STUDIES ON CELLULOSE FERMENTATION
I. THE CULTURE AND PHYSIOLOGY OF AN ANAEROBIC CELLULOSE-DIGESTING BACTERIUM[1,2]

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The relatively few studies which have been made on cellulose utilization by pure cultures of anaerobic bacteria have indicated that the cellulose anaerobes possess peculiar characteristics which set them apart from most bacteria. Among these characteristics may be mentioned the difficulty of their isolation, their inability to utilize sugars, and their tendency to lose the power of cellulose digestion in pure culture.

During an investigation of cellulose decomposition by protozoa in the rumen of cattle (Hungate, 1942, 1943a) attempts were made to demonstrate cellulose-digesting bacteria. One of the cultures was successful, and the causative agent was found to be a mesophilic bacterium which appeared to differ in some respects from the previously described cellulose-decomposing anaerobes (Khouvine, 1923; Werner, 1926; Cowles and Rettger, 1931; Meyer, 1935). The organism was isolated in pure culture and its characteristics studied.

CULTURAL AND MORPHOLOGICAL CHARACTERISTICS

Isolation. Evidence of the organism was first observed when rumen contents were inoculated into a dilution series of shake tubes containing agar and finely divided cellulose in a solution of inorganic salts. The cellulose was prepared by treating absorbent cotton with strong hydrochloric acid until it disintegrated into small particles, which were then washed free of chlorides. According to Farr and Eckerson (1934), these particles give an X-ray diffraction pattern similar to that of untreated cellulose. They were ground with water in a pebble mill to give a finely divided suspension. The salt solution was composed of the following: NaCl, 0.6 g; $(NH_4)_2SO_4$, 0.1 g; KH_2PO_4, 0.05 g; K_2HPO_4, 0.05 g; $MgSO_4$, 0.01 g; $CaCl_2$, 0.01 g; and tap water, 100 ml. Sterile, oxygen-free nitrogen containing 5 per cent carbon dioxide was bubbled through the medium after sterilization, and sufficient sterile sodium carbonate solution to give a pH of 7.4 was added. The tubes were stoppered without admitting air and incubated at 38 C.

After 6 days gas bubbles were present at several points in the first tube, so agar in the vicinity of one of these bubbles was inoculated into a dilution series of the same medium. After 10 days gas had formed, and from the fourth tube a new series was prepared. This series soon showed gas, and after 3 weeks small

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clear spots were present in tube 5. A small bacterial colony could be seen occupying the center of each clear zone.

One of these colonies was diluted into a series containing glucose instead of cellulose, and growth was soon evidenced by gas formation and the appearance of colonies in the first four tubes. A colony in the fourth tube was inoculated into another glucose series, and from the greatest dilution showing growth a colony was inoculated again into a cellulose series. Gas developed in this series, and after a few weeks small colonies surrounded by clear zones could be seen in the higher dilutions. Thus, the power to digest cellulose was not lost by passage through glucose. This alternation between glucose and cellulose substrates has been repeated many times, always with the same result. During these transfers the bacteria in the colonies preserve an approximately constant shape and size.

Several considerations indicate that a pure culture has been obtained. (1) The organism has been grown continuously in dilution series in agar for a period of 4 years. At each transfer a colony from one of the higher dilutions has been used for the next series. (2) The cells have exhibited an approximately constant morphology and physiology during this time. (3) Ability to digest cellulose is not lost by passage through dilution series containing glucose. (4) Each colony in the higher dilutions of a glucose series is capable of digesting cellulose when inoculated into a suitable cellulose medium.

The pure culture was always used in the following studies on the morphology and physiology of this organism.

Morphology. In glucose shake tubes with 2 per cent agar, young colonies are uniformly disc-shaped and compact, though older colonies become more complex in shape, often by growth of daughter discs at right angles to the original one. The colonies in cellulose agar are irregular in shape, even when small.

Microscopically, the vegetative cells appear (figure 1) as slightly curved, gram-negative rods 3 to 5μ long and 0.3 to 0.4μ wide. This general shape has been observed in all media tested, including cellulose and glucose in liquid and solid media. Two cells may remain attached, and on one occasion in a colony growing in cellulose agar a long spiral of cells was observed. Terminal spherical spores with a diameter of 0.9μ are formed in profusion on cellulose, and to a less extent on glucose. Their maximum heat resistance was not determined, but they survive an exposure of 12 hours at 85 C. The sporangium develops as a terminal swelling in which the spore differentiates. At maturity the spore breaks loose almost immediately and is rarely observed attached to the parent cell.

