STUDIES ON CELLULOSE FERMENTATION

III. The Culture and Isolation of Cellulose-decomposing Bacteria from the Rumen of Cattle[1]

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There seems little doubt that cellulose-digesting microorganisms are extremely important in the digestion of plant materials in cattle and related forms (von Tappeiner, 1884). However, attempts to grow them and to study them under controlled conditions have been almost uniformly unsuccessful. Certain of the rumen protozoa have been shown to digest cellulose (Hungate, 1942, 1943). But their removal from the rumen does not impair cellulose digestion (Becker, Schulz, and Emmerson, 1929), and it must be concluded that other organisms also exercise this function. It has been assumed on the basis of microscopic examination (Henneberg, 1922; Baker, 1942) that these other cellulose-digesters are bacteria.

The present study was undertaken to test for cellulose-digesting bacteria in the rumen by cultural methods. The aim of the experiments has been to culture the cellulose bacteria, isolate them, and to estimate the numbers in which they occur. The latter was deemed essential in order to eliminate those cellulose-digesting species entering the rumen with the food but not participating significantly in the digestion of the cellulose (Ankersmit, 1905).

Belief that the bacteria in the rumen could be grown *in vitro* arose from observations of the flask cultures of the cellulose-digesting rumen protozoa. These cultures were maintained for many months with no additions except dried grass and cellulose. The gas produced in the flasks was found to be chiefly methane and carbon dioxide, the same as the rumen gases. This suggested that the microbial processes in the flask cultures were similar to those in the rumen. It seemed probable that cellulose-digesting bacteria similar to those in the rumen were present in the flask cultures, and the fact that they had grown for an extended period outside their host encouraged the attempt at their isolation.

DEVELOPMENT OF A CULTURE MEDIUM

Agar shake cultures with serial dilutions were adopted as the means of estimating the number of cellulose bacteria. The inorganic medium initially used was the same as that employed for growth of the protozoa (Hungate, 1942). An aqueous extract of dried grass (sterilized by filtration through a Seitz filter) and a sterile filtrate of an active culture of the protozoa were added

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to the cellulose and agar in the inorganic salt solution. Nitrogen containing 5 per cent carbon dioxide was bubbled through the tubes of melted agar before and after inoculation. The gas was first passed through chromous oxygen absorbent to remove traces of oxygen. Two drops of 1 per cent $Na_2S \cdot 9H_2O$ were also added to each tube just before inoculation in order to absorb any traces of oxygen. The tubes were cooled rapidly under running cold water to give an even dispersion of the cellulose in the agar, and were then incubated at 37 C.

These initial shake tubes failed to show cellulose decomposition, in contrast to the flask cultures of the protozoa. However, the tubes contained a greater concentration of cellulose than did the liquid cultures. This was necessary in order to give sufficient white opacity to permit detection of cellulose digestion. It seemed possible that with this higher concentration of cellulose the low buffering capacity of the medium might not permit sufficient growth of the bacteria to digest a visible amount of cellulose. Accordingly, an inorganic solution containing more phosphate was substituted. This medium supported development of cellulose-digesting bacteria, as evidenced by the appearance of clear spots in some of the tubes.

These clear spots were transferred to new dilution series of similar composition in an attempt to eliminate noncellulose bacteria. As a rule, a clear spot was also inoculated into a parallel series containing a different medium in order to gain information on the essential cultural factors. Thus, in some series the grass extract was omitted, in others the filtrate of the protozoa culture. Some included yeast extract, others a mixture of B vitamins.

The results obtained by these various procedures were extremely conflicting. In one transfer there would appear to be a definite advantage in using protozoan culture filtrate. When the experiment was repeated, quite different results might appear. None of the media tested was found to give reliable growth.

By inoculating several parallel series at each transfer it was possible to subculture 11 successive times, but the twelfth inoculation failed to give further growth. A certain amount of purification of the cellulose decomposer occurred during this time, and when the culture was finally lost there appeared to be only two organisms present. One of these was a small spiral form. The other was a coccus which often was joined in chains, especially in young cultures. When a colony containing these two forms was diluted in a shake series containing glucose, diffuse colonies developed that on microscopic examination were found to contain the spiral. It was inoculated into a cellulose series, but it failed to show cellulose digestion. This suggested that it was not the cellulose decomposer. However, since the culture method was relatively uncertain, another means of ascertaining the nature of the cellulose digester was also employed.

A bit of the cleared agar was diluted in a glucose series, and an approximately equal amount of the uncleared, cellulose-containing agar from an adjacent region was diluted in a similar series. Growth of colonies of the spiral organism took place in both series, and the number was of the same order of magnitude. This

indicated that it was not associated primarily with areas showing cellulose digestion.

The morphology of the spiral is shown in figure 1. This organism has been repeatedly encountered in cellulose cultures inoculated with rumen contents. It has also been observed in direct films of rumen contents. It appears to be a usual inhabitant of the rumen.

