing the trial and error method of investigation and involving calcium chloride, magnesium sulfate, sodium chloride, ammonium sulfate, and ferrous sulfate. Concentrations of the salts were the same as in the transfer medium.

In preliminary experiments the salts were individually omitted from the basal yeast extract medium. With the exception of calcium chloride, none of the salts had any marked effect on cellulose digestion. When the calcium salt was omitted from the medium a pronounced drop in the rate of digestion was noted. Calcium chloride alone was found to give better digestion than when all five salts were present and it seemed logical to add
### TABLE III

The Effect of the Omission of Sodium Carbonate upon the Rate of CMC Degradation as Determined by Viscosity Changes

<table>
<thead>
<tr>
<th>Hours of Incubation</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Yeast Extract Medium</td>
<td>0.98*</td>
<td>0.86</td>
<td>0.80</td>
<td>0.75</td>
</tr>
<tr>
<td>Basal Yeast Extract Medium Less Sodium Carbonate</td>
<td>0.99</td>
<td>0.92</td>
<td>0.90</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*Viscosity Change.*
each salt to a medium containing only calcium in order to determine if the rate of digestion could be increased over that obtained with calcium alone. None of the other four salts provided such an increase in the rate of digestion. The addition of ferrous sulfate resulted in the same rate of digestion as was the case when calcium chloride was the only salt. The addition of either magnesium sulfate or ammonium sulfate gave a slight drop in the rate of digestion. Sodium chloride caused a much greater drop in digestion. The results are given in Figure 6.

Table IV shows the viscosity values for the curves plotted in Figure 6 as well as those values obtained for the other salts discussed in the foregoing paragraph.

Effect of the Concentration of Calcium. - Since the addition or omission of calcium had such a marked influence on the rate of cellulose digestion a study of the amount of calcium necessary for maximal digestion was indicated. Graded amounts of calcium were added to the yeast extract basal medium. The results of this study are given in Figure 7. Maximal cellulose digestion is seen to occur at a concentration of 3.25 mg per 100 ml of medium. No increase in the digestion rate occurred when the calcium level was increased beyond this amount. The level of calcium chloride necessary for optimal
A. Basal yeast extract medium plus glucose, butyric acid, biotin, and p-ABA
B. CaCl$_2$ as the only salt
C. CaCl$_2$ plus NaCl as the only salts

Figure 6. The Effects of CaCl$_2$ and NaCl Upon the Rate of CMC Degradation as Determined by Viscosity Changes
### TABLE IV

The Effect of the Addition of Various Salts to a Basal Yeast Extract Medium upon the Rate of CMC Degradation as Determined by Viscosity Changes

<table>
<thead>
<tr>
<th>Salts Contained in Basal Yeast Extract Medium</th>
<th>Hours of Incubation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td>All five salts present</td>
<td>1.0*</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl₂ / FeSO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl₂ / MgSO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl₂ / (NH₄)₂SO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl₂ / NaCl</td>
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</tr>
</tbody>
</table>

*Viscosity Value.
Figure 7. The Effect of the Concentration of Calcium Chloride Upon the Rate of CMC Degradation as Determined by Viscosity Changes

A. Basal yeast extract medium plus glucose, butyric acid, biotin, and p-ABA. No salts.
B. A plus 1.75 mg. or 2.5 mg. CaCl₂ per 100 ml. medium
C. A plus 3.25 mg, 5.0 mg, or 6.25 mg. CaCl₂ per 100 ml. medium
cellulose digestion was found to be between 2.5 mg and 3.25 mg per 100 ml of medium.

**Plating on Yeast Extract Basal Medium**

The yeast extract basal medium containing the substances found to be stimulatory to cellulose digestion and calcium in a concentration of 3.25 mg per 100 ml of medium was then used in an attempt to isolate pure cultures of cellulolytic bacteria. The mixed culture was transferred several times in the liquid medium and then plated out on the same medium containing 3 per cent CMC and 0.5 per cent agar as described in another section. All the isolated and morphologically different colonies were fished from the plates and inoculated into deep stab tubes. When the isolate had grown out as a stab culture it was tested for cellulolytic activity in the liquid medium. All of the single isolates were also tested together as a mixed culture by transferring each from the stab culture to a tube of the liquid medium. None of the isolates was cellulolytically active either as a pure culture or together in any combination with all the other isolates.
DISCUSSION

Lack of success using the isolation technique of Hungate was discussed in another section. No ready explanation can be offered for the failure to isolate cellulolytic organisms by this method since the technique as described in the literature was adhered to rigidly. That this is not an uncommon experience was verified by Bryant (1957). He cited two specific examples whereby investigators used this technique repeatedly for periods of time up to one year or longer before success was achieved. He states that success is dependent upon manipulations which are readily mastered when observed in the hands of one who has been successful with the method.