In a sample of the bacteria from a growing liquid culture, numerous motile cells may often be observed. When stained by the method of Zettnow, 1 to 4 peritrichous flagella can be seen on nearly every cell (figure 2). Preparations containing sluggish cells are particularly favorable for observing the manner of motion, and on close examination it can be seen that they often move in a spiral of small diameter and rotate on their longitudinal axis. An end view of a long motile cell in a fresh mount will often show clearly that the curvature is spiral in nature.

Cultural characteristics. The organism grows well in agar shake tubes containing only cellulose and inorganic salts, but transfers to a liquid culture show growth only when biotin is added. According to the results of Butler *et al.* (1941), agar contains significant quantities of biotin, and it may be concluded that it is sufficient to support growth in the shake tubes. Addition of small amounts of a folic acid concentrate (supplied by Dr. R. J. Williams) and calcium pantothenate, or of a cold water extract of dried grass, does not appreciably hasten the time of appearance of gas in a liquid culture.

Anaerobic conditions are necessary for growth. At first oxygen was removed by bubbling nitrogen with 5 per cent carbon dioxide through the medium immediately after autoclaving. Since some of these tubes failed to show growth, a drop of 10 per cent sodium sulfide solution was added, and with this precaution

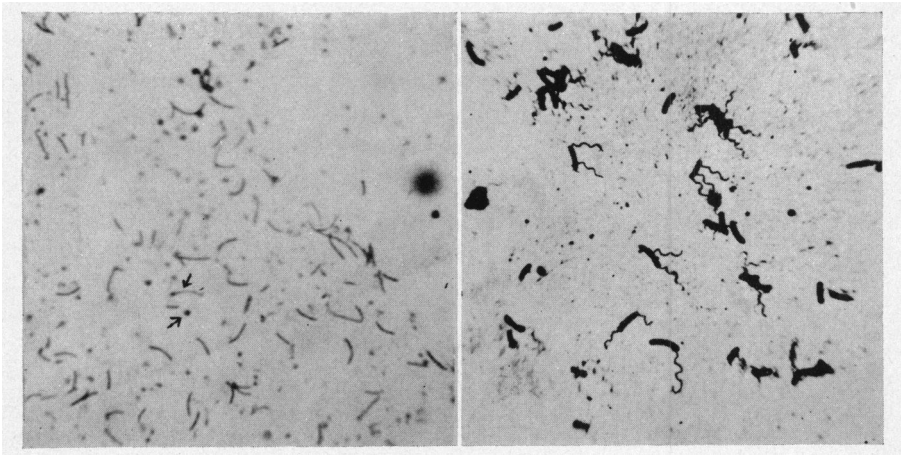

FIG. 1 FIG. 2

FIG. 1. PHOTOMICROGRAPH OF C. CELLOBIOPARUS FROM A CELLULOSE AGAR TUBE
Stained with Loeffler's methylene blue. The arrows point to a sporeforming rod and to a mature spore

FIG. 2. PHOTOMICROGRAPH OF C. CELLOBIOPARUS FROM A YOUNG CULTURE GROWN ON GLUCOSE AGAR
Stained for flagella by Zettnow's method. The flagella have been emphasized by tracing them with ink on the print

growth was regularly obtained. Sodium thioglycolate could be substituted for the sodium sulfide.

No evidence of sulfate reduction was observed when the organism was grown in a medium containing ferrous sulphate.

Good development occurs at a temperature of 38 C; at 25 C growth is slow; and at 18 C and 45 C it is inhibited.

The ability to ferment various substrates was tested in a concentration of 0.2 per cent in a solution containing biotin and the inorganic salts previously described. The pH was 6.8 to 7.0. The following substances were readily fermented with the formation of acid and gas: glucose, fructose, xylose, arabinose, mannose, cellobiose, melibiose, maltose, and a hemicellulose from birch. Occasionally, and more slowly, fermented were sucrose, lactose, raffinose, galactose,

mannitol, and dextrin. No acid or gas formation was observed with melezitose, trehalose, rhamnose, salicin, inulin, glycerol, and soluble starch.