An ability of the spiral cells to migrate rather rapidly through glucose agar was noted. Since it was necessary to incubate the cellulose cultures for 10 days before clear spots could be detected, there was ample time for it to move throughout the tube. This explains its frequent occurrence as a contaminant in the cellulose cultures. A number of repetitions of this attempt at isolation led to approximately the same results. None of the media employed gave any consistent indication of superiority. Some important variables were causing erratic results. It seemed possible that the noncellulose bacteria constituted

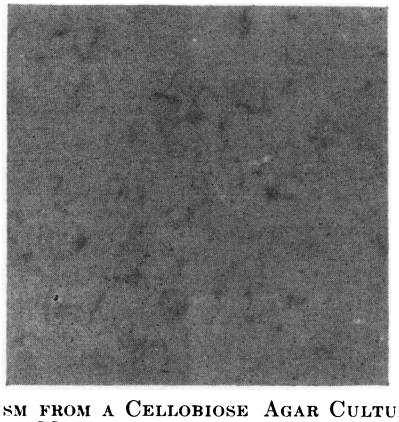

FIG. 1. SPIRAL ORGANISM FROM A CELLOBIOSE AGAR CULTURE. CARBOL FUCHSIN. MAGNIFICATION ABOUT 1,000 ×

one of the important variables and that fluctuations in their numbers and kinds might be of importance.

The presence of contaminants is usually considered favorable to the growth of cellulose-digesting bacteria. Many investigators have reported failure to obtain cellulose digestion with a pure culture, whereas mixed cultures were active. The success in using mixtures has usually been interpreted as indicating that the accompanying forms in some way aid the cellulose digestion. In the present experiments, however, the culture filtrate should have provided the helpful action of any "synergistic" forms, yet no consistent improvement through its use could be demonstrated. This finally led to the consideration of a different view of the influence of accompanying bacteria. It seemed possible that at least some of them exerted a deleterious effect on the cellulose decomposers and that failure to obtain growth in subcultures was due to overgrowth by other bacteria.

Following this line of reasoning, the culture method was modified in four ways.

(1) Much more careful attempts were made to avoid transferring contaminating forms. (2) The percentage of agar was increased to 2 per cent. This decreased the movement of bacteria in the medium but did not stop it entirely. (3) The inorganic medium was modified to include carbonic-acid bicarbonate as the principal buffer system. The high carbon dioxide content (70 to 75 per cent) of rumen gas coupled with the neutral reaction of the rumen contents indicates that considerable quantities of bicarbonate are present in the rumen. The saliva is the source of this salt. It seemed possible that bicarbonate would be a more natural constituent of the inorganic medium than was the phosphate. Carbon dioxide freed of oxygen was used to displace oxygen and to provide a suitable pH. (4) In order to give more rapid development of the cellulose decomposers, some liquid from the rumen was included in the medium.

For the earlier experiments rumen fluid was obtained at the abbatoir and transported to the laboratory with as little exposure to the air as possible. The samples were obtained by slitting the rumen within 10 minutes after death of the animal, inserting a tubular screen (12 meshes to the inch) into the rumen contents, and drawing into a pipette the liquid and particles which penetrated the screen. For later experiments the liquid was removed by a similar technique from a cow with a rumen fistula.[2]

The liquid rumen contents were immediately boiled, filtered through cotton, and then stored in the refrigerator under an atmosphere of carbon dioxide. In the preparation of the culture medium the agar was dissolved in 4 parts of the inorganic medium. Three parts of cellulose suspension were added and, after boiling, 3 parts of the rumen liquid were added, and the mixture was again boiled. Sodium thioglycolate (0.05 per cent) was added and the medium immediately sterilized at 15 pounds for 15 minutes. On removal from the autoclave the medium was cooled to about 50 C. A solution of sodium bicarbonate (sterilized by filtration) was then added (0.5 per cent final concentration), and the medium was held at 46 C until inoculated.

The composition of the mineral medium before being mixed with the other ingredients was, in percentages: NaCl—0.09; $(NH_4)_2SO_4$—0.03; K_2HPO_4—0.05; KH_2PO_4—0.03; $CaCl_2$—0.015; $MgSO_4$—0.015; in tap water.

The cellulose was prepared by treating absorbent cotton with concentrated hydrochloric acid, which caused it to break up into small particles. If the concentrated reagent caused browning, it was diluted with a little water. After 48 hours or more the cellulose was filtered off, washed with tap water, and air-dried. Before use it was suspended in tap water in a concentration of 5 per cent and ground for 72 hours in a pebble mill.