A certain amount of speculation seems justifiable from the results obtained in attempts to isolate a cellulolytic organism from the rumen and mixed cultures. An appreciable amount of evidence can be cited which points to the possibility of a synergistic effect being in operation which makes isolation of the cellulolytic organisms very difficult if not impossible in view of our present lack of knowledge of these organisms.

One such piece of evidence was the experiment conducted using the filter paper disc-Serratia plate method. In spite of the fact that the actively cellulolytic mixed cultures were originally isolated by this
technique, no lysis of filter paper occurred in subsequent experiments when these cultures were again plated. After an incubation period of three weeks, the growth was scraped from the agar plates and re-inoculated into a liquid medium. No lysis of filter paper occurred in the liquid medium. This suggested at least three possibilities.

First of all, it has been reported in the literature that agar may be inhibitory to cellulolytic organisms. Secondly, it may be simply a case of the cellulolytic organisms not developing on the agar plates due to lack of sufficient anaerobiosis. A third and seemingly the most likely possibility is that the breakdown of cellulose is a joint effort involving more than one organism. Not only might it be a case of the right combination of different organisms, but correct relative proportions of these different participants.

That agar was inhibitory in these experiments is unlikely since the mixed cultures were first isolated from agar. Addition of dry sterile agar to the liquid medium did not interfere with cellulolytic activity when this medium was subsequently inoculated with a mixed culture. This indicates that agar has no inhibitory action.

That anaerobiosis is the governing factor in the isolation of cellulolytic organisms likewise seems unlikely since the Serratia plate technique was adequate for the original isolation of the mixed culture.
Pointing to the possibility that cellulolysis is a joint effort is the fact that lysis on the filter paper discs occurred only in the low dilutions of samples taken from the sheep and could not be demonstrated in later experiments with the use of mixed cultures. This seems to indicate that correct proportions of certain organisms are necessary for lysis to occur. Samples taken directly from the sheep are more likely to give the correct proportions than is a mixed culture. Also, direct sheep samples are more likely to contain a necessary factor(s) than is an aliquot from a mixed culture where only a few types (relatively speaking) of rumen organisms are present.

When the mixed culture was diluted in the liquid transfer medium containing cellulose an interesting observation was made. After incubation of these tubes, digestion of the filter paper occurred in the first two tubes and in the fourth and fifth tubes. The filter paper in tube three was not attacked to the point of digestion. This same type of observation was made on at least two other occasions. This evidence also points toward the fact that correct proportions of organisms must be present to effect a breakdown of cellulose.

Further evidence for a synergistic effect was obtained from the plating experiments dealing with 3 per cent CMC, 0.5 per cent agar mixtures. As with filter paper discs, digestion of cellulose was evident only at
the low dilutions. On plates containing well isolated colonies no liquefaction of the CMC-agar occurred. Only a small percentage of the isolated colonies demonstrated a cellulolytic power. As these were purified into constituent pure cultures the ability to digest CMC became weaker with each successive step of purification. The pure culture ultimately obtained was only weakly cellulolytic in the one per cent CMC medium.

The fact that colonies picked according to Hungate's halo criterion failed to digest cellulose can be explained on the basis of purity. By picking such colonies with the aid of a dissecting microscope only well isolated colonies were picked and these, being pure, could not digest cellulose. It is also likely that a very large number, perhaps thousands of colonies must be picked in order to insure success in finding three or four which are cellulolytic and in reality the halo criterion is without any real merit since a random picking of colonies from an anaerobic plate would serve the same purpose.

In reference to the synergistic effect discussed above, admission must be made to the fact that at no point in this investigation could synergism be proved. When the mixed culture was plated out and all the morphologically different colonies picked and tested, none of the isolates was active by itself nor was any digestion
evident when all the isolates were combined. Success with this type of experiment would have supplied strong support if not absolute proof to the theory of synergism. On the other hand, failure to demonstrate the phenomenon of synergism certainly does not eliminate the possibility of its existence. By picking only those colonies of different morphological characteristics one or many different organisms of identical colony morphology may be overlooked and again it may be a question of recombining in such a manner as to achieve the correct relative proportions which were present in the original active mixed population.

Evidence is not lacking for the existence of synergism as a requisite for cellulose digestion. Pochon (1941) related a study made by Snieszko which indicated such a synergistic effect. Snieszko isolated a strictly anaerobic rod from a mixed culture but noticed that other organisms existed in the supposedly pure culture of this organism. The differences between the contaminants and the "pure" rod were slight and were manifested by dimensions of spores and the size of the rods. He felt the mixed culture consisted of three slightly different organisms and was successful in isolating two of these on ordinary media. The two organisms so isolated were non-cellulolytic, only the mixed culture was able to digest cellulose. Snieszko felt the organism he could
not isolate was the cellulolytic one, the other two being contaminants. Other such examples of impure cultures are cited by Pochon and by others and indicate the necessity for a synergistic relationship among organisms in order for cellulose breakdown to be accomplished. In order to determine the roles played by various organisms in this challenging and fascinating process many more extensive studies are required.