TABLE 1

Comparison of the mesophilic cellulose-digesting species of Clostridium

	C. werneri	*C. dissolvens*	*C. cellulosolvens*	*C. cellobioparus*
Spores	oval, 1–1.2 by 1.5–2μ	oval, 2 by 2.5μ	spherical 1–1.5μ	spherical 0.9μ
Flagella	peritrichous	none	none	peritrichous few in number
Sulfate reduction	yes	no	no	no
Ferments readily	cellulose	cellulose	cellulose xylose arabinose dextrin	cellulose xylose arabinose glucose fructose mannose maltose cellobiose melibiose hemicellulose
Ferments less readily				galactose sucrose lactose raffinose mannitol dextrin
Does not ferment	glucose	glucose fructose galactose arabinose xylose sucrose maltose lactose and other substrates	glucose fructose mannose lactose maltose sucrose melezitose and other substrates	melezitose trehalose salicin inulin soluble starch glycerol rhamnose
Other distinctive features	growth is grayish	growth is yellow		cellobiose and no glucose from cellulose digestion

Taxonomy. The taxonomic position of the present bacterium has been difficult to determine. Its characters relate it both to the genus *Sporovibrio* and to certain species of *Clostridium*. The curved shape, spiral motion and rotation,

and the gram-negative reaction, suggest a vibrio relationship, and the few flagella are irregularly inserted at various points in the same manner as described by Starkey (1938) for the sporeforming cells of *Sporovibrio desulfuricans*. However, a typical vibrio with a single terminal flagellum has not been observed as a preponderant form in any of the cultures, and because of this it seems inadvisable to assign the organism to the genus *Sporovibrio*.

The mesophilic, gram-negative, cellulose-decomposing bacteria, which are included by Bergey *et al.* (1939) in the genus *Clostridium*, are *C. werneri*, *C. dissolvens*, and *C. cellulosolvens*. The "stecknadel" form isolated by V. Meyer (1935) and the cellulose-decomposing organism in the cultures of Pochon (1935) also belong in this group. The present bacterium possesses many characteristics in common with these species of *Clostridium* and it is assigned to this genus on the basis of its anaerobic nature, spore formation, and peritrichous flagella. It may be pointed out, however, that if the curved shape and negative gram reaction were to be considered of more taxonomic significance than the arrangement of flagella, the organism would belong in the genus *Sporovibrio*.

Morphologically the spores and vegetative cells of the present organism closely resemble *C. cellulosolvens*, except that the latter was reported as nonmotile. Lack of motility may not indicate absence of flagella, however, since Werner (1926) could observe no motility in hanging drop cultures (aerobic) of *C. werneri* even though peritrichous flagella could be demonstrated by staining.

Although there is no reliable morphological basis for a separation of the present bacterium from *C. cellulosolvens*, the two can be distinguished by the different substrates which they ferment. These are listed in table 1. Because of the large number of carbohydrates which the present organism ferments, it is considered to be a distinct species, *Clostridium cellobioparus* n. sp., the specific name referring to its cellobiose-producing characteristic which will be described in a later section. It should be noted that the failure of *C. cellulosolvens* to ferment glucose cannot be ascribed to an inhibiting action of autoclaved glucose (Stanier, 1942) as cellulose was readily fermented by this organism in cultures which contained glucose (Cowles and Rettger, 1931).

Occurrence. After the method for growing the organism was well established, samples of the contents of several rumina were inoculated into suitable dilution series in order to determine the frequency of its occurrence. In the series thus prepared it was not possible to demonstrate this organism. This might be expected from Ankersmit's observations (1905) on the relative scarcity in the rumen of cellulose-decomposing anaerobes of this same general type.

PHYSIOLOGICAL CHARACTERISTICS

Nature of the fermentation. The dependability of growth of *C. cellobioparus* in pure culture has made it possible to carry out analyses on the fermentation products of cultures in which known quantities of glucose or cellulose were fermented. The inorganic medium, with addition of biotin, was employed in most of the experiments. This medium is favorable for studies on metabolic products since it contains negligible amounts of organic carbon.