ISOLATION OF THE BACTERIA

Rumen contents were inoculated into the new medium with serial dilutions. Growth of cellulose-decomposing bacteria, as evidenced by the appearance of

[2] The author is much indebted to Dr. R. W. Dougherty of the College of Veterinary Medicine for making two of these animals available and for aid in withdrawing the samples of rumen fluid.

clear spots, occurred much more rapidly than in any previous series. Cellulose decomposition was evident in the tubes of lower dilution after 2 days, and within a week clear spots 2 mm in diameter were present in some of the higher dilutions. These spots were present not only in the solid agar in the bottom of the tube, but were also in the thin agar lining the upper, gas-filled portion. This was one of the most important results of the new method because it made it possible to transfer only that agar in which the cellulose had been digested, and thus to reduce to a minimum the number of contaminants carried in the inoculum. It was also possible to obtain pure cultures using 1 per cent agar. This concentration was used in most of the later work because it gave more rapid development of the colonies.

Colonies were subcultured in cellulose dilution series using a colony in a high-dilution tube for transfer. As soon as the culture appeared to consist of only one kind of colony, it was also subcultured in a parallel glucose or cellobiose agar dilution series. The sugars were sterilized by filtration and added to the medium (0.1 per cent final concentration) after the heat sterilization. The composition of the sugar media was the same as that of the cellulose agar except that tap water and the sugar replaced the cellulose suspension.

If growth occurred in the sugar series, a colony in the highest dilution was subcultured again in a sugar series. Each series was examined for uniformity of the colonies. From a high dilution of this second series, a colony was picked to cellulose. If rapid and typical cellulose digestion occurred and there were no indications of any contaminants, it was concluded that a pure culture of the cellulose-digesting bacterium had been isolated. Four pure cultures were obtained in this way.

These cultures were purified relatively easily as soon as the technique of isolation had been worked out. However, other cellulose-digesting colonies occurring in agar dilution series inoculated with rumen contents have not been grown so successfully. Several of them have shown sporadic growth through a few transfers, but then have failed entirely. Many modifications of the culture medium have been tested in an attempt to obtain consistent growth of all cultures, but as yet no completely satisfactory method has been found. Improvement has sometimes been noted when 2 per cent horse serum was added to the medium just before it was inoculated. It has also been found helpful to carry some strains in a liquid rather than in an agar culture.

A total of 6 different strains of rumen cellulose bacteria have been obtained in pure culture, as judged by successful growth in cellulose after passage through 2 sugar dilution series. Three other cultures were apparently pure but were not grown in sugar.

DESCRIPTION OF ISOLATED STRAINS

In the pure cultures, and in the cellulose-decomposing colonies which have been examined but not isolated, there have been observed two morphological types, a coccus and a rod.

The coccus form was first encountered in the series inoculated from the pro-

tozoa cultures, but was not obtained pure from them. It has also been frequently observed in dilution series inoculated from the rumen. Four strains have been purified. The first (strain C) was obtained from the rumen of an animal slaughtered at Austin, Texas. It was transferred through 7 subcultures in cellulose and at the end of that time appeared to be pure, but its failure to grow in glucose prevented the application of the rigorous purity test. No bacteria developed in the glucose agar cultures. On the eighth transfer this strain failed to give further development.

Coccus strain A was obtained from a cellulose agar series inoculated with the rumen contents of a steer killed at Moscow, Idaho. The strain was carried through 4 cellulose agar series. It was then inoculated into a glucose series and showed the development of white, slightly opaque, lens-shaped colonies. One of these in a high dilution was inoculated into another glucose series and from this back into cellulose, in which the organism readily grew.

The other two strains of cocci were obtained from a cow with a rumen fistula. One of them (strain H) occurred initially as an isolated colony in the sixth dilution tube of a cellulose series inoculated with rumen contents. It was subcultured several times in cellulose agar, and appeared to be pure, but would not grow in glucose. The other (strain M) developed in the fifth dilution of a liquid culture series containing cellulose. It was subcultured and purified by repeated transfers in cellulose agar. It failed to grow in glucose but did grow in cellobiose and was pure-cultured. This strain has given more rapid digestion in cellulose agar tubes than has any other strain of bacteria isolated from the rumen, clear spots appearing within 2 days after inoculation.

In all of the strains of cocci the cells average about 1 μ in diameter, though there is much variation from one culture to another, and even within a single colony. Strain C showed a considerable tendency to form chains, as illustrated in figure 2. In some cultures of this strain a definite capsule was present. Strain A showed practically no chain formation in agar (figure 3) but in liquid cultures some loose filaments of from 4 to 8 cells were formed.

The coccus strains usually exhibit a gram-negative reaction. Films of strain A occasionally contain some gram-positive cells. Strains A and M have been observed to include cells containing gram-positive granules though most of the cell was negative. The negative reaction does not appear to be due to the age of the cells since young colonies (2 days old) of strain M have been stained *in situ* in the agar and the peripheral young cells were completely gram-negative.

