THE ANAEROBIC MESOPHILIC CELLULOLYTIC BACTERIA[1]

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Although much is known of the biology of many of the agents decomposing cellulose, the available information on the anaerobic bacteria is far from satisfactory because so few have been obtained in pure culture. Some of the strains studied were probably successfully isolated (18, 4, 5), but the techniques were indirect and the criteria of separation could not be rigorous, leaving some doubt as to culture purity. In other publications (10, 33), purity of strains is assumed on the basis of inadequate evidence, and attributes ascribed to the cellulolytic anaerobes may be due to contaminants. The difficulties and problems involved in the pure culture of cellulolytic anaerobes have been recently discussed by Sijpesteijn (38) and much of the pertinent literature reviewed.

In studying the symbiotic utilization of cellulose the need for pure cultures has been particularly evident and since 1941 this problem has received the attention of the author. Methods for pure culturing the cellulose bacteria have been developed and used for the isolation of numerous strains. The nature of the isolation procedure was indicated (13), but it was not described in detail. Subsequently some investigators have not achieved success with the method and it seems desirable to publish a more complete description. This is given in the first section of this paper.

The anaerobic cellulolytic bacteria which have been isolated fall into five categories: actinomycetes, thermophilic sporeformers, nonsporeforming rods and cocci, and mesophilic sporeformers. One species of actinomycete has been described (14). The thermophilic sporeformers have been studied by McBee (21, 22). Cultural and physiological characteristics of the other groups are presented in the following sections, and some of the literature on the mesophilic sporeformers is critically reviewed in an attempt to trace ideas rather than to include all publications. Considerations on the ecology of the cellulolytic anaerobes are discussed in the concluding section.

I. THE ISOLATION TECHNIQUE

Gases. Oxygen-free carbon dioxide, hydrogen, or nitrogen is obtained by bubbling the cylinder gases with low oxygen content through a chromous acid solution similar to that

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described by Stone and Beeson (39). Twenty-mesh metallic zinc (75 gm) is treated with 50 ml of 3N HCl and stirred vigorously for 30 seconds; then mercuric chloride solution (2.5 ml of a saturated aqueous solution diluted to 50 ml) is added and the mixture stirred for three minutes after evolution of gas ceases. The zinc is washed by decantation and placed in the gas washing bottle. Fifty gm of chromium potassium sulfate, $CrK(SO_4)_2 \cdot 12H_2O$, are dissolved in a minimal amount of distilled water. This solution is placed in the washing bottle and 10 ml of 5N sulfuric acid are added. The cylinder gas is passed through the bottle to displace all air. After standing 24 hours the slow evolution of hydrogen from the zinc reduces the chromium and the solution turns a brilliant clear blue. The reduction of the chromium can be speeded up by forcing the solution back and forth through the zinc several times.

The preparation effectively absorbs oxygen for weeks and even months if the gas contains only small quantities. This is usually the case with tank carbon dioxide and hydrogen. Tank nitrogen contains more oxygen and the absorbent is soon exhausted. In such cases hot reduced copper may be used instead of or preceding the chromous solution. Alkaline pyrogallol removes not only oxygen but also the carbon dioxide which may be required for the growth of some organisms and which stimulates many others. Bicarbonate-CO_2 buffers cannot be employed when pyrogallol is the absorbent.

Substrate. Finely dispersed cellulose is obtained by packing absorbent cotton into a liter Erlenmeyer flask containing HCl (270 ml conc. HCl diluted to 300 ml). Sufficient cotton is added to absorb all the acid solution. After standing at room temperature for 24 hours, the

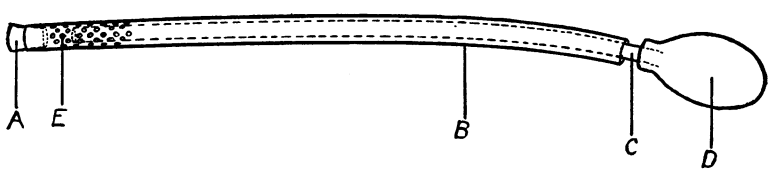

FIG. 1. Apparatus for removing rumen fluid from a fistula animal

fibers break up and a suspension of small particles is obtained. These are collected by filtration at reduced pressure, washed and air dried. This material (24 gm) is ground in a pebble mill with 600 ml water for 72 hours, giving approximately a 4 per cent concentration of very finely divided cellulose. Whatman filter paper (2 per cent) may be similarly ground without preliminary acid treatment, but it does not give as fine a suspension.

The concentration of cellulose used in an agar medium is usually 1.2 per cent if from cotton, or 0.6 per cent if from filter paper. In liquid cultures the concentration is usually 0.1 per cent.

When sugars are included in the medium they are first dissolved in water at 5 or 10 per cent concentration and filtered to remove all bacteria. This sterile solution is added aseptically to the medium after sterilization to give a concentration of 0.1 or 0.2 per cent.

Bovine Rumen Fluid. Rumen fluid has been found to be an essential culture ingredient for many bacteria. It may be obtained from a fistula animal or from one recently killed. In obtaining it from the fistula animal, the apparatus shown in figure 1 has been found convenient. B is a piece of Saran plastic tubing 10 mm inside diameter closed by a rubber stopper A. By perforating the tubing with a nail heated in the flame holes 2.5 mm in diameter are made along three inches of the tubing as shown at E. C is a piece of Saran tubing 6 mm in diameter which may be inserted so near the other end of B, and is supplied with a rubber bulb D. The plastic is sufficiently rigid so that it can be forced down between the rumen wall and the mass of ingesta until the perforated end dips in the rumen fluid. The latter is sucked into the bulb and transferred to a 500 ml flask. When the flask is completely filled, a rubber stopper is inserted without admission of air.

If the rumen liquid is to serve as an inoculum, it should be used as soon as possible after collection. If it is to be an ingredient of the culture medium, it should be refrigerated im-

mediately and the coarse particles allowed to settle out. The supernatant can be drawn off with a pipette as later described. It contains fine material in suspension, but the resulting cloudiness does not seriously impair its usefulness. In opening the flask of rumen fluid, oxygen-free gas should be passed into it, but stirring the sediment by bubbling the fluid should be avoided.

The Culture Medium. The final concentration of inorganic nutrients has been modified at different times during the course of the investigation and is not believed to be a highly critical factor. A successful combination contains 0.05 per cent $(NH_4)_2SO_4$, 0.05 per cent K_2HPO_4, 0.02 per cent KH_2PO_4, 0.005 per cent $CaCl_2$, 0.005 per cent $MgSO_4$, and 0.1 per cent NaCl. These and subsequent percentage compositions are based on the final volume.

The choice of buffer depends upon the gas used for anaerobiosis. When nitrogen or hydrogen is used, phosphate is the chief buffer and the concentration of the two phosphate salts is usually increased to a total of 0.5 to 1.0 per cent. The relative proportions of KH_2PO_4 and K_2HPO_4 are calculated from the dissociation constant and the pH desired. A pH of 7.0 has been found satisfactory for all the pure cultures which have been obtained. The most commonly used buffer combination has been pure carbon dioxide and 0.5 per cent sodium bicarbonate, which gives a pH of approximately 7.0.

The medium should contain a reducing agent and an oxidation-reduction indicator. Sodium thioglycolate (0.02 per cent), cysteine hydrochloride (0.01 per cent), and sodium sulfide have been used as reducing agents. The latter must be added after sterilization. Resazurin (0.0001 per cent) has been used as the oxidation-reduction indicator.

When liquid from the rumen is included in the medium extraneous reducing agents may be omitted, because the indicator therein often remains reduced if oxygen has been excluded throughout the process of preparation. Other media such as yeast extract, corn steep liquor, and to some extent dried grass, also reduce the medium whereas nutrient broth, proteose peptone, malt sprouts, and tryptone do not.

Triple distilled, or any other non-toxic, water (including tapwater) is suitable.

Preparation of Culture Medium. The details for the preparation of rumen fluid cellulose agar medium are as follows: 20 ml of an inorganic salt solution (same ingredients as given above, but two and one-half times as concentrated) are placed in a round bottom 100 ml pyrex flask. The agar is added and dissolved by boiling. Resazurin and the cellulose suspension (15 ml of the ground 4 per cent cotton cellulose) are added and the medium again boiled to drive off oxygen. At the same time CO_2 is bubbled through the mixture by means of a straight Pasteur pipette connected by rubber tubing with the gas source. The reducing agent (5 mg cysteine hydrochloride) and 15 ml of rumen fluid are then added without access of air.

A rubber stopper is held over the mouth of the flask while the gas is passing through it. As the pipette is withdrawn, the stopper is inserted without entrance of air and seated tightly. It is wired in place to prevent blowing out during sterilization. The round shape of the flask prevents breakage due to the sterilization pressure.

The exclusion of oxygen during preparation and sterilization of the medium permits use of less reducing agent than is required if air has free access. It is possible also that the rumen fluid may contain essential nutrilites which are destroyed by oxidation.

After sterilization, the flask is cooled to about 50 C under the tap without contaminating the stoppered portion. The wire is cut off and, as the stopper is removed, a straight Pasteur pipette with CO_2 passing through it via a rubber tube connection from one arm of a Y tube is inserted into the medium to minimize access of air. The outside of the pipette is sterilized by flaming. Sterile bicarbonate (2.5 ml of a filtered 10 per cent solution) is added, carbon dioxide is bubbled into the medium, and the flask shaken vigorously to aid in saturating the liquid and to break the surface gas bubbles which form. Equilibration for two minutes is usually sufficient to give a pH of about 7.0.

If sterile sugar solution or beef blood serum (1 ml) is to be included, it is added at this point.

Transferring Culture Medium with Minimal Access of Air. Empty culture tubes (16 x 150

mm Pyrex tubes without lip) are sterilized without cotton plugs and are closed with sterile rubber stoppers upon removal from the autoclave.

The medium is transferred as follows: A bent Pasteur pipette connected with the gas source via the second arm of the Y tube is inserted in the culture tube (see fig. 2) and all air flushed out. A sterile cotton-plugged 10 ml pipette provided with a mouth suction tube is inserted into the gas above the culture medium in the flask and by gentle suction the air in the pipette is replaced with CO_2. The taste will indicate when this has been accomplished. The medium is then drawn in and 10 ml quickly transferred to the culture tube. The last drop is expelled but no air is blown through the pipette. The tube is stoppered, and kept at 45 C until all tubes for a dilution series have been prepared.

The insertion of the rubber stopper without entrance of air requires a certain amount of digital dexterity. It is accomplished by holding the tube between the palm and last two fingers of one hand and the stopper between the index finger and thumb of the same hand.

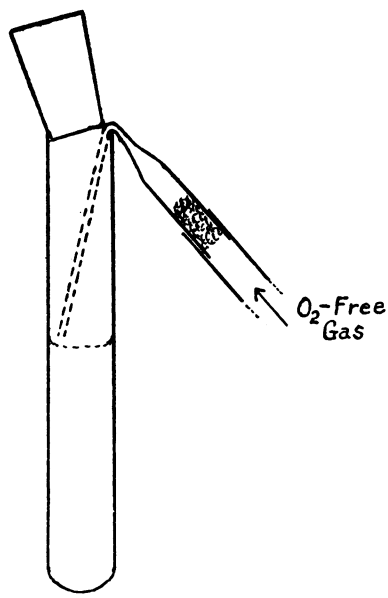

FIG. 2. Arrangement for preventing access of air to cultures

The stopper is inserted as the capillary is withdrawn (fig. 2). This manipulation may be, initially, somewhat awkward but becomes easy after a few transfers. The rubber stopper is finally tightly seated, using both hands and a rotary motion. If the medium is tipped so that the lower surface of the stopper is wetted after it is lightly inserted it can be seated more tightly and the film of agar which occupies the space between the stopper and the glass will effectively prevent the entrance of air. Culture tubes closed in this way have been found to remain anaerobic almost indefinitely. The reader should note that although the *absolute* exclusion of oxygen by means of a rubber stopper is not claimed or implied, any amounts gaining access are insufficient to change the color of the oxidation-reduction indicator.

Anaerobic Procedure for Inoculation and Dilution. In inoculating, the open end of a bent sterile capillary pipette is placed in the first dilution tube (as in fig. 2), extending 5 or 6 cm into the gas space above the medium. The stopper is stood upside down on a clean surface and left until it is to be reinserted. A second bent sterile pipette is inserted into the tube from which a colony is to be picked. The rubber tubing attached to this pipette should be long enough to allow the tube to be moved about while the colony is being picked. Gas is

passed through both tubes during the entire time that the stoppers are removed. The constant flow not only excludes oxygen but also reduces air contamination.

A Pasteur pipette which, after flaming, is cooled in the liquid to be inoculated, is used to pick a colony which is transferred to the first dilution tube. The stopper of the inoculum tube is flamed and reinserted as the bent capillary pipette is withdrawn. The bent capillary is flamed to the softening point and inserted into the second dilution tube. The first dilution tube is stoppered with minimal access of air and the contents thoroughly mixed. It is then opened and gassed again with the same capillary. A sterile 1 ml pipette with a fairly large opening in the tip is filled with the oxygen-free gas in the tube and 0.1 or 0.2 ml of this dilution transferred to tube 2. Tube 1 is closed aseptically with exclusion of air. The capillary is flamed thoroughly and used for tube 3. The dilutions are continued in this manner. It is helpful during these operations to have the tubes immersed to half their depth in a water bath which is maintained at 45 C.

The inoculated tubes are cooled under very cold running water. By repeatedly inverting them the medium is kept flowing over the inner surface of the tube and in constant motion until just before the agar is ready to set. This point must be ascertained through experience. The tubes are then momentarily inverted for the last time, forming a seal of medium around the stopper and leaving on the upper portion of the tube a thin film of cellulose agar which almost instantly solidifies. The tubes are held stationary in a slanting position under the tap until the rest of the agar has set. This gives all degrees of thickness of the agar, diminishing from the solid butt to a fraction of a millimeter at the top. The cultures may be incubated at temperatures up to 70 C. One to 1.5 per cent agar is satisfactory for incubation temperatures up to 40 C, but at higher temperatures 2 per cent agar is necessary (21).

Picking of Colonies. Colonies develop throughout the medium, but those in the thin agar adhering to the upper part of the tube are preferable for subculture because the cellulose-digesting colony can be better identified, and can be picked with less of the adjacent agar in which contaminants are present. The stopper is removed and gas is passed through the tube to be inoculated and also through the tube from which the colonies are to be picked. The selected colony is removed with a sterile Pasteur pipette, the tip of which is bent at a 90 degree angle and cut off squarely about 5 mm from the bend. A rubber mouth tube is attached to the capillary and the latter is flamed while blowing through it to prevent melting. It is cooled with the sterile medium in the tube to be inoculated and then filled with oxygen-free gas. The tip is placed over the colony and pressed in as gentle suction is applied to draw it into the capillary. It is highly desirable that the picking be performed under a binocular dissecting microscope because contaminating colonies may not be visible to the naked eye. Even if the microscope is not used during picking, it is advantageous to use it for a preliminary examination to detect the presence and location of contaminants.

Too much stress cannot be laid on the importance of picking only the cellulose-digesting colony with a minimum of surrounding agar. With certain types of cellulose bacteria, e.g., the actively cellulolytic rod found in the bovine rumen, it has not been possible to obtain a pure culture unless particular care was taken in the picking of the colony. The contaminating bacteria are often so numerous, even though dilutions in rumen fluid cellulose agar have been made through several transfers, that a sufficient preponderance of the cellulose digester is not obtained if much medium around the cellulolytic colony is transferred.

Modified Procedure for Less Sensitive Anaerobes. With the preceding technique it is possible to exclude oxygen sufficiently so that the oxidation-reduction indicator stays reduced throughout the operations of inoculation and dilution. Often a trace of oxygen gets in but it is absorbed by the reducing materials in the medium, giving the desired degree of anaerobiosis as indicated by the reduction of resazurin to the colorless state.