Hydrogen, carbon dioxide, acetic acid, formic acid, lactic acid, ethyl alcohol,

and other unidentified products are formed from both glucose and cellulose. The quantitative methods used in determining the amounts of each were as follows: Cultures were grown in round-bottom pyrex flasks (125 ml or 500 ml) with glass-sealed inlet and outlet tubes, which were drawn out and closed with a flame after inoculation and after oxygen had been removed by passage of oxygen-free gas. The all-glass container decreased the loss of carbon dioxide and hydrogen during the long interval required for fermentation. For measurement and analysis of the fermentation gases a tight rubber connection was made between the outlet tube of the culture flask and a mercury-filled gas-measuring burette. The capillary glass tip formed in sealing off the outlet tube was then broken inside the rubber tube and the excess gas allowed to pass into the burette until the pressure in both was atmospheric. The gas in the burette was measured and corrected for atmospheric pressure, temperature, and the vapor pressure of the water. The amount of carbon dioxide and hydrogen in it were determined by the customary methods of gas analysis. No evidence of carbon dioxide formation during combustion was observed, indicating the complete absence of methane. As the percentage of hydrogen in the collected gas was known, the amount left in the flask was calculated from the corrected volume of the gas phase within the flask. The amount of hydrogen dissolved in the culture liquid was also calculated from the solubility coefficient and the estimated partial pressure of the hydrogen before the flask was opened. Owing to the low solubility the amount dissolved was usually only a fraction of a milliliter.

The carbon dioxide remaining in the liquid and gas phases in the flask was determined in an absorption train immediately after the drawing off of the excess gas. The culture fluid was acidified with sulfuric acid in order to free all bound carbon dioxide. The carbon dioxide present in the excess gas was added to that found in the absorption train to give the total carbon dioxide in the flask. From this amount was subtracted the amount present in a control flask treated in the same manner as the experimental flask except that it was not inoculated, or, if inoculated, it was sterilized with mercuric chloride.

Ethyl alcohol was estimated by separation through alkaline distillation, oxidation to acetic acid with dichromate, collection of the acetic acid by steam distillation, and estimation of its amount from the Duclaux distillation.

After removal of the alcohol, formic and acetic acids were collected by acidifying and steam distilling. The formic acid was estimated in an aliquot of the distillate by its reduction of mercuric chloride. In a second aliquot the formic acid was oxidized with alkaline permanganate; the acetic acid remaining was steam distilled; and its amount was estimated from the Duclaux curve.

The residue of the steam distillation was neutralized, evaporated to dryness, and extracted with ether. After removal of the ether, the lactic acid was determined by the method of Friedmann, Cotonio, and Shaffer (1927).

Four representative experiments are summarized in table 2. Others have been completed with essentially similar results, though there is some variation in the fermentation products of cultures even when all conditions are thought to be identical.

Attempts to identify the unknown fermentation products have not been successful. They are probably not acids since the amount of recovered acids agrees fairly well with the total increase in titratable acidity during the fermentation. Tests for acetylmethylcarbinol and 2,3-butyleneglycol were negative.

Taking into account the state of oxidation of the products listed in table 2 and the fact that bacterial cells are usually more reduced than carbohydrates, it can be calculated that the proportions of carbon, hydrogen, and oxygen in the unknown fermentation products are approximately the same as in carbohydrates. No reducing materials are present at the close of fermentation, however, and attempts to demonstrate polyalcohols with the alkaline copper solution test have been entirely negative. Thus, only 70 per cent of the fermentation products have been recovered and identified. It is possibly significant that in quantitative balances for the fermentation of cellulose by *Clostridium dissolvens*, Khouvine, also, was able to recover only this proportion.

The excess of hydrogen over carbon dioxide shown in table 2 is of interest, since whether hydrogen is formed by decomposition of formic acid (Woods,

TABLE 2

Fermentation balances for C. cellobioparus

EXPT. NO.	SUBSTRATE	MG ATOMS C IN SUBSTRATE	FERMENTATION PRODUCTS (IN MILLIMOLS)						MG ATOMS C IN PRODUCTS	PER CENT CARBON RECOVERED
			CO_2	H_2	Acetic acid	Formic acid	Lactic acid	Ethyl alcohol		
1	100 mg glucose	3.33	0.28	0.55	0.51	0.29		0.22	2.03	60
2	100 mg glucose	3.33	0.32	0.53	0.44	0.49	0.03	0.22	2.22	67
3	455 mg glucose	15.15	2.36	2.98	2.29	0.97	0.55	0.55	10.66	70
4	462 mg cellulose	17.1	3.43	4.17	2.63	0.65	0.38	0.98	12.44	73

1936) or of pyruvic acid (Koepsell and Johnson, 1942) a mol of carbon dioxide should accompany each mol of hydrogen formed. In the experiments of table 2 and in all other experiments it has been found that the hydrogen exceeds the carbon dioxide by a significant amount.