Young colonies of the cocci give the iodophilic reaction which has been used by Henneberg (1922) and Baker (1942) to identify cellulose bacteria in the rumen by microscopic examination. The staining by the iodine is interpreted as due to glycogenlike material within the cells. Older cells do not exhibit this reaction, as may be seen from the photomicrographs of young colonies (2 days) of strain M in cellulose agar (figure 4). Only the peripheral portions of the colony containing the younger cells give the dark color with iodine.

At various times the coccus strains A, C, and M were inoculated into cellulose agar containing no rumen contents but with yeast extract (Difco) in concentra-

tions ranging from 0.1 to 0.5 per cent. Growth was about the same as in the cultures to which rumen contents were added. The colonies of strains A and C in yeast extract were distinguished by the production of a yellow pigment which diffused out from the colony for a short distance. A similar production

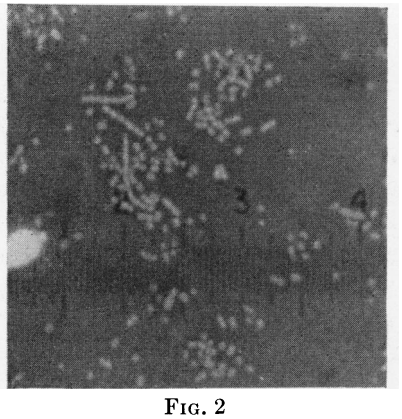

FIG. 2

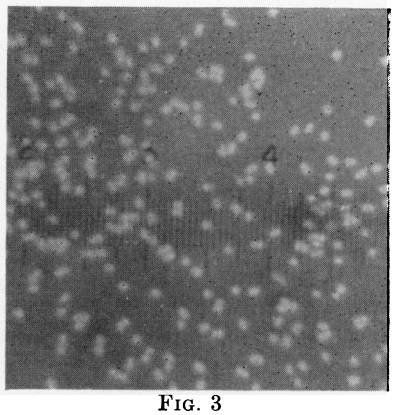

FIG. 3

FIG. 2. STREPTOCOCCUS, STRAIN C, FROM A CELLULOSE AGAR CULTURE. NIGROSIN. EACH SMALL MICROMETER DIVISION EQUALS 0.8 MICRONS

FIG. 3. STREPTOCOCCUS, STRAIN A, FROM A GLUCOSE AGAR CULTURE. NIGROSIN. MAGNIFICATION SAME AS FOR FIGURE 2

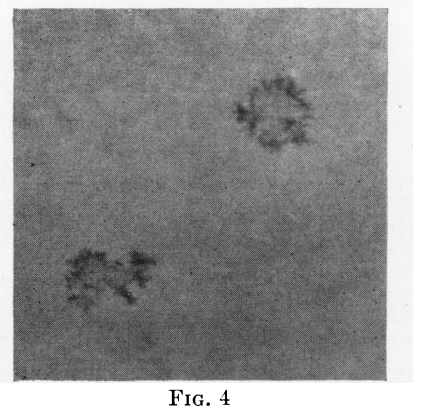

FIG. 4

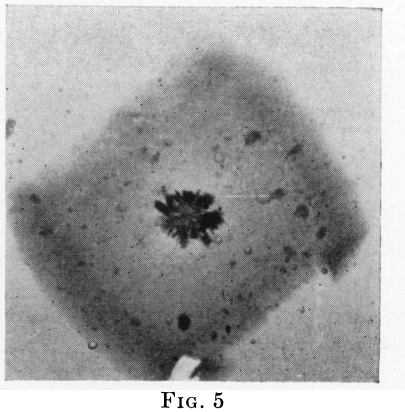

FIG. 5

FIG. 4. STREPTOCOCCUS, STRAIN M, 48-HR COLONY IN CELLULOSE AGAR. IODINE. MAGNIFICATION ABOUT 20 ×

FIG. 5. STREPTOCOCCUS, STRAIN A, OLD COLONY IN CELLULOSE AGAR. MAGNIFICATION ABOUT 6 ×

of pigment has not been observed in rumen cellulose tubes. The colonies of strain M did not form pigment even when grown in yeast extract.

The colony growth of the coccus strains in cellulose agar is quite characteristic. There is no sharp line of demarcation between undigested and digested cellulose such as is observed in *Clostridium cellobioparus* (Hungate, 1944) or *Micromonospora propionici* (Hungate, 1946). The appearance (figure 5) suggests that

the cocci are not as effective digesters of the more resistant cellulose. Very young colonies may show relatively little digestion (figure 6).

Reducing materials have been demonstrated in old cultures of strains A and C. The fact that a clearing of cellulose occurs at some distance from the colony (figure 5) shows that an extracellular enzyme is formed. In old cultures it presumably continues to act and produce sugar after the culture is too acid to permit growth of the cells.

