POLYSACCHARIDE STORAGE AND GROWTH EFFICIENCY IN *RUMINOCOCCUS ALBUS*

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ABSTRACT

HUNGATE, R. E. (University of California, Davis). Polysaccharide storage and growth efficiency in *Ruminococcus albus*. J. Bacteriol. **86**:848–854. 1963.—*Ruminococcus albus* strain RAM requires biotin, *p*-aminobenzoic acid, pyridoxamine, isovalerate, isobutyrate, 2-methylbutyrate, and either cysteine or sulfide. Rumen fluid and casein hydrolysate improve growth but are not essential. Up to 35% iodophilic polysaccharide is stored in cells from batch cultures and 17% in a continuous culture on a 10-hr cycle. The storage product is a polymer of glucose resembling starch. The yield of cells in continuous culture, corrected for stored starch, averaged 102 mg per mmole of cellobiose fermented to waste products. It is postulated that nine high-energy phosphates are derived from each cellobiose molecule. Conversions providing this number are discussed.

Investigations of overall rumen activities have dealt with the kinds and quantities of volatile fatty acids supplied the host, and the quantities of microbial cells formed. The cells are important to the ruminant, since they are digested in the true stomach and are the principal nitrogenous food.

Relatively few quantitative studies of cell synthesis by rumen bacteria have utilized pure cultures. *Ruminococcus albus* (Hungate, 1957), one of the important digesters of cellulose in the rumen, was chosen for a study of the relationship between fermentation and growth. This species appeared particularly suitable because the information on nutrients provided by Bryant and Robinson (1961*b*) permitted relatively easy determination of the growth requirements of the selected strain.

Early in the investigation, it became apparent that large quantities of iodophilic material were deposited in the cells during growth on cellobiose, necessitating a study also of stored polysaccharide.

MATERIALS AND METHODS

A strain of *R. albus* (Hungate, 1957), classified by Mah (1962) after he isolated it from a culture of *Ophryoscolex purkynei*, was used. This RAM strain was transferred daily by inoculating 0.1 ml of a 24-hr culture into liquid medium containing 0.2% cellulose, inorganic salts previously described (Hungate, 1950), 0.5% $NaHCO_3$ in an atmosphere of CO_2, and minimal defined organic nutrients.

The minimal medium as finally used contained, per 10 ml: 0.03 μg of *p*-aminobenzoic acid, 0.008 μg of biotin, 0.15 μg of pyridoxamine, and 750 μg each of isovaleric, isobutyric, and 2-methylbutyric acids. Cysteine hydrochloride and $Na_2S \cdot 9H_2O$ (each 0.025%) were used routinely in batch cultures.

The cellulose was a suspension of Whatman no. 1 filter paper (3%) ground for 24 hr in water in a large-pebble mill. To remove traces of oxygen, the carbon dioxide was passed over hot copper metal reduced with H_2. Air was excluded by previously described methods (Hungate, 1950), with the modification that inoculating needles were substituted for the Pasteur pipette capillaries, and red rubber stoppers for black ones. In carrying the routine culture, inoculation was often by hypodermic syringe injection through the rubber stoppers, the latter having previously been bored half through with a cork borer.

Optical density in the 16-mm culture tubes was measured in an EEL (Evans Electroselenium Ltd.) colorimeter.

Purity of higher volatile fatty acids was examined with the James and Martin (1952) gas chromatographic column. Total nitrogen was determined by the micro-Kjeldahl method. Reducing materials were measured with the copper reagent of Somogyi (1945) and the color reagent of Nelson (1944), with reference to a

standard curve constructed from analytical results with pure sugars.

Polysaccharide reserves in the cells were estimated as follows. To the dry cells, 0.2 ml of 30% NaOH was added. The cells were heated at 100 C for 3 hr to hydrolyze protein, and the polysaccharide was precipitated by adding 0.2 ml of water and 1.2 ml of ethanol. The insoluble material was centrifuged down, washed with 66% ethanol, and dried. Polysaccharide was dissolved by adding several small amounts of hot water and filtering from insoluble residues while the mixture was still hot. Hydrochloric acid was added to the filtrate to give a final concentration of 10%, and the polysaccharide was hydrolyzed by heating at 100 C for 3 hr. The solution was neutralized and made up to 1.5 ml, and a sample was tested for Cu reduction.