The developments in the field of heterotrophic carbon dioxide assimilation (Barker, 1936; van Niel *et al.*, 1942; Werkman and Wood, 1942) have shown that fixation of carbon dioxide is a common occurrence in many metabolic processes. Such an assimilation in the course of the fermentation by *C. cellobioparus* would account for the failure of carbon dioxide to appear in quantities equal to the hydrogen. Experiments 3 and 4 (table 2) were initially fairly free of carbon dioxide. Nitrogen instead of the nitrogen-carbon-dioxide mixture was used to displace the oxygen, and phosphate instead of bicarbonate was used as a buffer. The only carbon dioxide added was that present in the inoculum. In experiments 1 and 2 (table 2) an atmosphere of nitrogen and 5 per cent carbon dioxide was used with bicarbonate in the medium, and in these the carbon dioxide formed is considerably less than the hydrogen, the ratio between them approaching 2.

The relative decrease in the amount of carbon dioxide produced in cultures with greater initial carbon dioxide concentration is in agreement with the hypothesis that carbon dioxide is assimilated, as the amount of carbon dioxide assimilation would be expected to increase with increasing carbon dioxide concentration. The various reactions which are postulated (van Niel *et al.*, 1942) to explain the fixing of carbon dioxide are all equilibrium reactions, and an influence of carbon dioxide concentration on the rate of its reaction has been shown by Barker (1943) in the case of *Methanobacterium omelianskii.*

The nature of the digestion products. Relatively small amounts of cellulose were used in most of the quantitative experiments. If no additional buffers were added to the basal medium, approximately 0.15 g of cellulose per 100 ml of medium were decomposed before the acidity increased to a point inhibiting growth. In one of the early cultures containing 75 ml of medium, an excess of cellulose remained after active fermentation had ceased. The culture was left at room temperature for 6 weeks, at the end of which time all the remaining cellulose had dissolved, a total of about 250 mg. The culture liquid showed a strong reduction with Benedict's solution. This suggested that hydrolysis of the cellulose had continued after the medium became too acid to permit further growth. The products of digestion had accumulated without being fermented. The increase in acidity accomplished the same separation of the hydrolytic from the fermentative processes that was accomplished by Pringsheim (1912) through the use of antiseptics.

Subsequently it has been found that the reducing capacity of the medium increases gradually over a period of many weeks when an old, unopened culture is left with an excess of cellulose. As an example, a culture run in parallel with experiment 4 (table 2) contained initially 2.582 g of cellulose instead of the 0.462 g in experiment 4. Fermentation proceeded much as in experiment 4, but instead of being analyzed at the close of fermentation the culture (number 5) was allowed to remain in the incubator for 3 additional months.

Aliquots of this culture were tested for fermentation products, and the following were demonstrated (all figures as millimols): carbon dioxide, 3.71; hydrogen, 4.82; acetic acid, 2.52; formic acid, 0.52; lactic acid, 0.34; and ethyl alcohol, 0.67. These are approximately the same in amount as those formed in experiment 4 which was analyzed at the close of fermentation. This indicates that the same amount of cellulose was fermented. But whereas 0.462 g of cellulose disappeared in experiment 4, this culture had lost 1.883 g. The culture fluid showed strong reducing power, 10 ml reducing 66.3 mg of copper.

In order to test for products of hydrolysis, the culture solution of experiment 5 was filtered, evaporated to a small volume at reduced pressure, and alcohol added to precipitate the inorganic salts. The alcohol containing the reducing materials was separated, the alcohol evaporated, and the residue taken up in a small volume of water. This solution was used for tests on the nature of the reducing substances formed.

Samples hydrolyzed with dilute acid for 2 hours in the boiling water bath showed an increase of 32 per cent in reducing power. A preparation of the

enzyme emulsin was also effective in hydrolysis. Since emulsin splits the beta-glucoside linkage, this suggested the presence of cellobiose. An unhydrolyzed sample was heated with phenylhydrazine and sodium acetate, and copious quantities of an osazone, soluble in hot water but less soluble in cold water, were obtained. The osazone was recrystallized twice and found to have the same crystalline appearance and Kofler micro-melting point (192.5 to 194.5) as samples of cellobiosazone which were prepared in parallel. Elementary microanalyses of this osazone showed that its composition was similar to that of cellobiosazone. Values for duplicate samples of the unknown were: carbon, 55.15, 54.80; hydrogen, 6.06, 6.21; nitrogen, 11.02, 11.07. The theoretical values for cellobiosazone are 55.4, 6.16, and 10.77, respectively. These properties show that cellobiose is one of the products of cellulose hydrolysis by *C. cellobioparus*.