No two of the coccus strains that have been isolated have resembled each other in every particular. They have differed in the rate of cellulose digestion, growth in yeast extract, tendency to form chains and capsules, gram reaction, and in pigment production in yeast. However, it seems probable that they are all fairly closely related The morphology suggests that they belong to the genus *Streptococcus*. But their predominantly negative gram reaction is not

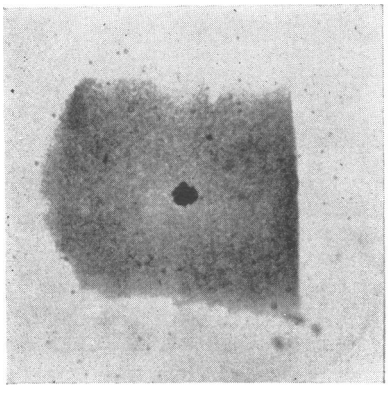

FIG. 6

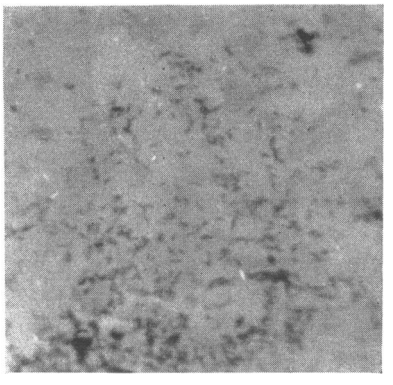

FIG. 7

FIG. 6. STREPTOCOCCUS, STRAIN A, YOUNG COLONY IN CELLULOSE AGAR. MAGNIFICATION ABOUT 6 ×

FIG. 7. ROD FORM, STRAIN A, FROM 4-DAY COLONY IN CELLULOSE AGAR. CARBOL FUCHSIN. MAGNIFICATION ABOUT 1,000 ×

consistent with this assignment, and for the present it seems preferable to delay naming the organisms. It is possible that the forms described as *Micrococcus ruminantium* and *Streptococcus jodophilus* on the basis of microscopic examination by Henneberg (1922) are the same as the cellulose-decomposing cocci isolated in the present investigation.

Six strains of rod-shaped bacteria capable of decomposing cellulose have been obtained from the rumen. Five of them have been obtained in pure culture (A, F, H, R, and S). Another (strain D) was lost before it could be carried through glucose series. All these strains have exhibited several features in common. In fresh mounts the cells are practically invisible, and it is necessary to stain in order to see them. They stain readily with carbol fuchsin but not with methylene blue. Young colonies in cellulose agar consist of small rods 1 μ by 0.3 to 0.4 μ (figure 7). At a later stage the rods almost entirely disappear, and instead are found minute and indistinct spheres of variable size.

The rods disintegrate very quickly after a colony has developed, and subcultures have not been successful when the rods were no longer present. This suggests that the minute indistinct spheres are degeneration products. The rod strains have been uniformly gram-negative. The cells even in young cultures do not stain with iodine except for a slight yellow color when the entire colony is stained. No brown reaction suggesting glycogen has ever been observed. This shows that iodophily alone is not adequate as an index of cellulose-digesting ability by rumen bacteria.

The rods grow readily when glucose or cellobiose is substituted for the cellulose in the medium. When growing on either of these sugars, however, the morphology is quite different from that exhibited with cellulose as the substrate. There is a much greater variation in size and most of the cells are much larger (figure 8) than when grown on cellulose. The large cells stain unevenly, with granules

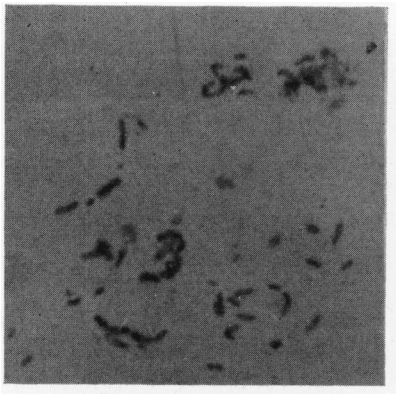

FIG. 8

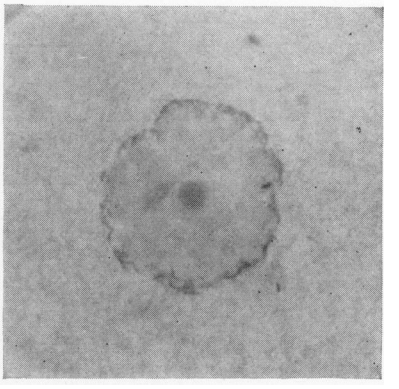

FIG. 9

FIG. 8. ROD FORM, STRAIN F, FROM 3-DAY COLONY IN GLUCOSE AGAR. CARBOL FUCHSIN. MAGNIFICATION ABOUT 1,000 ×

FIG. 9. ROD FORM, STRAIN R, FROM 3-DAY COLONY IN CELLULOSE AGAR. CARBOL FUCHSIN. MAGNIFICATION ABOUT 40 ×

within the cell showing affinity for the dye, whereas the main portions of the cell are barely visible. Old colonies in a sugar medium consist of an amorphous mass of material in which numerous small spheres of variable size predominate.