For less sensitive anaerobes such as *Clostridium* species, the culture medium may be transferred to the tubes in air and dilutions made without special precautions to avoid oxygen, provided the operations are performed quickly. After all the tubes have been inoculated, the air in them is replaced by oxygen-free gas. If too much exposure to air has not occurred, the cysteine or other reducing material reacts with the dissolved oxygen and

the resazurin decolorizes. However, exposure to air during preparation and inoculation occasionally results in failure of some of the tubes to show reduction and no growth occurs. Also, when a large number of parallel subcultures are inoculated from a single tube, more reliable results are obtained if procedures to exclude oxygen are followed during the entire process.

Criteria of Culture Purity. The following standard for culture purity has been used in isolating most of the bacteria described in the following sections. The organism must grow in solid cellulose medium and give isolated spots in which the cellulose is digested and in which the cellulose-digesting colony (or diffuse growth if no colony is formed) may be identified and picked. Picked colonies must give growth in solid cellulose medium when inoculated into dilution series and the numbers of colonies obtained must be approximately proportional to the number expected. From cellulose, the organism must be inoculated into a solid sugar or other clear medium in which all types of colonies which develop can be seen. A single colony in a high dilution must be subcultured in a second clear dilution series and this must be continued until two successive series give colonies of only one sort, the uniformity being determined not only macroscopically but also by microscopic examination. Any colony from this second sugar series must upon reinoculation into a solid cellulose medium give rise to cellulose-digesting colonies present in approximately the expected numbers in the various dilutions and containing cells microscopically similar to those in the sugar series. Cultures which successfully pass these tests under critical scrutiny are considered pure. In no case have any phenomena been observed which would suggest any impurity of cultures thus obtained, except that occasional chance contaminants have been encountered. These were eliminated by the procedure of the initial isolation.

The above are essentially the classical criteria for culture purity according to the plating method of Koch and differ only in that the organisms must be cultured not only upon cellulose agar but also upon a solid sugar medium. The reason that cellulose agar is included in the isolation procedure is to aid in obtaining pure cultures rather than as a criterion of purity. There are so many contaminating bacteria in a liquid enrichment of cellulolytic bacteria, even though cellulose is initially the only organic substrate, that after inoculating solid sugar medium the chance of picking a cellulolytic colony is slight, not only because the contaminants outnumber the cellulose organisms but also because the contaminants often grow faster in the sugar agar media. Dilution cultures in cellulose agar are necessary to assure the picking of a cellulolytic colony and to show whether the medium is satisfactory for growing the single cellulolytic cells separated by dilution. Unless the medium supports growth of an isolated cell from the impure cultures there is little chance that it will be adequate after purification.

Inoculation from cellulose agar into a sugar agar is a necessity because the opacity of the solid cellulose medium hampers the detection of contaminating colonies. Passage through at least two clear agar dilution series in which there are no indications of any second organism is a customarily accepted criterion of culture purity. It is this step in the isolation of cellulolytic anaerobes that presents the greatest difficulty and which in so many cases has led an investigator to believe that his "isolate" had lost its cellulose-digesting capacity. Actually a contaminating form morphologically quite similar to the desired organism was being picked.

Cellobiose is the preferred sugar substrate to be used in isolating cellulolytic

bacteria because all strains studied can utilize it. Some of the cocci from the rumen cannot utilize glucose, and McBee (21, 22) has shown that the thermophilic strains cannot grow on this monosaccharide. Undoubtedly other such strains exist.

Clausen (4) appears to be the only earlier investigator who has observed the formation of isolated cellulolytic colonies in solid medium. Though unsuccessful with streak plates, he obtained growth with poured plates if the atmospheric pressure was reduced below the vapor tension of water, and he noted the development of separate clear areas with a colony in each.

It is rather remarkable that so few other investigators have observed discrete colonies on cellulose agar. This is the chief factor which has prevented the application of classical isolation techniques. Explanations may be offered as follows: (*a*) The technique for anaerobiosis was inadequate. This caused growth to occur only at points of heavy inoculation and separate colonies were not distinguishable. (*b*) Surface streaking permitted confluence of growth through spreading. (*c*) Inoculations into pour plates and shake tubes were so heavy that numerous microscopic colonies developed and the clear area around each was unnoticed. The amount of cellulose in the solid medium was much greater than could be completely digested and consequently the culture gave no visible evidence that cellulose had been dissolved. (*d*) Failure to incorporate finely divided cellulose into a solid medium. Very few investigators have tried direct dilutions into solid cellulose media. One is led to suspect that a study of the literature has convinced most workers that only novel and untried methods could successfully yield pure cultures.

The present method involves no entirely new practices or techniques. A study of the literature discloses that all of the individual steps had already been tested and found useful during the very early periods of bacteriology. Thus, Liborius (20) used rubber stoppers with inlet and outlet tubes for air displacement. Roux (36) employed drawn out capillary pipettes for bubbling gases and incubated tubes on the side to give a thin layer of medium. Fuchs (9) passed hydrogen through the tube and then closed it quickly with a rubber stopper. Many early investigators suggested the use of reducing materials in the medium (reviewed by Kursteiner (19)).

Since these early workers reported success with relatively simple methods and apparatus available in most laboratories, it is surprising that during subsequent years such a variety of inadequate techniques and indirect methods designed to yield pure cultures of anaerobic cellulose decomposers have been developed. Perhaps, as stated by Roux (36), "Les descriptions des appareils même les plus simple donnent toujours l'idée de difficultés et de complications beaucoup plus grandes que celles qui exist réellement." Whatever may have been the factors leading to their discard, suitable combinations of the first developed techniques for direct isolation of anaerobic bacteria have led to the separation by the author and graduate and undergraduate students of more than twenty-five pure cultures of anaerobic cellulolytic bacteria. Most of the types encountered have been obtained in pure culture. In addition, purple and green bacteria, sulfate

reducers, an anaerobic spirochaete, and several obligately anaerobic bacterial contaminants have been isolated. Some of the cellulolytic strains have long been considered difficult or impossible to isolate by direct methods. In achieving success with these more difficult strains an implicit belief that the organism can grow in pure culture is often essential in order that isolation attempts be not abandoned during the more or less prolonged series of failures which may mark the path to a pure culture.

In addition to its success in yielding pure cultures, the present technique possesses the following advantages. Cultures may be repeatedly examined for growth, colony type, zones of cellulose digestion, contaminants, or other features, without opening the culture tube. If necessary to open it, sufficiently anaerobic conditions may be maintained until the tube is restoppered. The method is particularly adapted to slow-growing organisms. The tubes remain sufficiently anaerobic almost indefinitely and there is no drying out or air contamination. The use of a solid medium with the resultant discrete colonies allows picking of a preponderance of the cellulolytic cells even in the rich nutrient media required by many cellulolytic organisms.

II. NONSPOREFORMING RODS

By means of the techniques described in the preceding section some new types of nonsporeforming rods have been found active in the dissimilation of cellulose. Some of these have been isolated from the bovine rumen, others from sewage sludge, and one strain from soil.

A. From the Bovine Rumen

1. An Actively Cellulolytic Rod. Although many strains of sporeforming cellulolytic bacteria have been obtained from the rumen by enrichments, use of quantitative dilutions shows that such bacteria are relatively insignificant, a result already suggested by the microscopic appearance of the rumen contents (1, 11). The active agents which have been found are nonsporeforming rods and cocci.

Of the cellulose decomposers isolated from the rumen, the actively cellulolytic rod has been the most difficult to obtain in pure culture, though colonies are invariably observed in initial rumen liquid cellulose agar dilution cultures inoculated with rumen contents. The original pure cultures (15) were lost soon after their isolation when they were transported to Washington State College. At intervals reisolation was attempted with the previous technique, but as cultures became purer the growth was poorer.

A part of the difficulty was found to be due to the use of different batches of agar, one a domestic and the other Japanese. Parallel series with these two agars were inoculated with identical dilutions of an impure colony. The series in the Japanese agar medium showed excellent colony development in the first four dilutions whereas only a few slowly developing colonies appeared in the first tube of the other series.

Growth was found to be more consistent if 1 per cent beef blood serum was added to the medium, and the strain was transferred through a number of dilu-

tion series in rumen liquid cellulose serum agar. Each time a colony was picked from one of the higher dilutions showing growth. A single colony was then carefully picked to cellobiose agar. Growth occurred in all dilution tubes, and in the fifth were present thin lenticular colonies similar to those formed by the strains of the cellulolytic rods previously isolated. In addition, there were colonies of the spiral form (15). The lens shaped colonies were picked to cellulose medium but no cellulose digestion resulted. Microscopic examination of the colonies on cellobiose disclosed gram negative rods of approximately the same size and shape as the formerly isolated cellulolytic rods when the latter were grown on glucose or cellobiose.

Results such as these have been interpreted by many investigators as evidence that the cellulolytic organism had lost its ability to digest cellulose when grown on sugar. If the author had not previously isolated the cellulolytic rod and found that it grew readily on cellulose after culturing in sugar media, the idea of the loss of an adaptive enzyme might have been entertained. However, repeated dilution into cellobiose agar of colonies in cellulose agar finally yielded colonies, closely similar in microscopic and macroscopic appearance to those previously encountered, which exhibited active cellulose digestion when inoculated into cellulose agar. The strain was pure-cultured by transfer through two rumen liquid cellobiose serum agar series and then returned to rumen liquid cellulose serum agar. Cellulose digesting colonies developed in tubes of higher dilution, indicating that most of the cells which had grown on cellobiose gave rise to colonies of cellulose digesting bacteria.

This strain has been successfully maintained in stock culture for more than a year. It has been grown in a broth consisting of 30 per cent rumen fluid, 0.1 per cent cellulose, inorganic salts and bicarbonate. The broth is similar to the medium used in isolation except that the beef serum and agar are omitted. When 0.1 ml of a culture is transferred daily to 10 ml of fresh medium, almost all the cellulose is gone after 24 hours. This rapid digestion of cellulose has been maintained throughout subculture. Some batches of rumen fluid support slightly more rapid growth than others, but these variations are relatively minor.

Transfers made later than 24 hours show a delay in the speed with which the cellulose disappears. Subcultures made at 10 days may not digest all the cellulose until after 4 to 6 days of incubation. Cultures have been found to be viable up to 24 days in the rumen fluid cellulose broth. After that not all transfers show growth.

The optimum temperature is 38 to 42 C. Rapid growth occurs at 44 C but not at 48 C. Below 38 C growth falls off gradually until at 23 C it is very slow.

The rod is an obligate anaerobe. In those portions of agar cultures in which resazurin is not decolorized there is no growth of colonies.

A test was made to determine whether media other than rumen fluid would supply necessary factors for growth. The media tested were 0.5 per cent proteose peptone, 0.2 per cent malt sprouts, 0.5 per cent tryptone, 1.0 per cent yeast extract, 0.2 per cent dried grass (*Poa pratensis*), 5.0 per cent beef serum, 1.0 per cent corn steep liquor, nutrient broth, 0.2 per cent distillers dried solubles,

and the medium of Gall *et al.* (10) which contains 1 per cent each of proteose peptone, tryptone, beef extract, yeast extract, and milk. All tests were made in duplicate. Each tube contained 0.1 per cent cellulose, and the amount of cellulose disappearing was used as a rough index of the amount of growth. Sodium bicarbonate, CO_2, and both cysteine and thioglycolate were the other ingredients of the test media.

Six days after inoculation there was possibly a slight disappearance of cellulose in some of the media, but after 18 days only the tubes containing distillers dried solubles showed complete digestion. Further transfers in distillers dried solubles failed to show growth. If 2 per cent blood serum was added growth was slightly improved but could not be maintained in serial transfer.

Preliminary inoculation and incubation of the blood serum medium with the gram negative non-cellulolytic rod previously isolated from the rumen gave a medium in which, after resterilization, fairly satisfactory growth of the cellulose decomposing rod took place. The growth of the non-cellulolytic bacterium improved conditions for growth of the cellulolytic rod. Since dried grass and malt sprouts, materials quite similar to the natural food of ruminants, failed to support growth, it is highly probable that in the normal rumen the factors required for growth of the cellulolytic forms are in part produced by the accompanying microörganisms.

The utilization of various carbohydrates was tested by inoculation into the inorganic bicarbonate medium plus 10 per cent rumen fluid. The substrates were tested at a concentration of 0.1 per cent. Cysteine (0.01 per cent) and resazurin (0.0001 per cent) were included. Criteria of fermentation were increased turbidity and acid production. The latter was tested with brom thymol blue, carbon dioxide being bubbled to prevent entrance of oxygen which would cause the resazurin to turn red and mask the color of the brom thymol blue. It also minimized pH changes due to loss of CO_2. With the relatively heavily buffered medium and the low concentration of substrate, the pH drop due to substrate utilization was not large but was clearly detectable.

Cellulose, cellobiose, glucose, maltose, dextrin, starch, and trehalose were fermented. The substrates which were not attacked included: L-arabinose, L-xylose, D-arabinose, D-xylose, D-fructose, D-galactose, D-mannose, L-rhamnose, melezitose, sucrose, lactose, melibiose, raffinose, glycerol, sorbitol, dulcitol, mannitol, inositol, salicin, inulin, starch-free hemicellulose from oat hulls, hemicellulose from white birch wood, hemicellulose from black locust, and pectin from lemon. With the exception of trehalose, the fermentable carbohydrates are limited to cellulose and starch and the sugars resulting from their hydrolysis.

Several analyses of the products resulting from the fermentation of cellulose and sugar by the rod have been made. Strain H, isolated in Texas, was inoculated into two flasks of media, one containing glucose and one cellulose. Controls were similar but were not inoculated. The cultures were grown in round bottom flasks, with glass inlet and outlet tubes which were sealed with a flame after inoculation. The buffer was bicarbonate and CO_2, the gas being used also to obtain anaerobic conditions. Because of the large quantities of carbon dioxide

used, any production or utilization of this gas during fermentation was less than chance variations in the amount initially present. The gas at the termination of the experiment consisted almost entirely of carbon dioxide. No combustible gases were detected.

The glucose culture contained 0.174 meq more volatile acid and 0.4 meq more non-volatile acid than the control.

The experimental cellulose culture showed an excess of 0.143 meq volatile acid and 0.87 meq non-volatile acid. From the crystal form and its temperature of sublimation, the non-volatile acid appeared to be chiefly succinic acid. Five volumes of 95 per cent ethanol were added to the Ba salts of the non-volatile acids to precipitate Ba succinate. This first precipitate had a Ba content of 49.7 per cent. The recrystallized Ba salt contained 53.4 per cent Ba (calc. 54.2 per cent).

From the 82 mg of cellulose fermented were produced 51.3 mg of succinic acid and 8.6 mg of volatile acid, assumed to be acetic acid. The total amount of

TABLE 1

*Products of Cellulose Fermentation by the Actively Cellulolytic Rumen Rod**

	EXPT. 1	CONT. 1	DIFF.	EXPT. 2	CONT. 2	DIFF.
CO_2	0.31	0.435	−0.125	0.216	0.364	−0.148
Formic acid	Trace	Trace		Trace	None	
Acetic acid	0.92	0.64	+0.28	1.08	0.872	+0.208
Propionic acid	0.20	0.173	+0.027	0.212	0.186	+0.026
Butyric acid	0.136	0.120	+0.016	—	0.070	—
Lactic acid	—	—		None	None	
Succinic acid	0.458	None	0.458	0.501	0.077	+0.424
Cellulose (as glucose)			−0.554			−0.531

* The figures are in millimoles.

acid produced during the fermentation was estimated from the difference in the amounts of $Ba(OH)_2$ required to titrate aliquots of the experimental and control cultures between pH 2.5 and 8.5. This difference, 0.83 meq, is less than the 1.01 meq represented by the succinic and acetic acids found. The fact that the two values are of the same order of magnitude suggests that no other acids were produced in large quantity.