Unhydrolyzed samples of the culture solution showed no traces of glucosazone when heated with phenylhydrazine and sodium acetate. Control tests with as little as 5 mg of glucose in 5 ml gave a definite precipitate of glucosazone, but none could be detected in the experimental tubes even when seeded with a crystal of pure glucosazone. If 5 mg of glucose were added to 5 ml of the unknown sugar solution they gave the typical crystals of glucosazone in about the same amounts as when tested in water, showing that no factors were operating to prevent the demonstration of glucose in the culture material.

In corroboration of the negative osazone tests for glucose, unhydrolyzed samples of the culture solution supported very little fermentation when tested with yeast. Samples hydrolyzed with acid or emulsin were readily fermented and glucose could be demonstrated in such samples with phenylhydrazine. All these results show that glucose is not formed as a final product of the hydrolysis of cellulose by *C. cellobioparus*. The failure to obtain glucose as a product of cellulose digestion was unexpected in view of its demonstration as a product of cellulose decomposition by other workers. Careful, repeated experiments have failed to show any evidence of glucose.

The quantitative importance of cellobiose as a product of cellulose hydrolysis by *C. cellobioparus* was estimated in three different ways. (1) The osazone yield of the experimental solution was compared with that of an amount of cellobiose giving the same copper reduction. A sample of the unknown, showing copper reduction equivalent to 191 mg of cellobiose, formed 84.1 mg of crude osazone. A parallel experiment with 191 mg of cellobiose yielded 88.9 mg of osazone. (2) The acid hydrolysis of pure cellobiose under conditions similar to those used with the experimental material caused an increase in reducing power of 36.4 per cent as compared with 32 per cent for the unknown. (3) The fermentable sugar in an emulsin-hydrolyzed sample was estimated with the yeast method and the result compared with a similar experiment on pure cellobiose. Four tubes were prepared as shown in table 3. All were adjusted to a pH of 5.0 and incubated at 38 C for 24 hours. Tubes 2 and 4 were then boiled to precipitate the protein in the enzyme solution and all were filtered. The filtrate of each was made up to 4 ml, and 2 ml of the control tubes were used for a determination of copper reduction. The values obtained were used to calculate the sugar present, assum-

ing that it was all cellobiose. The glucose equivalent was calculated, together with the amount of carbon dioxide to be expected on fermentation. One ml of each filtrate was then equilibrated with carbon dioxide, and the gas production by a heavy yeast suspension was measured. The results are shown in table 3, all figures being on the basis of 4 ml.

It may be seen from table 3 that the carbon dioxide produced by the experimental solution was approximately that to be expected if its reducing power were due to cellobiose. Similar experiments with other culture solutions have given the same result.

Although there is a considerable error in each of the above methods of estimating cellobiose, the general agreement between them shows clearly that the major part of the reduction in digests of cellulose by *C. cellobioparus* is due to cellobiose. With this information, further approximations may be made in order to determine whether significant amounts of nonreducing substances appear during digestion of cellulose by this organism.

TABLE 3

Results of the yeast fermentation experiment

TUBE NO.	CONTENTS	CALCULATED GLUCOSE FROM Cu VALUES	CO_2 PRODUCED, CORRECTED	GLUCOSE ACCOUNTED FOR BY CO_2	PER CENT OF THEORETICAL YIELD
		mg	*ml*	*mg*	
1	Boiled emulsin and cellobiose	36.4	0.00		
2	Active emulsin and cellobiose	36.4	5.44	22.0	60
3	Boiled emulsin plus expt. sol.	47.4	0.48		
4	Active emulsin plus expt. sol.	47.4	8.16 (7.68 corr. for tube 3)	30.9	65

If it be assumed that the cellulose fermented in experiment 5 was equal to that in experiment 4 (0.462 g), it can be calculated that 1.883 − 0.462 or 1.421 g of cellulose were hydrolyzed but not fermented in experiment 5. If all of this were hydrolyzed to cellobiose, it should yield approximately 1.5 g. Assuming that the entire copper reduction value of the culture solution of experiment 5 (66.3 mg Cu per 10 ml) was due to cellobiose, the total amount present was 1.464 g. The closeness with which this figure approaches the value of 1.5 g of cellobiose to be expected on the basis of the weight of cellulose digested shows that cellobiose is the chief product of hydrolysis. Appreciable quantities of nonreducing materials are not formed. It may be concluded that intermediates between cellulose and sugar, the cellulose dextrins, do not accumulate in very large amounts during the cellulose digestion by *C. cellobioparus*. Similar conclusions were reached by Khouvine for *C. dissolvens*.