The rod-shaped cellulose decomposers have exhibited a type of colony growth in cellulose agar which has not been encountered in any other cellulose anaerobes. The first evidence of growth in cellulose agar is the development of a clear space in which the cellulose is completely digested. Within this space no colony can usually be detected, although with strain R a disc-shaped colony may be present in the center of the clear space. By removing a bit of the thin agar containing a young colony and staining the whole colony *in situ* with carbol fuchsin, the active bacteria can be seen as a thin ring in the outermost part of the clear spot immediately adjacent to the cellulose (figure 9). The inner part of the clear area contains few rod-shaped cells, though the indistinct spheres

previously mentioned are numerous. The rod form apparently migrates through the agar, and the cells in the center of the colony either become moribund or migrate to the periphery.

There is a sharp line of demarcation between digested and undigested cellulose around colonies of the rod. The location of the cells indicates that relatively close contact with the cellulose is necessary for its digestion, a situation reminiscent of that observed in *Sporocytophaga* (Stanier, 1940). Correlated with this behavior, no reducing materials have been found to accumulate in old cultures of these strains. The several strains of rods have been found to differ slightly from each other in the ease with which they can be cultured and in their rate of growth. However, they all appear to be strains of the same species.

In addition to the strains of cellulose decomposers from the rumen which have been isolated and carried in culture, numerous colonies developing in initial series inoculated with rumen contents have been examined microscopically. In all of these, the cellulose decomposer has seemed to be a rod or a coccus similar microscopically to the strains which have been isolated.

The macroscopic appearance of the thin cellulose agar in which a cellulose-digesting colony from the rumen is growing is sufficient to determine whether the coccus or the rod is concerned. The boundary of cellulose digestion around a colony of the rod is always sharp and distinct, whereas around the coccus it is indefinite. This has made it easy to determine which organism predominated in the quantitative dilution series inoculated with rumen contents.

THE NUMBER OF CELLULOSE BACTERIA AS DETERMINED BY CULTURE METHODS

It has always been possible to demonstrate cellulose-decomposing bacteria in cellulose dilution series inoculated with rumen contents. The numbers per ml of rumen fluid in different animals as found during the initial studies were as follows: A—480,000; B—1,300,000; C—210,000; D—40,000,000; E—18,500; F—13,000,000; G—1,500,000; H—1,200,000,000; and I—780,000.

In all of these original series, except D and H, the cellulose bacteria caused clear spots which gradually increased in size. Few additional colonies developed after further incubation. In the D series, colonies of this sort appeared, and after 13 days one of them in tube 4 was transferred to a new series. The air in tube 4 was displaced with carbon dioxide, and the tube was replaced in the incubator. Seven days later it was noticed that numerous small clear spots were present in the thin agar lining the upper portion of the tube. The volume of this agar was estimated by weighing it, and from the number of colonies present it was calculated that the original inoculum contained 40,000,000 cellulose bacteria per ml.

Previous experience with *Clostridium cellobioparus* had shown that clear spots and streaks in the thin layer of agar might occur if the tube had been laid in a horizontal position. This would allow the liquid which had collected on the surface of the solid agar to spread over the thin region and scatter any bacteria present. Although no indications of this were observed in tube 4 of

the D series, it was thought that a mishap of some similar nature might have occurred. However, the same phenomenon was encountered even more strikingly with the series from cow H, and it was concluded that also in the D series the clear spots which appeared after prolonged incubation represented cellulose bacteria which had been present in the original inoculum.

The series inoculated with rumen contents from animal H was incubated for 6 days, at which time clear spots and gas were present in the first three tubes. The colonies were similar in general appearance and behavior to those previously encountered. During the subsequent few days of incubation, colonies appeared in tubes 4 and 5, only one being present in the latter. When the tubes were examined 17 days after inoculation, however, the thin agar in the upper portion of tube 5 showed 152 clear spots. This tube had not been opened and had been incubated in an upright position during the entire period. Furthermore, the single precocious colony was near the bottom of the tube in such a position that there was little chance for bacteria to escape from it into the thin agar layer at the top. It was concluded that the numerous colonies in the thin agar represented cellulose bacteria in the inoculum. In both animals D and H the colonies appearing in such numbers contained only the rod. These initial series were inoculated from animals killed at the Austin abbatoir. The rod form predominated in most of these animals, although the coccus was also present in large numbers.

Four different experiments have been performed to estimate the numbers of cellulose bacteria in one of the experimental animals with a rumen fistula. In the first, there were found 40 million cellulose bacteria per ml. The coccus was the predominating form as determined by the type of colony. The rod was present in the tubes of lower dilution.

It seemed possible that, if the rod required close contact with the cellulose, there could conceivably be present in the rumen similar bacteria which were prevented from growing in agar because of inability to migrate through the gel. Accordingly, a liquid series was tested in parallel with the agar. Serial dilutions were made in the liquid tubes, and from each an inoculation was made also into agar. No significant differences in numbers were given by the liquid and agar series, about 40 million per ml being indicated by each. In this series, also, the coccus was more numerous than the rod.