A more thorough quantitative experiment has been run on the rod isolated at Pullman. The medium was rumen liquid cellulose broth with phosphate buffer. Nitrogen provided anaerobiosis during preparation and inoculation. The flasks were evacuated after inoculation to reduce the nitrogen and carbon dioxide initially present. The controls were inoculated in the same fashion but were refrigerated instead of incubated. Experiment 1 and control 1 were analyzed in parallel as were experiment 2 and control 2. A summary of the products recovered is given in table 1.

From table 1 it is again apparent that acetic and succinic acids are the chief products formed by the rod. There was no evidence of hydrogen, lactic acid, or ethanol. A trace of formic acid may have been produced.

Carbon dioxide appears to be utilized. It probably enters into the formation of succinic acid in a manner comparable to the fixation of CO_2 during the fermentation of glycerol by the propionic acid bacteria (47).

The evidence for production of propionic and butyric acids in these experiments is not very satisfactory. Due to their presence in the rumen fluid of the control tube in quantities almost as great as those of the experimental tube, it is possible that the excess propionic and butyric acids in the experiment are due to experimental error.

In experiment 1 and control 1, Elsden's chromatographic procedure (7) for separation of the volatile acids was employed. Its accuracy was tested on a known mixture containing formic, acetic, propionic, and butyric acids in about the same amounts as in the experimental cultures. The butyric and propionic acid bands were extracted from the column with the solvent, neutralized, and the amounts determined by Duclaux distillations. The formic and acetic acid bands left on the column were forced out into separate flasks containing a little water. After neutralization with $Ba(OH)_2$ each flask was boiled to drive off the chloroform and butanol. During this operation it was necessary to shake the flask to avoid bumping. The flask contents were then poured through a filter to separate the silica gel, which was washed several times until no brom cresol green showed in the filtrate. The volatile acid in the filtrate, freed with a slight excess of H_2SO_4, was determined in a Duclaux distillation. The yields of the acids, starting with 0.078 mmol of formic, 0.103 mmol acetic, 0.12 mmol propionic, and 0.13 mmol of butyric acid were 49, 85, 62, and 86 per cent, respectively. It seemed possible that the recovery of propionic acid was low because it evaporated from the chloroform-butanol during the rather long time that the latter was extracting it. The low formic acid recovery may be ascribed to an incomplete extraction from the $KHSO_4$.

In a second test on known amounts of the acids, they were separated on the column, and the latter was forced out before the butyric acid band had been extracted. The bands were separated, the acids washed out, and the amounts determined by Duclaux distillations as before. In this run the yields were: formic acid 56 per cent, acetic acid 78 per cent, propionic acid 86 per cent, butyric acid 86 per cent. This procedure was used in experiment 2 and control 2 (table 1), and the amounts of each acid found were corrected according to these experimentally determined yields. The excess of initially determined total volatile acid in the experiment over the control was 0.252 meq. The excess of the separately determined acetic and propionic acids in table 1 is 0.234 meq, a fair agreement, considering the small quantities and the numbers of acids present.

In another experiment acetic and succinic acids were again the chief products. The experimental flask contained a slight excess of propionic acid over the control but there was no apparent production of butyric acid.

From all these analyses of cultures of the actively cellulolytic rod from the rumen, it is quite clear that acetic and succinic acids are the chief fermentation products. It is possible that propionic acid is also formed in small quantities but more accurate analyses are desirable before final conclusions are drawn. The development of a culture medium free of the volatile acids would greatly aid in the unequivocal determination of propionic and butyric acids.

Numerous tests for copper reducing substances have been made on old cultures which contained excess cellulose and were incubated after fermentation had ceased. In no case has any copper reduction been observed. This is in contrast to most of the anaerobic cellulose decomposers which have been studied. The rod also is distinguished from the other cellulolytic anaerobes by the fact that distinct colonies with surrounding clear zones usually do not form in cellulose agar.

The bacteria migrate through the medium and are found in a thin layer at the junction between digested and undigested cullulose (15, fig. 9). Both of these observations indicate that the mechanism of cellulose hydrolysis by this rumen rod differs from that of the other strains. It is possible that an extracellular cellulase is not formed. However, in view of the insoluble nature of cellulose this is highly improbable. It is conceivable that the mechanism of hydrolysis may be linked with the energy yielding metabolism in some fashion which requires a close spatial connection between the two. This would explain the close proximity of the bacteria to the cellulose undergoing digestion. A few strains of the rod which have not been isolated but which were observed in initial dilution cultures were seen to appear in cellulose agar first as small lens shaped colonies with a slight zone of clearing around them, but very soon the colony became diffuse as the cells migrated out in the typical manner. That any clear zone was observed suggests that an extracellular enzyme may be produced.

The strains of the nonsporeforming rods from the rumen which completely digest cellulose have all appeared microscopically similar. In cellulose agar they are very small slightly curved rods 1.0 to 2.0 μ long and 0.3 to 0.4 μ wide. In fresh mounts with transmitted illumination they are not easily seen, but under the phase microscope the cells are clearly visible. Motility has not been detected but from the manner in which the cells migrate through the agar it seems possible that some method of locomotion is present. No spores have ever been observed; and since the cells die out in old cultures, it is probable that no resistant cells of any type are formed. The old cells in the center of a cleared spot in the agar no longer appear as typical rods but are indistinct rounded bodies, faintly staining, and interpreted as being degeneration forms. The fact that the old rods become spherical suggest that no rigid cell wall is present, and this may explain the relative invisibility of the cells in fresh mounts.

In glucose and cellobiose cultures, the cells are usually larger and may show uneven staining.

The minute size of these rods, the gram negative reaction, the occasional uneven staining, the obligate anaerobic nature, and their intestinal habitat are all characteristics of the genus *Bacteroides*. However, the following features suggest myxobacterial affinities: (*a*) the cells migrate through the cellulose agar in a manner resembling the cytophagas, (*b*) they cannot easily be seen in fresh mounts with ordinary illumination, and (*c*) in old colonies irregular spheres and oval bodies are present.

Because it has not been possible to detect any creeping or bending movements of this rod, it is assigned to the genus *Bacteroides* instead of to the myxobacteria. The formation of large quantities of succinic acid is the most outstanding characteristic of this rod and makes appropriate the specific name, *succinogenes*.

Bacteroides succinogenes n. sp. includes those gram negative, nonsporeforming, obligately anaerobic rods from the rumen of cattle which actively ferment cellulose with the formation of acetic and succinic acids. No hydrogen or carbon dioxide is formed and the latter may actually be used. In old cultures containing excess cellulose no copper reducing materials accumulate. In addition to cellu-

lose, the following are fermented: cellobiose, glucose, starch, dextrin, maltose, and trehalose. Numerous other carbon compounds, including several hemicelluloses, do not support growth.

The rods are very small when growing on cellulose, measuring 1.0 to 2.0 μ long and 0.3 to 0.4 μ in diameter. On sugar media they are somewhat larger. In old cultures the rods disappear and are replaced by small spheres and ovals of variable size.

The temperature range is 23 C to 44 C, with an optimum at about 40 C.

Rumen fluid supports rapid development in the presence of fermentable carbohydrate. No growth occurs on a number of common culture media.

2. A less actively cellulolytic rod. During the isolation of *Bacteroides succinogenes* cotton was the source of the cellulose used. It was treated with HCl and finely ground in a pebble mill as previously described. It was realized that the cellulose eaten by the cow was not identical with cotton cellulose (27), but the latter was used because of its relative purity, a factor which might reduce the number of non-cellulolytic organisms which could develop.

The incomplete digestion of cotton cellulose by the yellow cocci (see next section) and also by some of the sporeformers (Section IV) which were isolated suggested that all elements in the suspension were not equally attacked. Microscopic examination indicated that the smaller less dense particles were digested first. These differences were ascribed to variations in the degree of hydration of the cellulose during grinding, the smaller particles being more hydrated.

It seemed possible that other celluloses might be more hydrated than cotton and more easily digested by some of the rumen bacteria. Sijpesteijn (38) reported that a yellow coccus from the rumen readily digested filter paper but was inactive on cotton. Filter paper was accordingly used in place of cotton in some further experiments. Whatman no. 1 filter paper not previously treated with HCl was ground in the pebble mill. It was found that whereas the cotton cellulose could be ground at a concentration of 4 per cent, the same amount of filter paper matted up and had to be diluted to 2 per cent. Even at this concentration continuous grinding for 72 hours failed to break the fibers into the small particles characteristic of the ground cotton. However, the particles did not settle out in the suspension and occupied about the same space as the 4 per cent cotton.

Using rumen fluid agar dilution series with this filter paper cellulose a second type of cellulolytic rod was isolated from the rumen.

The isolation technique was similar to that employed for *Bacteroides succinogenes* except for the different cellulose. In the initial series slightly yellow disc colonies were found in the sixth dilution tube in numbers indicating 100,000 bacteria per ml of original sample. The cellulose was only incompletely digested around the colonies and it was believed that they were composed of the yellow cocci previously encountered (15). However, microscopic examination disclosed rods. By subculturing on rumen liquid cellobiose agar a pure culture was obtained. When it was returned to rumen liquid cellulose agar the colonies grew readily but at first did not appear to digest any of the cellulose. Later a gradual solution

of the particles in the immediate vicinity of the colony could be detected. In liquid cultures the total amount of filter paper cellulose did not visibly diminish after a few days of incubation, but it appeared more transparent than an uninoculated control. After prolonged incubation, a decrease in the volume of the cellulose was clearly evident.

This rod (strain W) digests either cotton or filter paper cellulose but not as completely as does *B. succinogenes*. It differs also in the following respects: it does not migrate through the agar as readily; there is an extracellular cellulase since cellulose is digested a short distance away from the colony; and in old colonies there is not a replacement of the rods by irregular spheres and oval bodies.

When grown in rumen fluid cellulose agar, the cells are slightly curved gram negative rods 0.8 to 1.5 μ long and 0.3 to 0.4 μ in diameter. On cellobiose the curvature is greater (fig. 3) and the cells slightly larger. The smear shown in

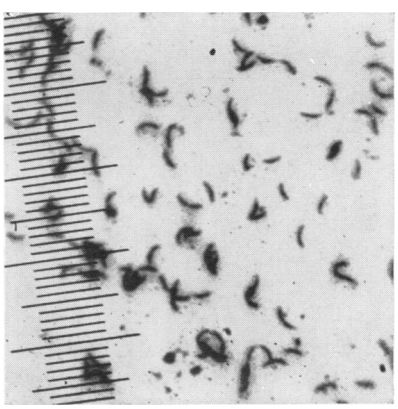

FIG. 3. Photomicrograph of a fixed smear of the less actively cellulolytic rod from the rumen. Carbol fuchsin. Each small micrometer division equals 0.82 μ.

figure 3 was from a rumen fluid cellobiose agar culture incubated for 20 hours. Growth was very rapid and large colonies had formed. Even at this young stage all cells were gram negative. Spores have not been observed and no motility has been detected.

In testing the effect of temperature, the time for disappearance of cellulose from the liquid medium could not serve as a criterion of growth because of the incompleteness of digestion. Consequently cellobiose was selected as the substrate and utilization was followed by periodic analyses of aliquots for copper reduction. The optimum temperature was 30 to 37 C. Growth was good at 41 and 47 C but absent at 55 C. Below 25 C little development occurred.

The following substrates were fermented with production of acid and gas: L-arabinose, D-xylose, L-xylose, glucose, D-galactose, D-mannose, D-levulose, maltose, lactose, cellobiose, sucrose, raffinose, dextrin, soluble starch, hemicellulose from oat hulls, hemicellulose from black locust, and hemicellulose from white birch. Development was very rapid on these substrates and copious gas was produced after 24 hours of incubation. Acidity was tested after 4 days.

Acid but little gas was produced from melibiose, salicin and inulin. Tested substrates which were not fermented included D-arabinose, L-rhamnose, melezitose, trehalose, mannitol, dulcitol, D-sorbitol, inositol, and glycerol.

Quantitative analyses of fermentation products were made on cultures containing rumen liquid and cellulose with phosphate buffer. An inoculated refrigerated culture served as a control. After incubation for 90 days, analyses were made with the results shown in table 2.

The materials formed are typical of a butyric acid fermentation. Carbon dioxide, hydrogen, and butyric acid are the principal products and account for about 65 per cent of the cellulose and all of the acetic acid disappearing. Acetic acid may have accumulated during the early stages of the fermentation but a later conversion into butyric acid caused a decrease in the total amount present at the end of the experiment. The deficit of propionic acid exceeds the experi-

TABLE 2

*Products of Cellulose Fermentation by the Less Actively Cellulolytic Rumen Rod**

	EXPERIMENT	CONTROL	DIFFERENCE
Carbon dioxide	2.11	0.82	+1.29
Hydrogen	0.313	0	+0.313
Ethyl alcohol	trace	None	+trace
Formic acid	0.016	None	+0.016
Acetic acid	0.886	1.78	−0.894
Propionic acid	0.383	0.467	−0.084
Butyric acid	1.48	0.314	+1.166
Lactic acid	0.11	0.057	+0.053
Succinic acid	None	None	
Residue (cellulose and cells) as glucose	0.17	1.23	−1.06

* The figures are in millimoles.

mental error encountered in other similar analyses. Traces of ethanol and formic and lactic acids were produced but no succinic acid was found.

Parallel experimental and control cultures on cellobiose showed essentially the same results except that the conversion of acetic to butyric acid was not as marked, due to a much shorter incubation period. There was a deficit of propionic acid in the cellobiose of the same magnitude as in the cellulose experiment. It is possible that the separation of propionic acid on the chromatogram is affected by the increased butyric acid and decreased acetic acid in the experiment as compared with the control, and that the seeming disappearance of propionic acid is due to analytical error. However, visual inspection disclosed no aberrations in the separation of bands that could account for the difference in amounts. The propionic acid band on the experimental chromatograms was about as distinct as on the control, though it did appear to be slightly less intense in color. If propionic acid is utilized, its conversion into other products is an interesting problem.

The supernatant fluid in a cellulose agar tube which showed digestion of a

major part of the cellulose after 3 months of incubation was tested for copper reducing substances but none were found. From this negative result it cannot be concluded that the bacterium does not hydrolyze cellulose to sugar, since conditions may still have been favorable for fermentation of the latter. Compared to many cellulolytic anaerobes, less total acid is produced in a culture of this rod because the conversion to butyric acid of the acetic acid already present in the rumen culture fluid reduces acidity and in part counteracts the increased acidity due to production of butyric acid from the cellulose. The total volatile acid in the experimental culture of table 2 only slightly exceeds that in the control. This low acidity favors the fermentation of greater amounts of cellulose. It is probable that the old agar cultures tested for sugar were still favorable for growth and fermentation and consequently no sugars accumulated. They must occur as intermediates, however, because clear areas form around colonies in cellulose agar. Cellulolytic enzymes diffuse from the cells to the substrate, and the sugars formed diffuse back to the cells. To demonstrate these sugars it will be necessary to use cultures containing higher concentrations of cellulose than have yet been tested.

Correlated with the low increase in total acidity of cultures of this rumen rod is an increased longevity. The experimental culture of table 2 when tested for purity after 90 days of incubation, yielded numerous colonies in the inoculated dilution tubes.

This less actively cellulolytic rumen rod differs from the genus *Butyribacterium* (3a) in being smaller, gram negative, more curved, and with slightly tapering ends. Its curved shape suggests affinities with the genus *Vibrio* but motility has not been observed. In many morphological respects it resembles *Bacteroides succinogenes*, but forms distinct colonies in cellulose agar, does not show uneven staining when grown on cellobiose, and does not form round degeneration bodies in old cultures. For the present it is not assigned to a genus but will be called the less actively cellulolytic rumen rod.

The significance of this strain in the rumen cannot be fully evaluated without additional data but some features may be mentioned. Its rapid growth is compatible with an active role in the rumen. The numbers in which it was found also suggest its importance.