Optimum reaction for cellulose digestion. Several experiments were run to test the effect of acidity on the activity of the cellulase. Samples of a culture were withdrawn at the close of fermentation, adjusted to various acidities, and incubated with toluene. The copper-reducing power of each sample was then

estimated either by inspection of the tubes after boiling with Benedict's solution or quantitatively with the Bertrand method. The most reduction was observed in the tubes at a pH of 5.5. Cellulose digestion was demonstrated at acidities as low as 4.4, but none at 4.0. On the alkaline side of the optimum, relatively rapid digestion occurred at pH 6.0 to 6.5, but fell off considerably at 7.0, and was very slight at 7.5. Fresh cellulose culture medium at a pH above 8.0 either failed to give growth when inoculated with *C. cellobioparus* or growth started only after a prolonged incubation. It is of interest that the optimum reaction for action of the cellulase lies at the lower limit for growth of the bacterium in the laboratory cultures.

DISCUSSION

The author is inclined to believe that the reduction and gradual loss of cellulose-digesting ability which has been reported for many pure cultures of anaerobic cellulose bacteria is in most cases due to an inadequacy of the culture method. Although it is possible that mutations in a strain might lead to a loss of cellulose-digesting ability, as is known to happen in the case of the enzymes required for some other substrates, it seems desirable to adopt this hypothesis only after exhaustive studies of growth requirements. The cellulose bacteria retain their cellulose-digesting capacity in crude cultures; and if the expression of this capacity is due to "symbiotic" activities of accompanying forms, it should be possible to analyze and duplicate the essential activity of the "symbiont." Although direct evidence on the subject is lacking, it seems most probable that the beneficial effects of accompanying forms are not a direct influence on the cellulase system, but rather the establishment of a suitable redox potential, the synthesis of essential nutrilites, the removal of toxic metabolites, or other activities which promote the general welfare of the cellulose organism without directly influencing cellulose digestion. According to this view the problems in pure-culturing cellulose anaerobes are similar to those of the many other anaerobes for which no satisfactory methods of isolation have yet been developed.

The reported inability of many cellulose-decomposing anaerobes to utilize simple sugars may be due to the toxic effect of autoclaved glucose. This has been reported by Stanier (1942) for aerobic cellulose bacteria, and, according to the studies of Lewis (1930), may be due to autoclaving glucose with phosphate in a slightly alkaline medium. During the present experiments no toxic effects of glucose have been observed, either because the organism is not susceptible or because the reaction of the medium during autoclaving was sufficiently acid so that no toxic substances were formed. The "stecknadel" form of Meyer (1935) was found to utilize glucose if a suitable medium was provided.

In view of the ability of *C. cellobioparus* to ferment glucose, it is of interest that this sugar is not formed as a normal product of cellulose hydrolysis by this organism. The first step in the utilization of cellobiose is apparently not an hydrolysis in which freely diffusible hexose is formed. An enzyme catalyzing this reaction, even though endocellular, would be expected to liberate glucose in the old cultures, provided no changes deleterious to the enzyme occurred in

the cells after death. Although such changes may have affected the enzymes in the above hydrolysis experiments, it does not seem likely that these changes would be sudden and complete; a certain continued action of the enzyme would be expected even though it might in time be inactivated. Thus, in view of the inability to find even traces of glucose in the old cultures, it seems reasonable to conclude that no enzyme catalyzing the reaction (cellobiose→glucose) was present.

Although *C. cellobioparus* does not appear to be of significance in the digestion of cellulose in the rumen, some of the results obtained from the study of it suggest the nature of the mechanism by which the ruminant utilizes cellulose. It has been observed that cellobiose did not accumulate in the medium until after fermentation ceased. Also, liquid media containing cellulose showed a somewhat slower development of the bacteria than the same media with glucose or cellobiose. The rate of formation of cellulose digestion products is apparently the factor limiting the rate of growth. If the rate of formation of the digestion products from cellulose is the limiting factor, it is evident that the concentration of these products (sugars) would always be low. On the other hand, the fermentation products would be present in relatively large amounts, the exact magnitude of their concentration depending upon their rate of production and the facilities for removing them. In the rumen, owing to the large volume of the contents, the surface-volume relationships are not favorable to a rapid absorption of fermentation products and an accumulation takes place. Since the digestion product, sugar, is present in a low concentration, whereas the fermentation products are present in relatively large quantities, it would be expected that if the host obtains any significant amounts of materials from the rumen they would have to be the fermentation products rather than the sugars. This has been postulated for ruminants by Woodman and Evans (1938) and has been demonstrated to be the case in the symbiotic utilization of cellulose by the termite and its wood-digesting protozoa (Hungate 1943b).