Glucose was identified by chromatography, using Whatman no. 1 paper with water-saturated phenol or the upper phase of *n*-butanol–ethanol–water (5:1:4) as the solvent.

Continuous cultures were grown in a 300-ml round-bottom flask, fed by a DCL Micro pump ($\frac{1}{16}$-in. diameter piston, 21 strokes per min; Distillers Co. Ltd., Great Burgh, England). Prior to use, the head containing the piston plunger was sterilized in an autoclave. The rest of the equipment was all glass except for rubber-tubing connections between containers. Separate parts were sterilized, with the connecting ends protected against contamination, and were assembled aseptically after sterilization. Prior to inoculation, the apparatus was gassed with CO_2 before liquids were added.

A 100-ml culture of strain RAM was grown in an all-glass culture flask with a 16-mm side tube which could be inserted into the EEL colorimeter to measure optical density. When the optical density reached the desired point, the culture was transferred to the continuous culture vessel and the pump was started.

During operation, a little CO_2 was kept flowing through the system to maintain a positive pressure and force the culture overflow into the collecting vessel. The latter was immersed in ice, and the collected culture was drawn off at intervals. A magnetic stirrer at slow speed kept the culture mixed. Occasionally, the speed was increased for a short period to break up any local accumulations of cells.

The continuous culture was operated three times, for periods of 32, 45, and 183 hr. In the first two runs, a minimal medium without rumen fluid was used. In the third, 10% cell-free rumen fluid was added. Cysteine was omitted, but Na_2S was included in the medium. The specific dilution rate was 0.1 per hr. If the feed rate was reduced below 15 ml per hr, a gas bubble developed in the pump and was not dislodged until the rate was increased.

Results

Nutrition. Of the wide range of inorganic nutrients and vitamins tested, including those of Bryant and Robinson (1961*a*), the following did not stimulate growth above the level obtained with a minimal medium: Fe, Mn, Cu, Co, Mo, Zn, ethylenediaminetetraacetate, nitrolotriacetate, riboflavine, folic acid, nicotinamide, pantothenate, cyanocobalamine, and thiamine. At one stage during the process of sorting out growth requirements, thiamine appeared to be stimulatory, but in the minimal medium as finally developed it was slightly inhibitory.

Pyridoxamine, biotin, and *p*-aminobenzoic acid were required. The minimal levels supporting growth were 0.0024 μg of biotin, 0.05 μg of pyridoxamine, and 0.013 μg of *p*-aminobenzoic acid per 10 ml of 0.2% cellulose broth.

Initially, a mixture of isobutyric, isovaleric, and valeric acids was tested. On the James and Martin (1952) gas chromatographic apparatus, each acid showed one principal band with only traces of impurities. Without valeric acid, slow growth occurred if acetate was supplied. Later, 2-methylbutyric acid was included in the medium and was found to give increased growth, which was not improved by acetate. There was no growth if isobutyrate, isovalerate, or 2-methylbutyrate was omitted. Either cysteine or sulfide could be omitted without diminishing growth, but, in the absence of both, growth was very poor, even with a 10% inoculum, and further subcultures failed to develop.

Dilution cultures from minimal cellulose broth into minimal cellobiose agar plus sulfide showed colonies in the thick agar at the base of the tube but not in the thin agar on the sides. If cysteine was included in the medium, colonies appeared in all parts, and their number per unit volume of agar was greater than in the base of parallel cultures containing only sodium sulfide.

Addition to the minimal medium of the volatile fatty acids from rumen fluid did not improve

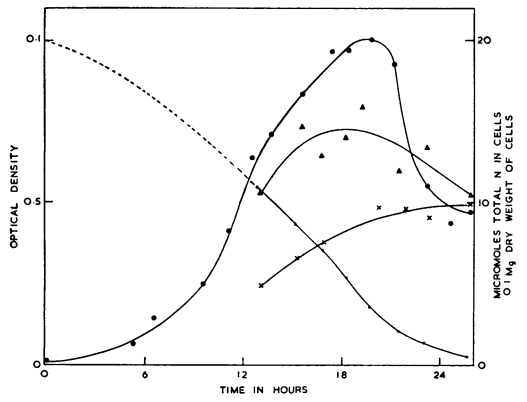

FIG. 1. *Cell nitrogen and dry weight compared with optical density and cellobiose disappearance at various stages of a batch culture. Symbols:* ● = *optical density*; • = *cellobiose*; ▲ = *dry weight*; × = *nitrogen content.*