In a second test the liquid media gave slightly greater numbers than the solid. At least 60 million bacteria per ml were indicated by the liquid series, whereas the agar tubes showed about 10 million.

In the last experiment with the fistula animal, two liquid series were compared, one with sodium thioglycolate and one without. The dilutions were made in the series without the thioglycolate. There were no differences in the number of cellulose bacteria demonstrated by these two series, 100 million per ml being found. This same number was also obtained from a parallel agar series without thioglycolate.

Cellulose was decomposed with great rapidity in liquid tubes inoculated with rumen contents. Tubes inoculated with 1/60,000,000 ml of rumen liquid

showed cellulose digestion within 3 days, and within 4 days it had all (10 mg) been decomposed. With 0.001 ml of inoculum, the cellulose was all digested within 24 hours.

During the experiments on culture and isolation, no attention was paid to the problem of obtaining a sample representative of the entire rumen. Much of the rumen contents consists of matted plant material with a high content of solids. It seemed probable that the number of bacteria in this material might be different from the number in the fluid. In order to gain information on this point, a fluid sample was withdrawn from a fistula animal and at the same time some of the matted solid contents were removed. The latter were mixed in a Waring "blendor," and samples were withdrawn after intervals of 1 minute, 6 minutes, and 11 minutes.

The numbers of cellulose bacteria in these samples, and also in the unmixed solid and liquid, were estimated by inoculation into solid and liquid dilution series. There were no significant differences between the agar series inoculated with the liquid rumen contents, with solid material, and with solid material mixed for 1 minute. All showed about 50 million colonies per ml inoculum. No cellulose-digesting colonies appeared in any tube of the agar series inoculated with the samples mixed for 6 and 11 minutes, respectively.

The results in the liquid series were essentially similar. No cellulose digestion occurred in any of the tubes inoculated with the material mixed for 6 or 11 minutes. The liquid and unmixed solid inocula showed growth in the fourth dilution tube. The 1-minute mixed material showed growth in the fifth dilution tube. This indicated 1 billion bacteria per ml. However, too much significance cannot be attached to this greater number, since the three agar series showed little difference and a single organism in the liquid culture would by chance occasionally be carried over.

The failure to obtain any growth at all with the 6- and 11-minute inocula was unexpected. It was improbable that the violent agitation had killed all of the bacteria, since most of the particles of plant material were not broken up to a very great extent. There was no attempt to exclude air during mixing in the Waring "blendor" and a great deal of it was whipped into the material. In view of the great susceptibility of the rumen protozoa to oxygen, it seemed possible that the bacteria were similarly affected.

In the next experiment carbon dioxide was passed into the Waring "blendor" during the mixing. Samples were withdrawn after one-half minute of mixing and after 2 minutes. These were inoculated into both liquid and agar series, as was also the unmixed material. All showed about the same number of cellulose bacteria. The dilutions in these experiments with different inocula were not sufficiently graduated to disclose minor differences in the numbers, but they demonstrated that samples of the rumen liquid give approximately the same number of cellulose bacteria as samples of the solid material.

As a summary of these studies on the numbers of cellulose-digesting bacteria in the rumen demonstrated by cultural methods, it may be stated that a count of approximately 50 million seems to represent the usual number present. In the

early studies in which the count was much lower, the technique had not been well worked out. The case in which high counts of over a billion were found are exceptional. Similar high counts have not been found in most of the experiments.

THE SIGNIFICANCE OF THE ISOLATED BACTERIA IN THE RUMEN

The demonstration of anaerobic cellulose digestion by the cocci is in agreement with the conclusions of other investigators on the nature of the cellulose decomposers. Henneberg (1922) correlated the formation of "*Frass-betten*" in cell walls with glycogen formation (iodophily) by the rumen bacteria and concluded, "*Die jodophilen Streptokokken gehören im Wiederkäuer zu den wichtigsten Zelluloseverzehrern.*" The cellulose-decomposing cocci which have been isolated apparently correspond to the streptococci which were seen by Henneberg. Baker (1942) has also reported the importance of iodophilic streptococci in the rumen, and it must be concluded that the cultural experiments fully substantiate the findings of these investigators insofar as the cocci are concerned.

Although the isolated cocci have a morphology similar to that of the cellulose decomposers which have been demonstrated by microscopic methods, it cannot be concluded from this evidence alone that the most important organisms have been isolated. Too many bacteria, differing in their cultural requirements, may be gram-negative, iodophilic, cellulose-decomposing cocci, and some of these may not grow under the cultural conditions that have been thus far provided. Additional evidence on the importance of the isolated bacteria can be obtained if the number in which they can be demonstrated culturally is compared to the number demonstrable by microscopic methods.