The low rate at which cotton and filter paper are digested in cultures does not necessarily imply that development in the rumen is thus limited. More easily digested celluloses and hemicelluloses in the normal cattle ration may be the substrates attacked. The wide variety of carbohydrates, including hemicelluloses, rapidly utilized by this strain suggest that many food constituents besides cellulose are attacked. Fermentation of these materials by this rod accounts in part for the butyric acid formed in the rumen.

B. From Sewage Sludge

Samples of sludge from the second anaerobic digestion tank at the Moscow, Idaho, sewage disposal plant were inoculated into tubes of rumen fluid cellulose agar. Clear spots developed in the lower dilutions and the cellulolytic organisms

were purified by diluting into further similar series. Cells inoculated into glucose agar dilution tubes gave colonies of one sort. Inoculation into cellulose agar after two transfers in glucose agar again gave active cellulose digestion. Cultures B and C were obtained in this way.

The numbers of cellulose decomposers at various stages of the anaerobic digestion process in the sewage plant were estimated. Samples were collected from (*a*) the undigested sludge as it entered the first anaerobic tank, (*b*) the fermenting sludge from the bottom of the first tank, (*c*) the sludge from the bottom of the second anaerobic tank, and (*d*) from the top of the second tank. Each of these was diluted (0.5 ml. inoculum) into a rumen fluid cellulose agar series from which quantitative counts could be made.

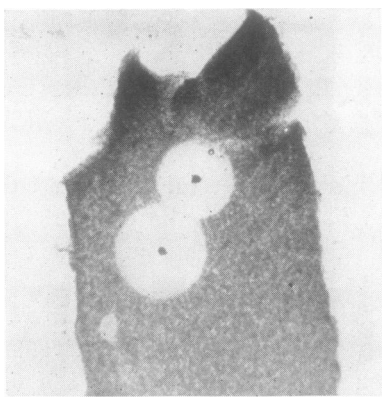

FIG. 4. Photomicrograph of a thin sheet of cellulose agar from the upper part of an anaerobic tube, showing colonies of sludge strain D and the surrounding area cleared of cellulose. Magnification 4×.

No cellulose digesting colonies were observed in the series inoculated with the raw sludge. The count for the bottom of the first tank indicated 880 per ml and for the bottom of the second tank 2600 per ml. The top of the second tank showed more colonies in the second dilution tube of the series than did the comparable tubes of the other inocula, but since conditions were apparently unfavorable in tube three and no colonies developed an exact count could not be made. The results of these several series indicate that the cellulose digesting bacteria multiplied during the passage of the sludge through the digestion tanks, and it is postulated that they are concerned with the decomposition of the cellulose.

In this series of quantitative inoculations most of the colonies resembled the strains already isolated. One of them, strain D, was grown in rumen fluid cellulose agar dilution series until the culture gave every indication of purity. No glucose or other sugar series was used. The clearing of the cellulose for relatively great distances around the colony (fig. 4) permitted ready detection of contaminating colonies. None were seen and it was concluded that the culture was pure. It has been transferred through more than 60 agar dilution series since first isolated and has been carried for more than three years.

Soon after isolating the sludge strains, it was found that they grew readily in cellulose yeast extract agar so this medium has been employed for routine transfers.

The three isolated strains consist of gram negative rods and diplorods, extremely variable in size, particularly when growing on cellulose (see fig. 5). The large rods may show internal granules, usually at each end of the cell. On cellobiose they are much more uniform and stain more evenly. Many of the rods are slightly curved. They are motile. No indications of spores have been observed; and on several occasions old cultures left untransferred have failed to grow upon subculture, suggesting that no resistant cells are formed. The vegetative cells occasionally show swollen portions but these have not been observed to give rise to spores. Agar cultures with cellulose are viable up to 4 weeks if

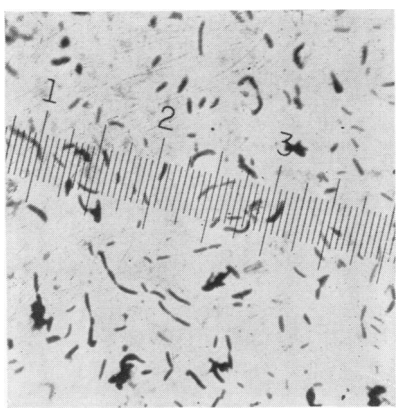

FIG. 5. Photomicrograph of a fixed smear of sludge strain C from cellulose agar. Carbol fuchsin. Each micrometer division equals 0.82 μ.

placed at 30 C as soon as colonies appear. If maintained at 38 C as long as two weeks they often fail to give viable subcultures. Strain C was lost in this way after being carried for six months in pure culture.

Strain B was used to test the effect of various concentrations of yeast extract. Liquid cultures containing 0.16 per cent cellulose received the following percentages of yeast extract: 0.05, 0.1, 0.2, 0.4, and 1.0. A control tube of 1.0 per cent yeast extract without cellulose was also inoculated. Eight days later all the cellulose had been digested in the 0.05 tube and most of it was gone in the 0.1 tube. The remaining tubes showed cellulose digestion in inverse proportion to the amount of yeast extract present. Turbidity in the control tube without cellulose showed that growth occurred on the yeast extract alone. This suggests that the latter spares the cellulose and explains the slower cellulose digestion with increased yeast extract.

In a second experiment, the yeast extract was maintained constant at 0.5 per cent and the amount of cellulose was varied from 0.02 to 0.4 per cent. In these cultures the cellulose disappeared first in the tubes containing 0.02 per cent and in subsequent tubes in the order of the amount of cellulose that they con-

tained. The tube with 0.4 per cent cellulose did not show complete digestion until after 40 days. All cultures were tested for reducing materials and no significant amounts found in any of them, indicating that all the digested cellulose had been fermented.

In old cellulose yeast extract agar cultures in which fermentation has ceased there is an accumulation of reducing substances. Much of the tube becomes cleared of cellulose. Old tubes of all three strains in yeast extract cellulose agar were used to obtain the sugars. These were either partially extracted from the agar by soaking it in water under toluene, or in some experiments the liquid which collected above the agar was removed aseptically and used without treatment of any sort.

The solution of sugars from strain C was tested with phenylhydrazine and sodium acetate. A hot water insoluble osazone was formed and also a hot water soluble one, but in microscopic appearance they were not typical for glucosazone and cellobiosazone, respectively, possibly because of impurities.

The reducing substances from strains B and D were added to nutrient broth and inoculated with a strain of *Escherichia coli* known to be cellobiose negative. An aliquot of each was immediately removed and refrigerated. The remainder was incubated at 37 C for 48 hours at which time samples were again removed and tested for copper reduction, as were the refrigerated controls. The copper reduction in the incubated samples decreased by 93 per cent for strain D and 90 per cent for strain B. Assuming that these decreases were due to glucose utilization the amount of glucose formed considerably exceeded the amount of cellobiose.

An aliquot of the sugars from strain D was hydrolyzed in 5 per cent H_2SO_4 for one hour in a boiling water bath and the increase in reducing power found to be 4 per cent. The unhydrolyzed and hydrolyzed samples were tested for their fermentability by yeast and by *E. coli*. For the unhydrolyzed material the yeast produced 48 μl of CO_2 per mg Cu reducing capacity. For the hydrolyzed culture 55.5 μl per mg Cu were formed. After action of the yeast the unhydrolyzed sample showed definite copper reduction when tested with Benedict's qualitative sugar reagent whereas the hydrolyzed material showed none.

Incubation with *E. coli* removed all copper reducing substances from the hydrolyzed sample and in this experiment caused a 91 per cent decrease in the unhydrolyzed material. In another experiment the sugars were fermented by the colorless rumen coccus (see Section III) which ferments cellobiose but no other sugars. The unhydrolyzed sample showed a distinct turbidity suggesting growth; its copper reduction decreased 35 per cent and the hydrolyzed culture 20 per cent.

In these experiments there was poor correlation between the amounts of glucose and cellobiose as estimated by the various procedures. With the unhydrolyzed material the decrease in copper reduction due to *E. coli* plus the independently determined decrease due to the coccus exceeded the original total copper reduction. These discrepancies prevent accurate quantitative comparisons of the amounts of glucose and cellobiose. However, the data suggest that the amount of glucose formed considerably exceeds the amount of cellobiose.

The fermentation products of strains B and D grown under nitrogen in cellulose, yeast extract, and phosphate medium were determined and are given in table 3.

The strain D culture was analyzed before any sugar had accumulated and gives a better indication of the amount of fermentation products recovered.

These constitute only one-half of the weight of cellulose disappearing. It is evident that other products in considerable quantities must have been formed.

The production of hydrogen and carbon dioxide by these sludge organisms clearly distinguishes them from *Bacteroides succinogenes*. The small colonies with wide zones of cellulose digestion, the motility, and the larger size are other differences. In their morphology and fermentation products they differ markedly from the less actively cellulolytic rumen rod and must be considered as a distinct type.

C. From Soil

In view of the presence of nonsporeforming rods in the rumen and in sewage sludge, their occurrence also in soil is of interest. In a single dilution experiment described more fully in section IV a colony of nonsporeforming cellulose digesting rods was encountered, the organism was isolated in pure culture, and

TABLE 3

Fermentation Products of Strains B and D on Cellulose

	STRAIN B	STRAIN D
Cellulose Disappearing	204.8 mg	177.9 mg
CO_2	0.45 mmol	0.26 mmol
H_2	0.62	0.48
Ethanol	0.34	0.61
Formic acid	—	None
Acetic acid	0.51	0.42
Lactic acid	None	None
Succinic acid	None	0.20
Glucose, as detmd. by Cu reduction after hydrolysis	90.0 mg.	None

carried as soil strain I. It digested cellulose more completely than did the sporeformers isolated from the same series. Almost all cellulose disappeared in tubes containing 1.5 per cent cotton cellulose and only a small amount of copper reduction was shown by the liquid on top of the agar. Some of this liquid was collected and its copper reduction determined before and after incubation with the cellobiose-negative strain of *E. coli*. All traces of copper reduction disappeared after incubation, suggesting that all of the sugar was glucose.

Hydrogen and carbon dioxide were shown to be the gases produced by soil strain I in the cellulose yeast extract agar medium but they were not determined quantitatively. Other products were determined by analyses similar to those described for the sporeforming strains C and F in Section IV. From 700 mg of cellulose fermented there were found 157 mg ethyl alcohol, 105 mg acetic acid, 9 mg formic acid and 10 mg lactic acid. In addition the copper reduction of the supernatant fluid indicated that 100 mg of glucose had been formed. These products are quite similar to those of the sludge strains B and C.

Morphologically this soil strain is quite different from the nonsporeforming rods isolated from the rumen and from sludge (figure 6). The cells are about 0.5

μ in diameter and vary in length from 1.5 to 5.0 μ. They are actively motile. The rods are not entirely regular in shape; the bends which occur at various points probably mark the sites of future divisions.

Summarizing, the preceding results show that anaerobic, actively cellulolytic, nonsporeforming rods are present in the rumen, in sludge and in soil. They are important in the rumen and in sludge but their significance in the soil is not established. All strains are gram negative. Their morphology and fermentation reactions indicate at least four distinct types. The fermentations by the sludge and soil strains resemble those of many cellulolytic sporeformers (Section IV) but the succinic acid production by *Bacteroides succinogenes* and the butyric acid production by the other rumen rod are unique among the described cellulose decomposers.

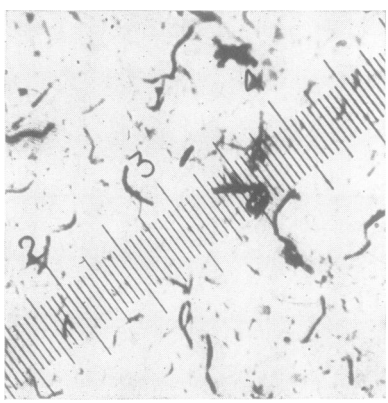

FIG. 6. Photomicrograph of a fixed smear of soil strain I from cellulose agar. Carbol fuchsin. Each micrometer division equals 0.82 μ.

III. COCCI FROM THE RUMEN

1. The Colorless Cocci. Judged by the numbers in which they can be demonstrated in cellulose agar dilution cultures the cocci of this group are the most important cellulolytic bacteria in the rumen. They are consistently found in numbers ranging from 10 to 100 million per ml. Three strains have been isolated in pure culture, M, U, and V. All three were closely similar in morphology, rate of growth, and colony type in cellulose agar. Strain M has been reported upon previously (15).

Clear spots showing cellulose digestion appear in rumen fluid cellulose agar tubes after only two days of incubation, the most rapid cellulose digestion that has been observed, except possibly for *Bacteroides succinogenes*. In liquid cultures the rate of growth is also extremely rapid. A tube of liquid rumen medium containing 0.1 per cent cellulose and inoculated with 1 per cent of a 24 hour culture will show most of the cellulose gone after 16 hours of incubation at 38 C.

The first two strains that were isolated, M and U, were subcultured by dilution of single colonies into rumen fluid cellulose agar tubes. The first one or two

transfers after picking from the original tube showed extremely rapid growth and cellulose digestion. When colonies in the thin agar on the upper part of the tube were examined under the dissecting microscope, the agar surface in the area cleared of cellulose was seen to be wrinkled and collapsed. The agar appeared to have undergone digestion. However, on probing the cleared area with an inoculating needle it did not seem to be entirely liquefied.

Later subcultures showed less or none of this effect on the agar. Additional changes in the behavior of the strains appeared at the fourth and fifth transfers. Growth became slower and clear spots did not appear until after four or five days of incubation. On further subculture the clearing was increasingly slow and less complete. By examining the cellulose agar tubes under the dissecting microscope colonies could be seen developing in the thin agar but at first they showed no signs of cellulose digestion. Later some of them developed areas of incomplete clearing. Dilutions into rumen fluid cellulose agar tubes finally failed completely to give any visible clearing of the cellulose. For both strains M and U this occurred after a total of seven or eight transfers.

Each strain had been carried through one dilution culture in rumen fluid cellobiose agar, which showed no signs of any contaminants and from which a colony in a high dilution was inoculated back onto cellulose and gave growth. Further transfers were in each case made by diluting a single colony from a tube of high dilution. The colonies appearing in the various tubes of the subsequent series occurred in approximately the numbers to be expected. They all consisted of cocci. These observations provide reasonably good evidence that the culture was pure. It may be concluded that under the conditions of culture the pure strains gradually lost their cellulolytic capacities. A loss of cellulose digesting power by other strains (32, 33, 37) similarly transferred to a sugar medium has been ascribed to a direct effect of the sugar on the enzymes of the bacteria. However, the evidence accumulated in the present studies indicates that a loss of cellulose digesting capacity after growth on sugar is usually due to impurity of the culture and picking of a non-cellulolytic contaminant. The failure of the coccus strains to digest cellulose was not an immediate consequence of growth on cellobiose since subcultures from the sugar series continued to show cellulose digestion through several transfers.

It is possible that the agar was in some way concerned with the diminution in cellulose digestion. After strain U had gone through the cellobiose agar series it was transferred back to liquid rumen cellulose medium and gave rapid growth and cellulose digestion. From the liquid tube it was inoculated into flask cultures for quantitative analysis of fermentation products. It was also transferred frequently to new tubes of liquid medium which continued to show growth and cellulose digestion through numerous serial transfers. But parallel subcultures by dilutions from the cellobiose agar series into rumen fluid cellulose agar showed the same progressive decrease in cellulose digestion exhibited by strain M. Strain U was lost when one of the liquid cultures was incubated too long before transferring and failed to give growth.

Strain V (fig. 7) was isolated by diluting through two cellobiose agar series

and then inoculating into liquid medium. It was carried by transferring every 24 to 48 hours in liquid culture and was also passed through several transfers in rumen fluid cellulose agar dilution series which showed some diminution of cellulose digestion; but the transfers were not carried long enough to test for complete loss of cellulase activity.