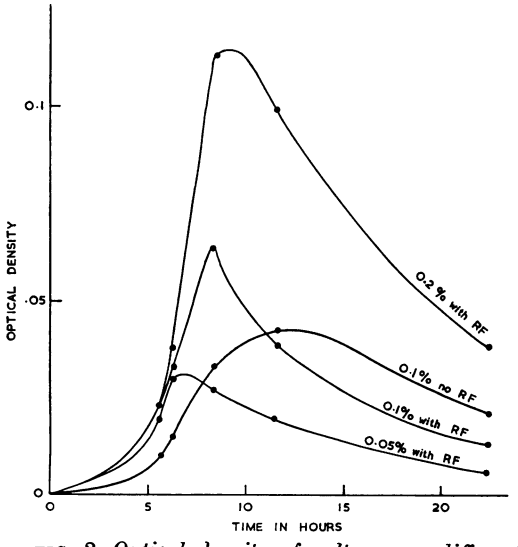

FIG. 2. *Optical density of cultures on different amounts of cellobiose, with and without rumen fluid (RF).*

growth, but it was increased by cell-free rumen fluid or acid-hydrolyzed casein.

Reserve polysaccharide. Batch cultures of *R. albus* strain RAM on cellobiose showed the growth curve typical of rumen cellulolytic cocci (Fletcher, 1956; Bryant and Robinson, 1961*b*), with a marked drop in optical density soon after the maximum. Phase microscopy of a culture prior to maximal density disclosed chains of 10 to 30 refringent cells. Stained with iodine and examined under ordinary transmitted light, they appeared slightly darker than unstained controls. Sedimented and treated with iodine, they showed the purple-black color characteristic of starch. Soon after optical density decreased, the cells were single or in twos, and on sedimentation showed a reddish-violet to purple color. Masses of cells from old batch cultures gave no color with iodine.

Cell nitrogen and dry weight at various stages of a batch culture were determined, in parallel with optical density and cellobiose disappearance (Fig. 1). Dry weight changed with optical density but was not proportional, the stored polysaccharide being more refringent than the remaining cell material. In another batch experiment, cells removed at the stage of maximal optical density weighed 4.1 mg per 10 mg of cellobiose supplied.

Changes in optical density with time, in batch cultures containing various concentrations of cellobiose, are illustrated in Fig. 2. This experiment employed all-glass sealed culture tubes. Tilting of stoppered tubes, prior to reading the optical density, exposed the cells to oxygen which had penetrated the red rubber stoppers, causing extremely variable results.

The proportionality between optical density and initial concentration of cellobiose is apparent in Fig. 2, both at maximal optical density and in the final crop of cells at 22 hr. In this experiment, the concentration of the minimal nutrients was doubled, and 20% clarified rumen fluid was added, to see if a greater concentration of nutrients diminished the quantity of reserves stored. Comparison of the 0.1% cellobiose curves with and without rumen fluid (Fig. 2) discloses that improved nutrition caused a more rapid increase in optical density and a higher maximum but gave a lower final optical density.

Cells in a broth culture on minimal medium, 20% rumen fluid, and 0.2% cellobiose were killed with formalin when the optical density reached 0.13. They were centrifuged, washed by resuspending in 70% ethanol, and recentrifuged. The dry weight was 5.43 mg, or 27% of the sugar in the culture. Since the cellobiose was not exhausted, the dry weight of cells probably constituted an even higher proportion of the substrate actually used.

The sugar from hydrolysis of the polysaccharides was identified as glucose by paper chromatography with the two solvents. In addition to the glucose, a very faint spot with an R_F 1.57 that of glucose was observed with the

butanol solvent, and an R_F 1.88 that of glucose was observed with phenol. The glucose recovered after hydrolysis of the polysaccharide amounted to 980 μg, equivalent to 880 μg of starch, which would constitute 16% of the cell weight. An older parallel culture without rumen fluid showed an optical density of 0.07 when killed, fixed, and similarly analyzed for reserve polysaccharide. The cell weight was 3.25 mg with a starch content of 188 μg, calculated from the recovered glucose.

Determinations of cellobiose, optical density, cell weight, and nitrogen content, on samples from a large batch culture, are shown in Table 1 for three different times of sampling.