One of the more gratifying aspects of this investigation resulted from the success obtained in using CMC as a substrate. CMC provides a very convenient tool for measuring the rate of decomposition of cellulose. In addition since the hydrolysis of CMC by cellulolytic organisms occurs at a much faster rate than is the case with native cellulose, a CMC medium could serve as a type of screening medium for prospective cellulose decomposers even though, as was pointed out by King (1956), there is at present no known quantitative relationship between the hydrolysis of native cellulose and the hydrolysis of CMC.

In this study investigations were carried out on compounds known to be stimulatory to cellulose digestion. Amounts of these compounds were chosen to correspond with amounts found to be effective by other authors. This method was felt to be necessary in order to determine the basic requirements for the digestion of soluble cellulose by a mixed culture, the assumption being that with
such a medium it would be possible to isolate pure cellulolytic cultures. More complete studies need to be made of those substances found to be stimulatory since in most cases no attempt was made at quantitation. The effects given using the five salts was particularly interesting. Various combinations of these salts at different concentrations might reveal that an increased stimulation is possible. The inhibition by calcium plus sodium chloride is especially one phase which merits further investigation. Evidence from the present study suggests that the inhibition is due to a high level of chloride ion. The pH of the medium containing calcium chloride plus sodium chloride had been adjusted by adding a milliliter of normal hydrochloric acid. Also, inoculum for the media was prepared using 0.85 per cent sodium chloride so that a high level of chloride was present in the medium and could have brought about an inhibitory effect. How the chloride ion may have caused this effect is obscure.
SUMMARY

1. Several anaerobic techniques were employed in an attempt to isolate pure cultures of cellulolytic bacteria from the rumen. Repeated platings were necessary in order to obtain pure cultures; none of those obtained was capable of the digestion of cellulose.

2. A method was devised to facilitate the dispensing of media under anaerobic conditions.

3. Media containing three per cent soluble carboxymethylcellulose (CMC) combined with 0.5 per cent agar-agar were found to be ideal for the plating of rumen organisms. Such media provided a convenient material for plating organisms in the presence of cellulose substrate.

4. The digestion of CMC by mixed rumen cultures was determined by following the change in viscosity produced in a one per cent CMC medium.

5. A mixed culture obtained from the rumen of a fistulated sheep was transferred in a one per cent CMC medium. By adding various substances to a basal medium a study was made to determine which of the added factors was stimulatory to the digestion of CMC by this culture.
12. The incorporation of urea into a medium containing no yeast extract or rumen juice resulted in practically no decomposition of CMC.

13. Calcium chloride in a concentration of 2.5 mg per 100 ml of medium gave better digestion than when no calcium chloride was added to the medium. Calcium chloride alone gave better digestion than when this salt was present with four other salts commonly employed in media for culturing cellulolytic bacteria.

14. The concentration of calcium chloride necessary for the fastest rate of cellulose digestion was found to be in the range of from 2.5 mg to 3.25 mg per 100 ml of medium.

15. The possibility of cellulose digestion occurring only as a result of a synergistic action was discussed.
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I, Harvey Junior Stiffler, was born in Salem, Ohio, on August 24, 1925. I received my secondary school education in the public schools of Salem, Ohio. After graduation from high school I served with the U. S. Navy for thirty months during World War II. My undergraduate training was obtained at Kent State University, Kent, Ohio, from which I received the degree of Bachelor of Science in Education in June, 1950. In September of that year I was recalled to active duty by the Naval Reserve, and served fifteen months during the Korean War. Upon release from active duty I returned to school at Western Reserve University and received a Master of Science in Microbiology from that institution in 1954. While in residence at The Ohio State University I served in the capacity of graduate assistant and research fellow in the Department of Bacteriology.
6. An indication of the necessity for carbonate in the digestion of CMC by the mixed culture was obtained when sodium carbonate was omitted from the medium. Practically no CMC digestion occurred in the absence of the carbonate.

7. The addition of calcium chloride plus sodium chloride to a basal medium resulted in an inhibition of CMC degradation.

8. An increased rate of CMC digestion was obtained by the addition of valeric, butyric, or iso-butyric acids. Butyric acid was slightly more stimulatory than was either valeric or iso-butyric. A combination of all three acids in the medium gave no better digestion of CMC than did butyric acid alone.

9. Glucose in a concentration of 0.15 per cent was found to be stimulatory to the breakdown of CMC.

10. Biotin and p-ABA had only a slight stimulatory effect on the process of CMC breakdown in the complex media employed.

11. A medium containing yeast extract but no rumen juice gave nearly as fast a rate of CMC digestion as did a medium containing only rumen juice.