When liquid transfers were made at 48 hours, cellulose digestion in the new cultures was less rapid than when the inoculum came from a 24 hour tube. This suggests that some of the bacteria failed to survive in the older culture. They are not all killed, however, as cultures in rumen fluid plus 0.1 per cent cellulose were still viable after two or three weeks of incubation. Strain V has been successfully carried in liquid culture over a period of seven months.

The ability of strain V to attack various carbohydrates was tested. The inorganic medium plus 10 per cent rumen liquid was used as the basal medium to

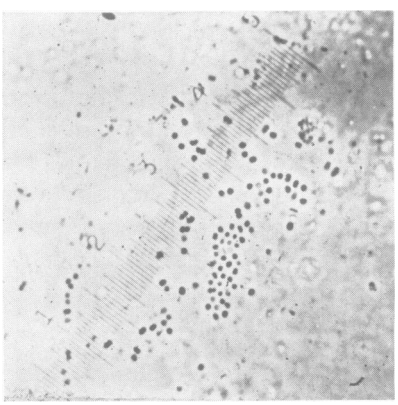

FIG. 7. Fresh mount of the colorless rumen coccus, strain V, from a cellobiose agar tube. Dark contrast medium phase, oil immersion objective. Each micrometer division equals 0.82 μ.

which 0.2 per cent of each substrate was added. The inoculum for each tube was 0.1 ml from a 24 hour liquid culture. The following substrates were tested: xylose, glucose, galactose, levulose, mannose, rhamnose, trehalose, melibiose, maltose, lactose, melezitose, sucrose, cellobiose, raffinose, inulin, dextrin, soluble starch, birch hemicellulose, black locust hemicellulose, oat hull hemicellulose, mannitol, sorbitol, inositol, dulcitol, salicin and glycerol.

Turbidity and acid were evident in the cellobiose tube after 24 hours of incubation. The birch hemicellulose showed a few gas bubbles but the substrate did not dissolve and no acid was formed. In none of the other substrates was there any indication of growth, even after extended incubation.

Dilution cultures of strain V into rumen fluid glucose agar failed to give any colonies. During the isolation of strain M two dilutions into rumen fluid glucose agar were made and in neither case did any growth result, though parallel series in cellulose or cellobiose developed typical colonies.

The question of utilization of glucose as compared with cellobiose was further

investigated by testing cultures of strain V for copper reduction. Tubes of rumen fluid with each one of the following substrates were prepared: 0.2 per cent cellulose, 0.2 per cent glucose, 0.2 per cent cellobiose, or no substrate. All were inoculated with 0.1 ml of a liquid culture. Two ml of each were then removed and refrigerated. The rest was incubated at 38 C. When all the cellulose was gone in the incubated tube, the refrigerated controls and a 2 ml sample of each incubated tube were tested for copper reduction, using Bertrand's method with 2 ml of each copper reagent. The results are shown in table 4.

This experiment shows conclusively that glucose is not attacked by strain V. Cellobiose is the only tested sugar that is utilized. Of all the cellulose digesting bacteria adequately studied thus far, the colorless coccus from the rumen most closely approximates the concept of an obligate cellulose digester. Since cellobiose probably does not occur free in nature to any extent except as a digestion product of cellulose, the latter is the only natural substrate found for these strains.

TABLE 4

Mg copper reduced by 2 ml of Incubated and Refrigerated Cultures of the Colorless Cocci

SUBSTRATE	INCUBATED	REFRIGERATED
Cellulose	None	None
Cellobiose	None	4.4
Glucose	6.06	5.70
No addition	None	None

The growth of strain V on several media was tested. One-tenth ml of a 24 hour culture in rumen liquid cellulose broth was inoculated into each of the following: 0.2 per cent proteose peptone, 0.2 per cent tryptone, 1 per cent corn steep liquor, nutrient broth, 1.0 per cent yeast extract, 0.2 per cent dried grass, 0.2 per cent malt sprouts, 0.5 per cent distillers dried solubles, inorganic medium with no additions, Gall's medium, and rumen fluid broth. Cellulose (0.1 per cent), 10 per cent rumen fluid, 0.01 per cent cysteine, 0.0001 per cent resazurin and 0.5 per cent $NaHCO_3$ were other constituents of each medium. CO_2 was used to regulate the pH and remove oxygen.

In 24 hours most of the cellulose was gone in the rumen fluid broth. There was gas production in the grass medium. None of the other media showed signs of growth. After 2 days the cultures in grass, malt sprouts, nutrient broth, and Gall's medium showed some growth and after 4 days the cellulose was almost all digested. Cultures in proteose peptone and distillers dried solubles showed a slight amount of cellulose digestion.

The culture in the grass medium was subcultured to a tube of similar medium but no growth resulted. Of all the media, the rumen fluid cellulose was far the best. This result resembles that found with *Bacteroides succinogenes*.

Strain U was inoculated into liquid cultures for quantitative analyses of fermentation products. Nitrogen was used for anaerobiosis, phosphate as buffer,

and in addition 100 mg $CaCO_3$ was added to each tube. The usual 100 ml round bottom flasks with glass sealed inlet and outlet tubes were employed. Cysteine (8 mg) was used as a reducing agent and 10 ml rumen fluid were added to provide nutrients. Two ml of a 24 hour culture served as inoculum, giving a total volume of 87 ml of culture medium. The control was inoculated and then heated to boiling. The cellobiose flasks were set up exactly like the cellulose flasks except that 200 mg of cellobiose served as the substrate. The analyses were in general similar to those for the rumen rod.

The total volatile acids were determined by a Duclaux distillation, in which ten 10-ml fractions of distillate were collected, leaving an 11th residual fraction of 10 ml. The formic acid was determined in this residual fraction by mercuric chloride reduction. The correction factor necessary to give the total formic was determined from a Duclaux distillation of pure formic acid.

TABLE 5

Fermentation Products of the Colorless Coccus, Strain U

	CELLULOSE FLASK 1	CELLULOSE FLASK 2	CELLOBIOSE FLASK 3	CELLOBIOSE FLASK 4
Substrate disappearing...	174.8 mg	180.0 mg	200.0 mg	200.0 mg
CO_2.....	0.51 mmol	0.55 mmol	0.12 mmol	0.125 mmol
H_2.....	0.25	0.20	0.05	0.06
Ethyl alcohol.....	0.52	0.63	0.25	
Lactic acid.....	—	0.11	0.72	
Total volatile acids.....	1.45 meq	1.24 meq	1.00 meq	
Formic acid.....	0.27 mmol	0.30 mmol	0.29 mmol	
Acetic acid.....	1.18	0.94	0.71	
Propionic acid.....	—	None	None	
Butyric acid.....	—	None	None	

The Ba salts of the volatile acids in the first 10 fractions were obtained by evaporation to dryness, and the acetic, propionic, and butyric acids separated on the partition chromatogram according to the method of Elsden (7). The butyric and propionic acid bands were extracted and the amount of each determined by a Duclaux distillation. The results obtained are shown in table 5. The values represent the difference between the amounts in the experimental and in the control vessels.

One of the most striking features in table 5 is the difference between the amounts of the various products formed from cellobiose as compared with the amounts from cellulose. Cellobiose produced a much larger amount of lactic acid but less hydrogen, carbon dioxide, and ethyl alcohol. The agreement between the two cellulose fermentations is quite close; and the duplicate CO_2 and hydrogen values for the cellobiose fermentations agree, indicating that the differences between cellobiose and cellulose are not due to experimental error.

The determination of propionic and butyric acids in these experiments was not entirely satisfactory due to the presence of 0.18 and 0.085 mmols, respectively, in the control. The differences between control and experimental flasks were within the experimental error. The major portion of the volatile acid was

acetic; and the chromatogram showed a strong band of acetic acid. The amount was calculated as the difference between the total volatile acids and the amount of formic acid.

The products of the first cellulose fermentation amount to 130 mg as compared with 174.8 mg of cellulose disappearing, and in the second the values are 133.6 and 180 mg. In one cellobiose fermentation, the values are 137.8 and 200 mg, respectively.

The non-gaseous fermentation products of strain V were analyzed in a second experiment. Ethanol, and lactic, formic, and acetic acids were again the chief products found. No evidence for production of propionic or butyric acid was obtained.

Traces of copper reducing materials were found in some strain V cultures containing an excess of cellulose but were not found in others. The sugar in two cellulose agar tubes was collected by treating with alcohol, filtering off the precipitate of agar, and distilling off the alcohol at reduced pressure. A sample of the sugar solution was hydrolyzed for one hour at 100 C with 5 per cent sulfuric acid and then neutralized. Copper reduction values were determined on this hydrolyzed material and on an equivalent amount of the unhydrolyzed. They showed the same reduction, namely, 10.2 mg of Cu per ml of the original solution. If due to glucose this would indicate 5.8 mg per ml.

The hydrolyzed and unhydrolyzed sugars were tested for yeast fermentability in the Warburg respirometer, in parallel with an amount of glucose giving the same copper reduction. From 1 mg of glucose were produced 289 μl of CO_2 (theoretical 249 μl). From an amount of unhydrolyzed solution with a copper reduction equivalent to 1 mg of glucose were obtained 163 μl of CO_2 and from the hydrolyzed 191 μl. The same discrepancy between Cu values and the CO_2 produced by yeast that was noted for the sludge strains is again evident here. Copper reduction tests on the yeast suspensions at the end of the run showed that all the sugar had been used. These results indicate that the major part of the sugar was glucose. In this respect, the coccus resembles the thermophilic cellulolytic bacteria studied by McBee (22) which also produce glucose but are unable to ferment it.

The identity of these cocci with previously described types is not clear. The direct microscopical examination of rumen contents as practised by Henneberg (11) and Baker (2) disclosed the presence of cellulolytic cocci of various sizes which were assigned an important role in cellulose digestion. The morphology and iodophilic reaction of the present coccus agree with those of certain of the forms seen by Henneberg and Baker. If it is actually important in the rumen it must be identical with one of them. The identity will perhaps best be established through microscopic studies of the pure culture under conditions closely approximating those in the rumen. The form studied by Sijpesteijn and called *Ruminobacter parvum* may be similar to our colorless coccus. Until pure cultures are obtained, however, the relationship cannot be definitely determined.

The rapidity with which cellulose is digested by the coccus and its frequent presence in large numbers favor the assignment to it of an important role in the fermentation of cellulose in the bovine rumen. These features, together with its restriction to cellulose and cellobiose as substrates, the characteristic shape of the colonies in rumen liquid cellulose agar, its iodophilic reaction, its obligate anaerobic nature and special growth requirements mark it as a unique cellulolytic bacterium.

2. The Yellow Cocci. Two strains, A and C, of cellulolytic cocci which form a yellow pigment have been isolated from the rumen and grown in pure culture. These strains were similar in colony appearance and in the incompleteness with which they digested cellulose (15). The colony shape in cellulose agar is usually first a simple lens, later becoming more complex with secondary discs at various angles to the original. For a considerable time there may be little indication that the colony is cellulolytic. Later a slight clearing of the cellulose appears in the immediate vicinity of the colony and in very old cultures an almost complete digestion may be evident. There is not a sharp line of demarcation between the cleared area and the adjacent medium.

The yellow color does not always serve to distinguish these colonies since they are often white when growing in rumen fluid cellulose agar. It is rather the incompleteness of the cellulose digestion. The compactness of the colony in cellulose agar distinguishes it from the previously discussed colorless cocci which show a typical branching colony form. The yellow cocci are usually slower to develop than are the other forms.

Both strains of the yellow cocci were carried for a considerable interval in pure culture, but more complete studies were made with strain A. It was isolated from rumen contents which were relatively inactive in fermentation and in which few protozoa were present. In the initial dilution series from which strain A was obtained cellulose decomposing colonies appeared only in the first tube, indicating that there were extremely few in this particular rumen. During the first several dilution series, single colonies were picked to rumen fluid cellulose agar. Later 0.1 to 0.2 per cent yeast extract was substituted occasionally for the rumen fluid in the medium and good growth resulted, though possibly a little slower. From cellulose agar, the colony was diluted into two rumen fluid glucose agar series and from the second sugar series back to cellulose yeast extract in which colonies appeared after 7 days of incubation. Subsequent transfers were made to cellulose yeast extract medium. The subcultures from the agar series became less dependable as time passed and it was finally no longer possible to obtain growth. Transfers in liquid culture continued viable for an additional period but these, too, finally failed to grow after seventeen months of culture.

Cells in young cultures of strain A showed a strong affinity for iodine, giving a bluish brown color when stained with this reagent. Cells in older colonies show very little or no iodophily. The gram reaction is variable. A 2-day culture on rumen glucose agar was negative; a 7-day culture was also negative; a 9-day liquid culture on cellulose yeast extract showed a few positive cells, and an 11-day culture on cellulose yeast extract agar showed portions of a great many cells gram positive. This suggests that the medium may influence the Gram reaction.

Data on the temperature characteristics and the substrates fermented by strain A are incomplete. In one test it was found that no growth occurred in an agar dilution series incubated at 30 C during a period of 12 days, whereas a parallel series at 38 C showed growth.

Other than cellulose, glucose was the only carbohydrate tested for fermentability, and it was readily attacked. From the fact that the coccus produces cellobiose in old cultures it may be inferred that also this sugar is fermented.

A slight production of yellow pigment is often noted when strain A is grown on rumen fluid cellulose agar but it is much more marked when yeast extract is used. In tubes of yeast extract cellulose broth the undigested cellulose becomes increasingly colored and is finally a distinct yellow. Upon addition of acid to the medium the yellow color is greatly intensified and more orange. The pigment is confined chiefly to the bacterial cells since it collects in the sediment when an acidified culture is left undisturbed.

Cultures for the determination of fermentation products were grown in a liquid medium of yeast extract, cellulose, and inorganic salts. Phosphate was the buffer. An excess of cellulose was employed and the cultures were incubated for some time after fermentation had ceased (total time: sample A, 180 days; sample B, 67 days). This gave an opportunity for digestion of cellulose without accompanying fermentation and led to the accumulation of copper reducing substances. In test tube cultures it was established that the reducing substances did not appear until fermentation was practically complete.

TABLE 6

CO_2 Produced During Yeast Fermentation of Hydrolyzed and Unhydrolyzed Culture Medium

	0.5 ML UNHYDROLYZED SAMPLE		0.5 ML HYDROLYZED SAMPLE		0.5 MG GLUCOSE	0.475 MG CELLOBIOSE
	A	B	A	B		
μl CO_2 formed	32.4	29.4	80.5	78.6	120	16.7
μl excess over amt. formed in cellobiose control	14.2		62.9		103.3	

A sample of the flask culture when hydrolyzed with acid gave a 44 per cent increase in the amount of copper reducing substances. This indicated that cellobiose was present. The copper reduction after hydrolysis was equivalent to 1 mg glucose per milliliter of culture. The amount of yeast-fermentable sugar (glucose) in 0.5 ml of the culture before and after hydrolysis was estimated manometrically. Parallel vessels containing 0.5 mg glucose, and 0.475 mg cellobiose, respectively, were also run. The CO_2 production in the various vessels is shown in table 6.

This experiment indicates that most of the sugar is cellobiose. A small amount of glucose is suggested by the gas production from the unhydrolyzed culture. However, in view of the small amount and the fact that the culture medium may have contained other yeast-fermentable materials, the evidence for glucose is not conclusive.

The amount of CO_2 produced from the hydrolyzed sample of table 6 is only 60 per cent of the amount that would be expected if all the reduction were due to glucose. Similar discrepancies have already been noted in experiments with the sugars from the sludge bacteria and the colorless cocci.

An osazone experiment also failed to demonstrate the presence of glucose. Lacking conclusive evidence for glucose, it was assumed that all the copper reduction was due to cellobiose and the amount present was calculated on that basis.