Rumen contents were diluted 100 times and films were prepared as for the direct microscopic method for counting bacteria in milk. Iodine was first used to stain them, but very few of the bacteria stained intensely. A gram stain was tried but the affinity of the negative cells for the counterstain was so slight that enumeration could not be made. Minute droplets of dye left on the slide after use of Ziehl-Nielsen's carbol fuchsin prevented direct use of this stain. However, it was found that by diluting the carbol fuchsin 50 times the cells were visibly stained, with no deposition of dye except on the bacteria.

One sample of rumen contents from a fistula animal gave a clump count of 8 billion per ml rumen contents. Most of the bacteria were cocci, often occurring in pairs and short chains. Many were slightly elongated and might be classed as short rods. Some minute slender rods were seen and a few large ones. The spiral form was present to the extent of about 300,000 per ml. A few sarcinae were present. Another sample from the same animal at a different time gave 2 billion as the clump count, but the clumps were larger and included more bacteria than the first sample. Otherwise the microscopic appearance was quite similar.

These direct count figures show that the figure of 50 million derived from the culture experiments represents less than 1 per cent of the total bacteria (clumps) present. Under these circumstances, can it be concluded that the isolated

cellulose bacteria are representative of the ones active in the rumen? Although the evidence on this point is by no means conclusive, several considerations indicate that the isolated bacteria are actually the ones chiefly responsible for the digestion of the cellulose in the rumen.

(1) The numbers in which they have been demonstrated culturally, while small in comparison with the total, are nevertheless of significant magnitude.

(2) The same kinds of cellulose bacteria, and only those kinds, have been encountered in every dilution series inoculated from rumen contents. If cellulose bacteria with other characteristics were important in the rumen, it seems remarkable that they would not have been at least occasionally encountered.

(3) The cellulose bacteria which have been isolated are extremely active in cellulose decomposition under conditions closely similar to those present in the rumen.

(4) In the thin cellulose agar in the upper part of cellulose dilution tubes inoculated with rumen contents, it is possible to see not only cellulose-digesting colonies, but also noncellulose colonies which develop. By adding water to the tube and loosening the thin agar, it can be dumped out into a petri dish without being torn or mashed. A measured area of uniform thickness can then be cut out, surplus water dried off, and the agar weighed. It is then possible by microscopic examination to obtain not only an idea of the relative number of cellulose and noncellulose colonies, but also of the total number of colonies developing per ml of inoculum.

This was done with a cellulose series inoculated from a fistula animal. Microscopic examination showed that the non-cellulose-digesting colonies greatly outnumbered the cellulose digesters. The proportion was about 1 to 1,000. The total number of colonies was approximately 300 million per ml of rumen contents. This shows that in addition to the cellulose bacteria in the rumen a great many other viable bacteria are present, but not digesting cellulose under the conditions of the experiment. It is probable that the noncellulose bacteria are concerned with the breakdown of hemicelluloses, starches, and soluble materials, with the formation of methane, and with other activities supported by the diverse chemical substances present in the food and derived from the food by the activities of other microorganisms. It is possible that the use of a more fully hydrated and less resistant cellulose than that employed in the present experiments would yield additional numbers of cellulose digesters. However, there is a more or less continuous gradation in the degree of digestibility of various cellulose preparations, and an arbitrary line must be drawn at some point.

The rumen fluid used in the previous experiment had been filtered twice through a Seitz filter. This was necessary in order to remove bacteria and particles which might be mistaken for colonies. Apparently as a result of the filtration, the medium did not support so good growth of cellulose bacteria as in experiments using fresh unfiltered rumen contents, and the number of cellulose bacteria was relatively low. In dilution tubes containing the usual medium an accurate count of the noncellulose colonies could not be obtained, but observations indicated that they greatly outnumbered the cellulose digesters.

Culture series using noncellulose substrates have yielded values of 700 million per ml.

If most of the bacteria seen microscopically are not cellulose decomposers, the numbers in which the cellulose organisms have been demonstrated culturally become more significant and suggest that they are the principal ones concerned.

The significance of the cellulose-decomposing rod in the rumen has not been generally realized. The rod mentioned by Dougherty (1941) is possibly the same. The great activity of the rod in digesting cellulose and its occurrence in significant numbers favor the interpretation that it also plays an important role in the rumen fermentation. The fact the iodophilic reaction is lacking in the rods shows that this feature alone is not an adequate index of cellulose-digesting ability. This might be expected. Materials other than carbohydrates can serve as the substrates from which glycogen is synthesized. The formation of this reserve material depends on the dissimilatory and assimilatory capacities of the organism as well as on the nature of the substrate.

The rumen is an exceedingly complex (and interesting) microcosm and the cellulose bacteria are only one of many groups performing significant functions. However, they are an important group because they participate in the initial attack on an important substrate and provide products which must profoundly influence the course of metabolism of the associated organisms. Knowledge of their activities should aid in understanding the functions of the other organisms.

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