Microscopic observations of the organisms which are actually concerned with cellulose digestion in the rumen have been made by Ankersmit (1905) and Henneberg (1922). Henneberg describes the "frassbetten" which characterize the accumulations of cellulose bacteria. A similar close association with the cellulose has often been reported for the cytophagas. This closeness, coupled with the failure to demonstrate a cellulase in the cytophagas, has given rise to speculation on the possibility that an extracellular cellulase may not be formed and that the cellulose is metabolized without preliminary hydrolysis. This view has previously found some support in the seeming inability of the cytophagas and most cellulose anaerobes to utilize glucose. With the demonstration by Stanier (1942) that the cytophagas can utilize glucose, and the finding that glucose is also utilized by certain cellulose-decomposing anaerobes (*C. cellobioparus* and the "stecknadel" form of Meyer), one of the arguments for cellulose attack without hydrolysis is removed.

The closeness with which cellulose bacteria adhere to the cellulose particles can also be explained upon the hypothesis that an extracellular cellulase is

formed. The closer the bacterium approaches the substrate the greater the benefit it derives from its extracellular enzymes. This follows from a consideration of the several steps by which the cellulose is hydrolyzed and the products become useful to the cell. These are: (1) the cellulase must be secreted and diffuse to the substrate; (2) it must adsorb on the cellulose and liberate sugar; and (3) the sugar must diffuse back to the cell. It is evident that the farther the bacterium is from the cellulose the less the chance that the hydrolytic products will diffuse back to it. This would be particularly true in impure cultures where organisms, themselves unable to utilize cellulose, would readily absorb the hydrolytic products. Thus, the closeness with which cellulose-digesting bacteria cling to the cellulose fibers undergoing disintegration can also be interpreted as an adaptation which provides maximum returns to the cell from its hydrolytic enzymes.

The extracellular nature of the cellulase formed by *C. cellobioparus* is clearly demonstrated in cellulose agar tubes containing only one or two colonies. Around each colony a clear area develops in which the cellulose has been digested. No bacteria are present in this surrounding region. The outer boundary of the clear area is very sharp, indicating that proximal cellulose particles are completely digested before more distant ones are attacked. This shows that the cellulase is strongly adsorbed to the cellulose and is not free to diffuse until the immediately adjacent substrate is completely dissolved and no longer presents an adsorptive surface.

During the early stages of cellulose digestion by *C. cellobioparus* in a liquid medium, the fluid above the cellulose remains very clear, even though bubbles liberated from the cellulose in the bottom of the tube show that fermentation is in active progress. This situation is little changed until most of the cellulose is digested, when the supernatant liquid develops a marked cloudiness. The same appearance was reported by Clausen (1931) for his cultures. In a glucose culture the medium becomes uniformly cloudy as development progresses. These observations indicate that the cellulose substrate exerts a tactic influence on the bacteria, causing them to accumulate in its vicinity. Though possessed of an active motility, and giving off an extracellular cellulase, the bacteria nevertheless remain near the cellulose. Microscopic observations of living cultures indicate that there is not a close attachment of the cells of *C. cellobioparus* to the substrate, but that in their movement they tend rather to remain in the vicinity of the cellulose. This suggests an adaptive behavior on the part of the bacterium. With less motile cellulose-decomposing bacteria a similar adaptive response might result in a more permanent attachment to the substrate.

SUMMARY

An anaerobic cellulose-digesting bacterium, *Clostridium cellobioparus*, has been isolated from the rumen. It differs from other bacteria of this group in being easily isolated and in growing well on both glucose and cellulose. No tendency toward loss of cellulose-digesting capacity during four years of pure culture has been observed. The growth requirements are satisfied by an inorganic

medium with the addition of biotin and a carbohydrate. A variety of sugars are decomposed.

The fermentation products are acetic, formic, and lactic acids, ethyl alcohol, carbon dioxide, and hydrogen. Other products, amounting to 30 per cent of the substrate, have not been identified. Cellobiose is the chief digestion product of cellulose; no glucose is formed.

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