The culture medium was analyzed for other fermentation products. Chromatographic analysis of the volatile acids showed the presence of only acetic acid. No fatty acids higher than acetic could be detected. Lactic acid was the principal non-volatile acid formed. The estimation of succinic acid by manometric technique with succinic dehydrogenase showed only traces present. Carbon dioxide, hydrogen, and ethyl alcohol were the other products identified and measured. The amounts of all products recovered in the experiment are shown in table 7. Since the control contained only 0.2 per cent yeast extract negligible quantities of fermentation products were formed in it.

Inspection of table 7 shows that lactic acid is the principal fermentation product of the yellow coccus, strain A. This feature distinguishes it from the other cellulolytic bacteria which have been studied.

Strain C, the other yellow coccus, was also isolated from a rumen in which fermentation was relatively slow and in which few protozoa were present. There was more activity, however, than in the rumen from which strain A was ob-

TABLE 7

Summary of Products Formed by the Yellow Coccus

	EXPT. A		EXPT. B	
	mg	mmol	mg	mmol
CO_2	13.5	.307	9.4	.214
H_2	.53	.264	.3	.15
Acetic acid	33.0	.55	15.7	.252
Ethyl alcohol	11.6	.252	14.5	.315
Lactic acid	61.3	.68	49.0	.545
Cellobiose	80.0		167.0	
Cellulose disappearing	259.6		298.8	

tained. The agar series yielding strain C showed cellulolytic colonies in the first 5 dilutions and gave a count indicating 200,000 cellulolytic units per ml.

The initial dilution medium contained cellulose, agar, inorganic salts, thioglycolate, and, for each 10 ml, 2 mg asparagine, 0.012 γ biotin, 1 γ each of thiamine, Ca pantothenate, nicotinic acid, and riboflavin, and 3 γ pyridoxine. A parallel series using grass extract in place of the vitamins gave about the same number of cellulolytic colonies. The colonies appeared sooner (11 days) in the grass extract and grew faster, but subcultures failed to develop after a few transfers.

Colonies appeared in the lower dilutions of the vitamin series and grew very slowly. Six months later there was a single clear spot in tube 5 with a colony in the center. Microscopic examination showed cocci and some irregular rods. This colony was subcultured 8 successive times into dilution series of rumen fluid cellulose agar, usually with parallel inoculations into glucose series. At the 9th transfer it failed to grow and additional attempts to subculture were unsuccessful. Throughout the period of culture the colonies in the center of the area of cellulose digestion were composed of cocci.

On two occasions distinct capsules were observed. The cells occurred singly, in twos, in short chains, and in longer chains up to 16 to 24 cells. Different cultures varied in the extent of chain formation and in some the cells were chiefly single or in twos with only an occasional short chain. The diameter of the cells varied also, but usually ranged between 0.7 and 1.0 μ.

At the first subculture of this coccus a parallel cellobiose series was inoculated but the colonies developing were found to consist only of rods. At the second subculture a parallel glucose series showed colonies of rods and also smaller colonies of cocci, but these latter failed to grow upon subcultures into cellulose and glucose. At subsequent transfers a glucose dilution was always inoculated along with the cellulose series but on no occasion was any growth observed. This indicated that the culture was purer than before and the technique of diluting single colonies into solid medium probably gave a pure culture, but due to its failure to grow alone in the glucose medium the purity was not thoroughly established.

The failure to obtain growth on cellobiose and glucose media may not exclude these as substrates which can be attacked. In one culture cocci were found growing in the glucose series though their identity as cellulose-decomposers was not fully established. The cultural conditions were apparently not well suited to growth of this strain as evidenced by the long interval before colonies appeared and by failure to grow continuously in subculture. It is possible that lack of growth on glucose was due to unsuitability of the medium. The fact that appreciable quantities of copper reducing materials were formed in old cellulose agar tubes strongly suggests that at least cellobiose, and possibly glucose, were attacked.

Attempts to isolate additional yellow cocci have not been successful. Several rumen fluid cellulose agar series inoculated with rumen fluid have failed to show colonies of yellow cocci. Until more strains are studied it does not seem advisable to draw final conclusions as to whether strains A and C are variants of the same species. They are somewhat similar to the yellow coccus isolated by Sijpesteijn (38) and called *Ruminococcus flavefaciens*, but until additional pure cultures have been isolated and more complete studies made of the range of variation exhibited by these yellow cocci of the rumen it seems preferable to consider them as a group without assigning generic and specific status.

The isolation in pure culture of the cocci of the rumen, and the demonstration that they are numerous and active in cellulose digestion, fully substantiate the conclusion, based on direct microscopic examination, that cocci are important in the decomposition of crude fibre in the bovine rumen. Anaerobic cellulose decomposing cocci have thus far been found only in the rumen and their rather specific growth requirements suggest that the isolated strains are limited in their distribution to this habitat or other similar ones. Further studies on the extent to which they occur in the alimentary tracts of other mammals will be of great interest.

IV. SPOREFORMING RODS

This group of anaerobic cellulolytic bacteria must be widespread in nature because most of the strains encountered by various investigators have been

sporeformers. Soil, sewage, compost, manure, river mud, and the contents of the alimentary tract of numerous kinds of animals have all readily yielded sporeformers capable of digesting cellulose. The prevalence of this type in these habitats and the relative scarcity of nonsporeforming cellulolytic anaerobes may be due to the fact that the periods in which conditions (of temperature, moisture, anaerobiosis, and substrate) are favorable for growth occur only intermittently. Sporeformers are better fitted to survive in abundant numbers and take quick advantage of the next favorable period for anaerobic cellulose fermentation.

There is another factor which may explain the observed predominance of sporeformers among the strains isolated. Most investigators have inoculated enrichment cultures. Fairly large concentrations (1 to 2 per cent) of cellulose were used and subcultures made after most of the cellulose had disappeared. Under these conditions the medium becomes quite acid and nonsporeforming cellulose digesting bacteria tend to die out. If not eliminated by the first transfer they are after several.

That enrichment cultures are poorly suited for the identification of types and relative numbers of cellulose decomposing bacteria in their natural habitat is most clearly illustrated by the experiences with those in the rumen. Starting with Ankersmit in 1905, and continuing with the work of Clausen (4), Khouvine (18), R. Meyer (25), Pochon (28) and Sijpesteijn (38), the only cellulolytic bacteria obtained by the use of enrichment cultures were sporeformers. Ankersmit (1) recognized that these were not the important organisms in the rumen and this was the conclusion reached by Henneberg (11), Baker (2), Hungate (13) and Sijpesteijn (38).

The enrichment method alone is inadequate for estimating the importance of bacteria in nature unless it is accompanied by dilution techniques. In certain types of problems in microbial ecology as, for example, in examining animals and plants for pathogens, the establishment of their presence may be all that is desired, and suitable enrichment cultures are adequate. But for most activities of microörganisms this method is of limited value as has been repeatedly emphasized by Winogradsky (46).

One of the first steps in studying the ecology of organisms in a given habitat is to count them. This does not tell whether the cells are in a resting or active state, nor assay the degree of activity, but it is a first approximation. It is highly desirable that increased study be made on reliable methods for determining the numbers of those microörganisms which are of importance in their natural habitats.

Counts of the cellulose digesting bacteria are relatively easy. The insoluble substrate makes easy the detection of colonies produced from cellulose digesting units in a natural inoculum. Use of known dilutions gives semi-quantitative estimates of numbers.

It has seemed desirable to repeat some of the examinations for the types of cellulolytic bacteria active in soil. Two culture experiments were performed. The first involved quantitative dilutions to test whether sporeforming cellulose bacteria actually predominate in the usual enrichment cultures inoculated with soil.

Soil was inoculated into a glass stoppered bottle containing some filter paper and filled with an inorganic solution composed of 0.1 per cent $(NH_4)_2SO_4$, 0.05 per cent K_2HPO_4, 0.03 per cent KH_2PO_4, 0.01 per cent $CaCl_2$, 0.01 per cent $MgSO_4$ and 1 per cent $CaCO_3$. Active cellulose digestion was evident after two weeks of incubation at 30 C at which time quantitative dilution series of cellulose 0.2 per cent yeast extract agar were inoculated. After ten days clear spots were present in the tubes of higher dilution and a small colony visible in each. Some were yellow and others white. Microscopic examination of several colonies disclosed sporeforming rods. An attempt was made to purify the yellow colonies but no growth was obtained. A typical white colony was subcultured until pure. It has been designated as soil strain E.

This quantitative study on an enrichment culture from soil indicated that sporeformers were the most numerous cellulolytic bacteria. But from this result alone it cannot properly be concluded that the sporeforming cellulose digesters are most numerous in the soil. They may have outgrown and suppressed the nonsporeforming types in the enrichment culture. In order to obtain information on the kinds actually present in soil a second experiment using direct dilutions was run.

In this experiment 0.5 gm of soil was diluted into 10 ml of an inorganic medium containing 0.2 per cent yeast extract, 1.5 per cent agar, and 2.0 per cent finely divided cellulose. Inocula of 0.5 ml were transferred in each dilution. After 85 days the stopper had blown out of tube 1. In tube 2 about 20 colonies were counted, indicating about 800 cellulolytic units per gm of soil. Eight strains which seemed slightly different were subcultured into a fresh agar series. In tube 3 were found four colonies of sporeformers and a single nonsporeforming rod, indicating about 4000 units per gram of soil. The nonsporeformer was subcultured.

After 30 days clear spots were visible in the subcultures from tube 2, and in the center of most of them could be seen a very much branched colony with the filaments radiating out irregularly from the point of initiation of the colony. In the subculture from tube 3 there developed non-filamentous spherical colonies with an irregular surface. Microscopic examination disclosed that the thready colonies consisted of gram negative rods with terminal spherical spores whereas the spherical colonies contained gram negative nonsporeforming rods. Two of the filamentous colonies were subcultured continuously in cellulose agar dilution series. The remaining strains were discontinued as they appeared identical. The nonsporeforming rod was also subcultured and has been described in a preceding section.

The thready colony type is possibly identical with one of the strains isolated by Clausen (4) from cattle feces. His pictures show a close resemblance to these strains isolated from soil, and his remark, "Wir haben in keinem Fälle Aufhellungszonen um die Kolonie auf zellulosehaltigen Nährboden beobachten können" also is in partial agreement. The filamentous colonies in cellulose agar are at first visible among the particles of cellulose before there is any outward indication of digestion. Upon longer incubation, however, the cellulose around the colony begins slowly to disappear and finally almost all of it in the tube is

digested. This indicates that the cellulase is not strongly adsorbed on the cellulose in the immediate vicinity of the colony but is free to diffuse away from the substrate before the latter is completely dissolved.

In addition to *Clostridium cellobioparus*, whose characteristics have already been described (13), a total of seven other sporeforming strains of anaerobic mesophilic cellulose decomposers have been isolated in pure culture. Two of these strains were isolated from the termite which also yielded *Micromonospora propionici* (14). Two others were encountered in the course of trying to culture the active cellulose digesting bacteria in the rumen. Soil strains C and F were obtained from the cellulose agar series inoculated with soil and used for counts. Strain E was the isolate from the cellulose enrichment culture inoculated with soil. Also, a subculture of *Bacillus cellulosae dissolvens* was obtained from Khouvine by Dr. R. H. McBee who kindly sent it to the author. The results of studies on some of these strains will be discussed.

The cellulose agar dilution series inoculated with the soil C and F cultures showed a slow development of the colonies, and the visible cellulose digestion was even slower. The digestion continued over a long period until finally almost all of the substrate disappeared. This slow and continued solution of the cellulose had been due in most other strains to continued digestion after fermentation had ceased. In such old tubes a reduced pressure developed, possibly due to the outward diffusion through the rubber stopper of carbon dioxide or hydrogen. But the old tubes of C and F showed a positive gas pressure. On analysis, the gas was found to consist of carbon dioxide and hydrogen. This suggested that fermentation might have occurred even in old tubes, and in confirmation of this a test for copper reducing materials was entirely negative. Of all the strains of cellulose bacteria thus far encountered, these two sporeforming strains from soil and the one nonsporeforming strain are the only ones which ferment most of the cellulose in the usual cellulose agar tube. With all the others, [2] fermentation ceases after 0.1 to 0.5 per cent cellulose has been decomposed, and they either hydrolyze the cellulose after fermentation has ceased or fail to hydrolyze further as was the case with *Bacteroides succinogenes*.

The other fermentation products of soil strains C and F were determined by analyzing old agar tubes. The agar was forced out into a flask and weighed to determine the amount of culture medium. Some NaOH was added to neutralize fermentation acids and the alcohol distilled off. An aliquot was oxidized with $K_2Cr_2O_7$ to an acid which, by its Duclaux distillation curve, was identified as acetic acid. The agar residue of the alkaline distillation was acidified and steam distilled but the foaming due to the agar was so excessive that the solution was again neutralized and the agar precipitated with 8 volumes of ethanol. The alcohol was distilled off at low pressure and the residue acidified and steam distilled without excessive foaming. The volatile acids were titrated and an aliquot run on the chromatogram. Only acetic acid was present.

The non-volatile acids were extracted with ether from the residue of the volatile acid distillation, titrated, and then tested for lactic and succinic acids. Some lactic acid was present but no succinic. The results of the analyses on soil strains C and F are shown in table 8.

[2] The less actively cellulolytic rumen rod has not yet been adequately studied in this respect.

Considerable quantities of hydrogen and carbon dioxide must have been produced, which, if included in table 8, would materially increase the total recovery of products.

Strain E from the enrichment culture inoculated with soil was grown on two yeast extract cellobiose agar series and returned to yeast extract cellulose agar to obtain a pure culture. It was inoculated into yeast extract cellulose broth but failed to grow, suggesting that the agar provided some essential factor. A number of other liquid media were tested; dried grass, rumen liquid, proteose peptone, and nutrient broth were found to support excellent growth in liquid medium. Distillers dried solubles, tryptone, and malt sprouts were fairly satisfactory.

TABLE 8

Fermentation Data for Soil Strains C and F

	C	F
Weight of medium	30.7 g	48.5 g
Estimated cellulose fermented	460 mg	730 mg
Acetic acid	97	131
Ethanol	79	83
Lactic acid	4	8

TABLE 9

Fermentation Products of Soil Strain E

Cellulose decomposed (as $C_6H_{10}O_5$)	0.43 mmol
CO_2	0.57
H_2	0.75
Ethanol	0.07
Acetic acid	0.48
Lactic acid	0.10
Succinic acid	0.21

No growth occurred in corn steep liquor, yeast extract or inorganic solution alone. In later work nutrient broth was used for routine transfers and quantitative cultures.

Strain E grew rapidly at 37 to 45 C. Some development took place at 26 C but none at 20 C. No growth occurred at 55 C.

The sugars and other carbon substrates tested for fermentability by *Bacteroides succinogenes* were also inoculated with strain E. L-arabinose, L-xylose, D-glucose, cellobiose, and the three samples of hemicellulose were the only substrates fermented. These results suggest that in nature the strain is almost exclusively concerned with the decomposition of cellulose and hemicellulose.

Quantitative determinations of fermentation products were made and the results are shown in table 9.

The amount of carbon dioxide found is probably too high since the carbon recovered exceeds the amount initially present and the average state of the

products is more oxidized than carbohydrate. In other respects the materials formed are similar to those encountered in cellulose fermentations by other sporeformers.

Reducing substances accumulate in old cellulose agar tubes. These were extracted as previously described and tested for increase in copper reduction upon hydrolysis and for yeast fermentation of the hydrolyzed and unhydrolyzed samples. Hydrolysis caused a significant increase in copper reduction and in CO_2 production by yeast indicating the presence of cellobiose. The CO_2 production by yeast from the unhydrolyzed sample showed the presence of glucose. The same discrepancies between copper values and carbon dioxide from fermentation that were noted for sludge strain D and the colorless coccus were also found for soil strain E.

Having obtained a number of strains of sporeforming cellulose anaerobes in pure culture, and having studied the characteristics of a number of them it seems profitable to review previous work in the light of recent information.

Trécul (43) appears to have been the first to call attention to the importance of microorganisms in the decomposition of plants, but he did not demonstrate specifically that cellulose was attacked. Popoff (34), in studying the production of methane from sewage mud plus various substrates, observed that plant fibers became covered with microörganisms and that methane production was more closely correlated with cellulose than with the decomposition of sugars or protein. This was the first observation which related methane production and cellulose decomposition, a relationship which has been repeatedly noted in subsequent studies. Popoff reported that in old cultures layers of rose to violet-red bacteria appeared near the surface, and below them a layer of deep grass green bacteria. He assumed that these were the agents decomposing the cellulose, but in the light of subsequent knowledge (44) it is evident that they were photosynthetic purple and green bacteria. The author has confirmed Popoff's observations and found that sewage sludge placed in a transparent container in diffuse light is an excellent enrichment culture for both purple and green bacteria. They presumably live in part at the expense of the decomposition products from the cellulose in the sludge. Quantitative dilutions of the zoogloea on the rocks of a trickling filter have shown purple bacteria in numbers exceeding 10,000 per ml.

Van Tieghem (42) studied the microbes associated with the fermentation of cellulose and named *Bacillus amylobacter* as the agent which attacked the cell walls of decaying plant material. His observations were the first which called attention to the importance of the sporeforming anaerobes in cellulose decomposition. Hoppe-Seyler (12) was the first to obtain quantitative data proving the anaerobic fermentation of cellulose. The products recovered were chiefly CO_2 and CH_4. He reported that these gases were produced by a culture at a relatively constant rate for about one year.

The mesophilic cellulose bacteria associated with animals were first demonstrated by von Tappeiner (40, 41) when he showed that microörganisms were responsible for the cellulose digestion in the rumen. No cellulolytic enzymes secreted by the host could be detected. Bacteria were believed responsible for cellulose digestion and it was shown that acetic and higher fatty acids, CO_2 and methane were formed when cellulose disappeared *in vitro*. These products were assumed to be available to the host. It is of interest to note that, although the conclusions which von Tappeiner drew regarding the mechanism of cellulose utilization in the rumen were correct, it is very doubtful that the cellulolytic bacteria active in his cultures were the same as those in the rumen. His cultures showed a delayed fermentation starting 6 days after inoculation which continued for several weeks. The bacteria grew readily on meat infusion broth. These phenomena are so similar to those which have been encountered by subsequent investigators in cultures in which the cellulose digesters were

sporeformers, that it is almost certain that also in von Tappeiner's cultures the active bacteria were sporeformers rather than the nonsporeforming rods and cocci which have since been found. However, the products in the rumen cultures and in other cellulose enrichments are similar, and the conclusion of von Tappeiner that microbes in the rumen fermented the cellulose was entirely correct.

Omelianski (27) used enrichment cultures for demonstrating cellulolytic bacteria in various inocula. In some of these hydrogen was formed whereas others produced methane. He concluded that the cellulose was decomposed by two different sporeformers, one producing methane and the other hydrogen. Omelianski was well aware that he was working with impure cultures but assumed that, with cellulose as the only organic substrate, the other bacteria would be eliminated to a considerable extent. The invalidity of this assumption was demonstrated when Kellerman and McBeth (17) isolated from Omelianski's hydrogen culture two species of cellulose decomposers which were different in morphology from the ones observed by Omelianski; and in addition they demonstrated the presence of five aerobic non-cellulose decomposers. Kellerman and McBeth made no attempt to determine the number of kinds of obligately anaerobic bacteria in the hydrogen culture so it is evident that the demonstrated contaminants were only a part of the total. Contaminants were also found in the methane culture and it must be concluded that the evidence presented by Omelianski was inadequate to prove that either methane or hydrogen was produced from cellulose by the organisms he described.

The chief contribution of Omelianski was that he developed enrichment culture methods by which crude cultures of cellulose bacteria could be readily obtained, and substantiated the observations of van Tieghem that the active cellulose decomposers were curved rods with terminal spores. Most of the subsequent workers have obtained similar results with enrichments.

Ankersmit (1) studied the cellulose bacteria in the rumen by means of direct microscopic examination and also by inoculating various dilutions of rumen contents into the enrichment medium of Omelianski. He obtained cellulose decomposition but noted that the numbers of bacteria indicated by his dilutions were too small to account for the active cellulose decomposition in the rumen. He further noted that the cellulose bacteria in his enrichment cultures were the curved sporeforming rods similar to those obtained by Omelianski and did not resemble the cocci and nonsporeforming rods seen in the rumen fluid. Ankersmit's paper is significant because he recognized that mere cultural demonstration of cellulose decomposers is not sufficient evidence to prove that the cultured bacteria are important in the rumen.

This defect in the enrichment technique was also pointed out by Henneberg (11). "Durch Anreicherung, sowie durch Anlegen von Kulturen stört man sofort das natürliche Nebeneinandervorkommen." Henneberg concluded from microscopic observations that cocci instead of sporeformers were the active cellulose decomposers in the rumen and from the morphology alone described *Micrococcus ruminantium* and *Streptococcus jodophilus*. He emphasized that the bacteria staining with iodine were the cellulose decomposers, assuming that stainable reserve polysaccharide could be formed only if a carbohydrate food was available, and assuming further that cellulose was the only carbohydrate present. The same criterion of cellulose digestion has been used by Baker (2) in his microscopic studies on intestinal cellulose bacteria. Although this criterion applies to certain species of anaerobic cellulose digesting bacteria, it is not applicable to all (15).

Pringsheim (35) was the first to show that cellobiose and glucose are intermediates in the anaerobic breakdown of cellulose. Thermophilic, hydrogen producing, methane producing, and denitrifying cultures, when treated with antiseptics, showed accumulation of sugar. Both glucose and cellobiose were identified. His experiments were of great significance in that they constituted the first successful attempts to analyze the steps in the process by which cellulose is converted to fermentation products. However, Pringsheim attached more importance to glucose as an intermediate than has been substantiated by subsequent work. He concluded that glucose was the product of cellulose decomposition

supporting the development of nitrogen fixing bacteria, and that glucose was absorbed by those hosts with symbiotic cellulose bacteria in their alimentary tract. His findings so influenced work on the role of cellulose bacteria in ruminant nutrition that for 25 subsequent years the alternative theory of von Tappeiner that the fermentation acids were the useful materials for the host was almost completely ignored. This latter theory has recently received factual support through the investigations of the Cambridge workers (6).

The papers appearing in the period following Pringsheim emphasize the problem of the isolation of the cellulose anaerobes in pure culture. One of the most thorough investigations was by Khouvine (18). She isolated *Bacillus cellulosae dissolvens* from the contents of the alimentary tract of man, guinea pig, rabbit, horse, sheep, and cattle. It could also be demonstrated in many soils. The technique of isolation was to remove from an enrichment culture a piece of filter paper on which the cellulose bacteria were growing and to wash it thoroughly in several changes of sterile water. The paper was then inoculated into a liquid medium containing filter paper. The washing was repeated in two subcultures and after the third treatment no further growth resulted. By adding fecal extract to the culture

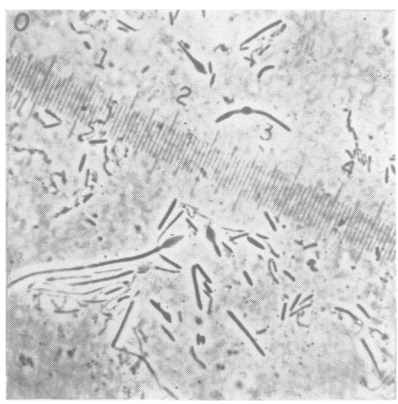

FIG. 8. Fresh mount of *Bacillus cellulosae dissolvens* from a 30 day culture in rumen fluid cellobiose agar. Dark contrast medium phase, oil immersion objective. Each micrometer division equals 0.82 μ.

medium, growth was obtained and a sporeformer and a diplococcus were found microscopically to be the only organisms present. The diplococcus was killed by pasteurization and the culture then considered pure.

Occasional doubts have been expressed regarding the purity of the strain isolated by Khouvine. The culture sent by her was diluted into rumen fluid cellulose agar tubes in the same fashion used with other cellulolytic anaerobes. After two weeks incubation colonies surrounded by clear spots developed. There were no indications of any colonies besides those fermenting the cellulose and it must be concluded that the culture was pure (see fig. 8).

The fermentation products reported by Khouvine for *B. cellulosae dissolvens* are those typical for other sporeforming cellulose bacteria with the exception of small quantities of butyric acid. This has been reported for a number of mixed thermophilic cultures, but the studies of McBee (22) indicate that the pure cultures produce only acetic acid. It is desirable that further investigations of the metabolic products of *B. cellulosae dissolvens* be made using the chromatographic method for identifying the volatile acids. As with the rumen rods and cocci, it is also desirable that a culture medium containing no volatile acids be developed since their presence in the fecal extract or in rumen fluid makes difficult the identification of small amounts which may be produced.

Although Khouvine demonstrated *B. cellulosae dissolvens* in a wide variety of intestinal habitats, its role and significance in the symbiotic decomposition of cellulose is still un-

known. It has not been found in dilution cultures inoculated with rumen materials, and its slow growth makes doubtful any important role in the rumen. It is highly desirable that dilution methods be used to ascertain its numbers in the various habitats from which it has been isolated.

The work of Werner (45) is quite comparable to that of Khouvine except that the cellulose organism studied was an inhabitant of the alimentary tract of the beetle, *Potosia cuprea*. The bacteria were demonstrated in the nest material of the anthills in which the beetle lived as well as in the alimentary tract, and it was concluded that they were derived regularly from the food. It is equally probable that those in the nest material were derived from the alimentary tract. The significance of the bacterium would be much clearer if the numbers present had been determined.

Clausen (4) contributed notably to knowledge of the cellulolytic anaerobes when he developed techniques for anaerobic plate cultures and isolated a number of active cellulose decomposers. It is unfortunate that he did not give more detailed information on the procedures by which pure cultures were obtained and of his criteria of purity, because it would lend greater weight to his data. However, he was the first to report obtaining discrete colonies of cellulose digesting anaerobes in cellulose agar; and from the illustrations of colonies and stained smears of the isolated organisms it seems fairly certain that he had pure cultures of several cellulolytic anaerobes.

Clausen was particularly interested in determining whether the methane and hydrogen producing cultures of Omelianski actually contained different cellulose decomposers. He enriched crude cultures according to Omelianski's methods and obtained simlar results, i.e., a methane fermentation of cellulose when the inoculum was not pasteurized and a hydrogen fermentation with pasteurized inoculum. From each of these cultures he isolated the identical cellulolytic anaerobe. This was the first experimental evidence that the cellulose bacteria in methane producing cultures did not form methane, though this had been suggested by several investigators. Clausen named this organism *Bacillus Omelianskii*. It was only slightly cellulolytic in pure culture in liver infusion broth, but with a contaminant was fairly active. These characteristics and the illustration of a colony which Clausen gives agree well with the soil strains C and F.

In addition Clausen isolated *Amylobacter navicula* which was much more active in decomposing cellulose than was *B. Omelianskii*. *A. navicula* grew well on a variety of sugars as well as on cellulose. From this it would seem highly probable that a pure culture was obtained since by growth in sugar media the contaminating colonies could be detected. Clausen does not report further testing of purity by dilution into solid sugar media and picking isolated colonies, a proof which is extremely desirable. In addition to digesting cellulose *A. navicula* fixed nitrogen and was actively proteolytic. These strikingly diverse characteristics make it additionally desirable that the purity of the culture be established beyond doubt. *A. navicula* can be distinguished by its morphology alone from the other sporeforming cellulose digesters which have been isolated. Its spindle shape and central spore, together with the rapidity of cellulose digestion mark it as a striking cellulolytic anaerobe.

Cowles and Rettger (5) undertook an intensive study of the organism which they obtained from a variety of cellulose enrichment cultures. They isolated a pure culture by repeated pasteurization and streaking on aerobic plates. The areas of no growth were inoculated into a fecal extract cellulose medium and upon addition of *Bacterium aerogenes* cellulose was digested. By pasteurizing, they could obtain a pure culture which grew and digested cellulose in meat infusion broth. The purity of the culture was substantiated when it was found that it would produce colonies on meat infusion cysteine dextrin agar plates incubated anaerobically. The colonies gave cellulose digestion when inoculated into liquid medium. This is the first reported instance in which isolated colonies of cellulolytic anaerobes on non-cellulose media were picked and found to give cellulose digestion.

Pochon (28) reported the isolation of *Plectridium cellulolyticum* from rumen contents and assigned to this bacterium an important role in the digestion of cellulose. The enrich-

ment technique was used and the inocula were pasteurized. The procedure used for obtaining a pure culture is of doubtful adequacy and from the characteristics given, it is probable that a mixed culture was concerned. Two sizes of spores were produced, and both gram negative and gram positive rods. In view of the evidence against an importance of sporeformers in cellulose digestion in the rumen, *P. cellulolyticum* cannot be accepted as playing a significant role unless further evidence is provided.

Pochon has reported on several other cellulolytic cultures (29–32) obtained from various sources. Although it was assumed that the cultures were pure the evidence is not convincing. Repeated subcultures in liquid media with intervening pasteurization were the sole means of purification for most of the cultures. Under these circumstances the reported adaptations to different media could equally well have been selection of suited cellulolytic strains from the mixture present. It must be reemphasized that results obtained with questionably pure cultures cannot but cause confusion as to the true characteristics of the cellulolytic anaerobes.

V. Meyer (26) attacked the problem of cellulose digestion in much the same manner as Clausen, in fact both studies were performed at the Kiel laboratory. Starting from similar enrichment cultures, he was able to isolate a plectridium which decomposed cellulose slowly. It was facultatively anaerobic and could be isolated in pure culture by the usual methods of diluting into solid media and picking isolated colonies. The obligate anaerobes which Meyer encountered were of a "Stecknadel" type, either with oval or round spores. He tried a number of classic methods to obtain a pure culture but without success and finally resorted to the inoculation of portions of aerobic plates which showed no colonies. This "Ausschneideverfahren" had previously been employed by Cowles and Rettger (5) and Werner (45). Meyer recognized the inadequacy of the method, "Denn es ist theoretisch stets möglich, dass bei Anwendung des Ausschneideverfahrens verschiedene Organismen, nicht nur die eigentlich Zellulosezersetzer auf dem Agar nicht zum Auskeimen kommen, so dass man ohne weiteres doch noch nicht zu Reinkulturen kommt, eine Vermutung, die sich leider bewahrheitete." But by combining it with a dilution method he was able to obtain cellulose digesting cultures which showed no aerobic growth when inoculated onto plates. With these cultures he demonstrated that the active cellulose decomposer grew in inorganic media only when accompanying bacteria were also inoculated. A wide variety of "symbionts" supported the growth of the cellulose decomposer in inorganic media. Addition of meat extract and peptone permitted growth without symbionts. These results, together with those of Khouvine, Clausen, and Cowles and Rettger are of significance since they show that cellulose bacteria in an enrichment culture of cellulose and inorganic salts cannot grow on this medium in pure culture. This may explain in part the failure of earlier attempts by Omelianski and others to obtain pure cultures.

R. Meyer (23–25) obtained a number of cultures which were assumed to be pure, but the criteria of purity leave much to be desired. He washed the fibers with their adhering cellulose bacteria according to the procedure of Khouvine and after a few transfers concluded that the culture was pure. A change in the odor from one of proteolysis to a pleasant one suggestive of an ester was an important criterion of purity. His cultures were extremely slow in cellulose decomposition, the most active one being transferred only once every 30 days.

Sijpesteijn's (38) results on the use of the enrichment method for detecting the cellulolytic bacteria in the rumen is the most thorough demonstration of the unsuitability of this method for identifying the active bacteria in their natural habitat. Using more direct methods, she demonstrated the presence of cocci and nonsporeforming rods, in agreement with other recent studies.

From the study of the literature and the isolates of sporeforming cellulolytic bacteria obtained during this investigation it must be concluded that there are a great many strains, varying sufficiently to be distinguishable, but not easily separated as species on the basis of present information. Insofar as general

morphology is concerned most of the strains are gram negative, curved rods with terminal spherical spores. Some have terminal oval spores and a few are gram positive. By their morphology alone, *B. cellulosae dissolvens* and *Amylobacter navicula* stand out as distinct species. The thready colony of *Bacillus Omelianskii* and the slow cellulose digestion also make it distinguishable. It will be necessary to separate most of the others on the basis of physiological characteristics. Before attempting such a classification it will be extremely advantageous to have data for all forms on many more physiological reactions than have been examined thus far. In addition the absolute purity of each strain should be established.

In their fermentation products the sporeforming cellulose anaerobes are remarkably uniform. Carbon dioxide, hydrogen, ethyl alcohol, and acetic acid have been found in all studied cases in which culture purity was definitely established. In addition, formic, lactic, and succinic acids may be present. In view of the possible diverse origin of the various strains as evidenced by their morphological differences, it is surprising that so little variation in type of fermentation products is found. Differences in fermentation products do not appear to be a suitable basis for separation into species.

In spite of the classical assumptions, based on enrichment cultures, that the cellulolytic anaerobic sporeformers can grow on inorganic media, all results with pure cultures indicate that complex organic nutrients other than carbohydrate are required. This can explain some of the difficulty in growing them in pure culture if it be assumed that contaminating forms produce growth substances required by the cellulose decomposers, a view which has already received some confirmation.

The nutritional requirements may offer a better basis for separating the various strains than do the fermentation products. Not only the carbohydrates fermented but also the types of nitrogen assimilated and the required accessory nutrilites need to be determined. These physiological characteristics should also provide clues as to the role of the various species in their habitat, and the factors which affect their natural growth.

V. ECOLOGY

One of the most striking features of the cellulolytic anaerobes is the low total concentration of substrate which can be fermented in cultures. In the medium buffered with carbon dioxide and bicarbonate, the growth of most strains is inhibited when 0.1 to 0.3 g of cellulose per 100 ml medium has been decomposed. A few strains ferment a larger amount, e.g., the sludge strains (0.4 per cent), the less actively cellulolytic rumen rod, and some of the sporeformers from soil (1.0 to 1.5 per cent). These values can be increased by adding $CaCO_3$ to the medium, but the concentration of cellulose which can be fermented under laboratory culture conditions is still relatively low for most strains.

Enzymatic hydrolysis of cellulose may continue for a long time in old cultures in which fermentation has ceased, but the rate is slow and the additional amount of cellulose hydrolyzed is small.

The accumulation of acid fermentation products probably accounts for the inhibition of fermentation. Environmental factors which remove these products will increase the concentration of cellulose which can be decomposed. This hypothesis can be used to explain in part the relation of the rumen cellulolytic bacteria to accompanying forms and to the host. It may explain in part the improved cellulose fermentation which many investigators have reported in crude cultures as compared with partly purified or pure ones. It also may account for the common association between methane and cellulose fermentations.

The cultures of Hoppe-Seyler (12) showed a total conversion of cellulose to CO_2 and CH_4. Recent studies have found no cellulose decomposing bacteria which produce methane. In Hoppe-Seyler's cultures many unknown organisms in addition to methane and cellulose bacteria were concerned with intermediate transformations and through them the cellulolytic and methanogenic bacteria acted in complementary fashion in such a way that natural cellulose underwent a complete anaerobic dissimilation.

The multiplicity of substrates attacked by the equally numerous kinds of microörganisms in nature suggests that any type of energy yielding substrate will be attacked provided other necessary nutritive ingredients and a suitable temperature are present. If it be assumed that in the anaerobic soil environment in which cellulose is fermented to CO_2 and CH_4 there are present all types of bacteria capable of further degrading any fermentation products from which energy can be derived, it may be concluded that no further energy can be derived by anaerobic fermentation of the final products. If this be correct, the results of Hoppe-Seyler's experiments suggest that under anaerobic conditions the maximum free energy is liberated from a cellulose substrate when no fermentation product contains both hydrogen and oxygen, and these elements are separately combined with carbon in the symmetrical molecules, CO_2 and CH_4.

It may be asked why the same complete dissimilation to gaseous products does not occur in the rumen. The answer probably lies in the time relationships. The soil fermentation of cellulose to CO_2 and CH_4 occurs slowly and over an extended period. The rumen fermentation is by contrast very rapid, and many of the intermediate processes which aid in the complete fermentation must be too slow to keep pace with the primary cellulose decomposition. But certain of them apparently are sufficiently rapid to convert some intermediates to other final products.

Some idea of the nature of these conversions in the rumen may be derived by comparing the cellulose fermentation products of the normal rumen with those of the pure cultures. The analyses of rumen fluid by Elsden (6) have shown volatile acids, chiefly acetic, with some propionic, and still less butyric acid also present. Nonvolatile acids (lactic and succinic) were demonstrated in much smaller amounts than the volatile acids. Analyses of the rumen fluid used in control cultures during the present investigation have consistently shown the presence of these same acids in quantities comparable to those found by Elsden. Carbon dioxide and methane are also always formed in a normal rumen fermentation. No evidence of hydrogen as a significant final product has been

observed nor has it been reported in the literature. Tests for ethanol and formic acid in rumen fluid have been uniformly negative.

Although carbon dioxide is apparently absorbed by *Bacteroides succinogenes*, it is produced by most of the pure cultures of rumen cellulolytic bacteria; and it is highly probable that a significant part of the CO_2 appearing as a final product in the rumen is formed by the organisms attacking the initial substrate.

Similarly, acetic acid is formed by most pure cellulolytic anaerobes whose fermentation products have been identified. In most instances it is a quantitatively important product. If the metabolic products of these isolated bacteria are representative of the products formed by all microbes attacking cellulose, hemicellulose, starch, and other fermentable carbohydrates in the intact rumen, it is evident that much of the acetic acid in the rumen is formed during the primary fermentation of the substrate, rather than as a result of secondary processes. The demonstration of acetic acid as an important product of many rumen organisms which attack cellulose accounts in part for the abundant production of acetic acid in the normal rumen.

The finding of butyric acid as a product of the less actively cellulolytic bacterium suggests that the butyric acid occurring as an end product in the rumen is also formed in part during the primary cellulose fermentation. In addition it is possible that non-cellulolytic bacteria in the rumen convert acetic to butyric acid, using hydrogen from the sources mentioned below.

The ethanol, formic acid, and hydrogen formed by pure cultures of rumen cellulolytic bacteria are not end products in the natural fermentation. Also, the concentrations of lactic and succinic acids in the normal rumen are less than might be expected from the analyses of the pure cultures. Other products occur in greater concentrations in the mixture than can be accounted for by the amounts formed by the pure strains, namely, methane and propionic acid.

The absence of hydrogen in the rumen and the presence of methane could be nicely explained if the former were used in producing the latter. This has been suggested by numerous investigators and has been shown to be accomplished by certain methane bacteria obtained from sources other than the rumen (3). A rapid utilization of hydrogen with production of methane by rumen contents has recently been demonstrated in this laboratory by Julia Schmitz and provides experimental support for this explanation.

The formic acid produced by the pure cellulolytic strains may be reduced to methane or dehydrogenated to carbon dioxide. The alcohol is possibly dehydrogenated to acetic acid. The hydrogen, formic acid, and ethanol constitute a source of hydrogen in the primary products that may be used for the reduction of other intermediates.

The propionic acid in the rumen may be accounted for by the reduction of lactic acid and the decarboxylation of succinic acid. Elsden (6) has isolated from the rumen a bacterium which forms propionic from lactic acid, and Johns (16) has demonstrated the formation of propionic from succinic acid by another strain. Sijpesteijn (38) has suggested that succinic acid formed in the rumen may be converted to propionic acid and has found succinic acid formed in a

mixed culture of *Ruminococcus flavefaciens* and *Clostridium* sp. The finding of large amounts of succinic acid as a fermentation product of *Bacteroides succinogenes* lends support to the proposal that decarboxylation of succinic acid accounts for a significant part of the propionic acid in the rumen.

These hypotheses account for the disappearance of some primary products of the cellulose decomposers, and for the appearance of some materials not formed from cellulose by the isolated pure cultures. It should be emphasized that these postulated intermediate steps are largely speculative and are not based on quantitative determinations of rates at which the various substances can be converted in the rumen. But they do provide a coherent picture, are consistent with present results, and suggest reactions the significance of which deserves further investigation.

Suggestions may also be offered to explain the prevalence of nonsporeforming cellulolytic bacteria in the rumen as compared with the predominance of sporeformers in soil. It seems highly probable that the cellulolytic rumen bacteria are symbionts which have undergone a considerable period of evolution in the host. They grow best when rumen liquid is included in the culture medium; and most strains require it. Similar bacteria have not been reported from outside the rumen. It is a common experience of veterinarians that when the microflora of an isolated ruminant has been lost, repopulation with the typical flora may not readily occur and normal digestion is greatly hastened by feeding normal rumen contents. Although it is possible that the rumen cellulolytic bacteria occur elsewhere in nature, other types may be better fitted for these other habitats, and if the rumen forms exist outside the host they do not find there the same optimal factors which support their abundant development in the rumen.

The qualities which make the rumen bacteria particularly well adapted are their rapid growth and accompanying active cellulolysis. Since the feeding and absorptive activities of the host keep their environment favorable, there is little value in their possessing the capacity to survive an unfavorable period through sporulation. Since sporulation confers little survival advantage it either has never evolved or has been lost. The fact that food in the rumen is not retained indefinitely places a high survival value on rapidity of cellulose digestion, and selection has been in this direction rather than toward survival through sporulation.

In the sludge fermentation as in the rumen there is a continuous feed of substrate and the conditions for fermentation remain constant over considerable periods of time. This favors the development of nonsporeforming types and may account for their predominance in sludge. But the holding period in the sludge tank is much longer than in the rumen and there is a more complete fermentation similar to that in Hoppe-Seyler's experiments. The sludge does not usually become acid. Methane and CO_2 probably account for a much larger proportion of the cellulose fermented than is the case in the rumen. The cellulolytic bacteria which come to predominate in the sludge fermentation probably occur elsewhere in nature as well. The single nonsporeforming strain isolated from soil is distinctly different from the sludge forms as well as the rumen rods.

But other free-living types undoubtedly exist. It is possible that the sludge forms may come in part from the human intestine.

There is a marked prevalence of obligately anaerobic bacteria among the cellulolytic strains isolated from the rumen and from sludge. No facultatively anaerobic strains have been encountered. Both these habitats are highly anaerobic, and it is probable that their oxidation-reduction potential is maintained at a constant low value. This may account for the prevalence of obligate anaerobes in these environments. Organisms operating over a wide range of oxidation-reduction potentials might be expected to require a greater variety of enzymes than those living within narrow limits, and their protoplasmic machinery would be correspondingly more complex. The maintenance of this more complex machinery in an environment in which it would not be used could constitute a handicap which would account for the predominance of obligately anaerobic bacteria over those able to grow also in air.

During the present investigations, the possibility of using pure cultures of cellulose bacteria for industrial fermentations has been kept in mind; but none of the strains encountered appears to possess characteristics suitable for an industrial process. First, the concentration of cellulose which is fermented is very low in the case of the rapid fermenters such as the rumen rods and cocci; and second, each of the diverse products is present in an even smaller concentration. This precludes their economic recovery with the industrial processes at present available. The possibility of continuously extracting fermentation acids with an immiscible non-toxic solvent from which the acids could be removed by passage through an alkaline solution has been considered as a means of removing and concentrating fermentation acids. A preliminary test by Dr. R. H. McBee showed that extraction was too slow and that there was too much emulsification of the solvent.

Those bacteria such as soil strains C and F decompose larger concentrations of cellulose but their growth is exceedingly slow, and again the diversity of products does not favor an industrial process. The butyric acid producing rod from the rumen may decompose a larger concentration of cellulose but does not rapidly digest the more resistant types, such as the cellulose in cotton and wood.

Only a small number of strains of cellulolytic bacteria have been discovered thus far, and it is quite possible that there exist types which can ferment larger concentrations of cellulose with less diversity of products. Workers in the field may well be watching for such strains.

The variety of fermentation types discovered thus far is not very great, since most strains produce H_2, CO_2, ethanol, formic acid, acetic acid, lactic acid, and succinic acid in varying proportions. An exception is the fermentation by *Micromonospora propionici* in which CO_2, acetic and propionic acids are formed, but this fermentation is very slow. The fermentation of cellulose by *Bacteroides succinogenes* produces succinic acid equivalent to a large fraction of the cellulose fermented, but the total concentration is still not high. The fermentation to butyric acid by the less actively cellulolytic rod needs further exploration of its industrial possibilities.

The use of controlled cultures containing mixtures of several pure strains has been considered as a possible means of influencing the nature of the products and increasing the concentration of cellulose which can be fermented. *Saccharomyces cerevisiae* was inoculated with sludge strain D into a cellulose yeast extract agar series. A parallel control contained no yeast. Both were incubated at 30 C, at which temperature growth of the sludge form was slow. No differences in the initial rate of cellulose digestion could be detected. The tubes were kept for two years at which time all cellulose in the tubes with yeast had been hydrolyzed. The control cellulose was almost all hydrolyzed. A test for copper reduction by the fluid collected above the agar was negative for the yeast series whereas the control showed copious copper reduction. The results suggest that the yeast fermented the sugar formed after fermentation had ceased. In another experiment *Bacteroides succinogenes* was inoculated with yeast into a liquid tube of rumen cellulose. After the cellulose had been fermented, the culture was tested for ethanol but none was found. This indicated that the presence of the yeast had not modified the cellulose fermentation in the direction of alcohol production.

A more extended study of the interrelationships between the cellulolytic bacteria and accompanying forms might lead to valuable industrial processes, but in view of the low concentrations fermented and the problems concerned with the recovery and separation of products and in view of the cost of gathering cellulosic materials to a central fermentation plant, it does not seem feasible at present. The cost of collection could be decreased by having numerous small fermentation plants but the expense for capital outlay and operation would be prohibitive. The concentration of cellulose might be increased if the inhibitory fermentation products could be continuously removed, but no process for this is available. In summary, an industrial cellulose fermentation might be profitable if the cost of collection of raw materials could be minimized through use of numerous small plants, if these small plants could be cheaply constructed, if operation could be made automatic to decrease necessary personnel, and if the concentration of cellulose fermented could be increased by continuous removal of fermentation products. Although such a situation is at present quite out of the question as an industrial process, it is almost an exact specification of the ruminant animal, a small fermentation unit which gathers the raw material, transfers it to the fermentation chamber and regulates its further passage, continuously absorbs the fermentation products, and transforms them into a few valuable substances such as meat, milk, etc. To these advantages must be added also the crowning adaption: the unit reduplicates itself.

There seems little possibility or desirability at present of substituting artificial machinery for the ruminant. It would appear rather that improvements in biologic cellulose utilization will lie along lines of analyzing the ruminant mechanism and determining ways in which it may be modified to provide a more profitable utilization of available materials. Numerous studies have already been made on partial substitution of wood pulp for hay and of ammonia for protein in the nutrition of ruminants. Further improvements based on exact studies of the nutri-

tional requirements of the rumen microörganisms appear highly desirable. In approaching the problem it should be kept in mind that the first step in ruminant nutrition is microbial nutrition. Microörganisms are first fed and they in turn feed the host. This emphasizes the need for continued investigations in order that the present results may be extended to give a complete and coherent knowledge of microbial processes in the ruminant and in related forms.

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