Results and calculations from the third continuous culture are shown in Table 2.

Discussion

Nutrition. The vitamin requirements of the RAM strain are identical with those of strain 7 of Bryant and Robinson (1961*b*), except that a slightly higher concentration of *p*-aminobenzoic acid is needed. The requirement for 2-methylbutyrate supports the view (Allison et al., 1962) that rumen cocci need branched-chain precursors for synthesis of valine, leucine, and isoleucine. The replacement of acetate by 2-methylbutyrate suggests a role for the former in synthesis of 2-methylbutyrate.

The experiment on sulfur nutrition indicates that sulfide or cysteine is required for growth, sulfate being unsuitable. Unfortunately, after the experiment was performed, it was discovered that red rubber stoppers were more permeable to oxygen than the black stoppers previously used. Instead of inability to use sulfate, entrance of oxygen into the cultures without cysteine or sulfide may have prevented growth. In later experiments, cysteine gave more colonies in cellobiose agar, even when black stoppers were used and sulfide was added, suggesting that cysteine or similar compounds improve conditions for initiation of growth. Continued cultivation by daily transfer of 1% inoculum into media without cysteine shows that such compounds, if essential, can be synthesized.

The unexpected permeability to oxygen of the red rubber may explain the difficulties experienced by some workers in using the rubber-stoppered roll tube method to grow anaerobes. Particularly with cellulose agar cultures, during the relatively long period required for appearance of clear zones, inhibitory concentrations of oxygen could enter. This problem is also serious during storage of sulfide, cysteine, or other reducing solutions.

Polysaccharide reserves. Bryant and Robinson (1961*b*) noted the marked rise and rapid fall in optical density of cultures of *R. albus* strain 7.

TABLE 1. *Weights and nitrogen contents of cells from a batch culture on minimal medium*

Age of culture	Optical density	Cells in 25-ml culture	Fermented cellobiose in cells	N in cells	Cell N per mg of cellobiose
hr:min		*mg*	%	%	*μg*
24:10	0.082	4.72	22.1	6.85	15.0
28:45	0.102	5.12	15.6	7.64	11.9
44:50	0.037	5.45	11.7	10.5	12.3

TABLE 2. *Cell yields in relation to cellobiose utilized*

Time	Cu-reducing substance in effluent, as cellobiose	Cellobiose used	Cell wt	Cellobiose available for ATP production	Nitrogen content of cells	Reserve starch in cells	Cell wt recalculated to a 10.5% N basis	Corrected cell yield per mmole of cellobiose for ATP production	Cell N-total cellobiose ratio
hr:min	*mg/10 ml*	*mg/10 ml*	*mg/10 ml*	*mg/10 ml*	%	%	*mg/10 ml*	*mg*	*μg/mg*
5:25	0.44	9.56	2.81	6.75	8.6	18	2.30	116	24
16:25	0.27	9.73	2.32	7.41	8.9	15	1.96	90	20
19:45	0.22	9.78	2.64	7.14	9.5	9	2.39	114	25
23:20	0.16	9.84	2.28	7.56	9.0	14	1.95	88	20
75:15	0.19	9.81	2.42	7.39	9.4	11	2.16	100	23
100:30	0.11	9.89	2.70	7.11	7.6	28	1.95	94	29
126:45	0.10	9.90	2.56	7.34	8.4	20	2.05	96	22
135:50	0.17	9.83	2.94	6.89	8.4	20	2.35	116	25
Average		9.79	2.58	7.20	8.7	17	2.14	102	22.4

No microscopic evidence of lysis or reserve storage was detected. Cells removed after the period of maximal optical density may still have contained sufficient storage product to prevent microscopic detection of a difference in iodophily. The iodophily and marked drop in optical density encountered with many strains of *Ruminococcus* suggest that abundant polysaccharide storage is a common characteristic.

The value of 10.5% cell nitrogen at 44:50 (Table 1) resembles the percentage of N (10.44%) found by Block, Stekol, and Loosli (1951) for the total rumen bacteria. On the assumption that the value of 6.85% at 24:10 was lower owing to stored polysaccharide, the latter composed 35% of the cells.

The value of 35% for the polysaccharide stored during stages of maximal optical density is somewhat greater than the 20 to 25% glycogen in N-starved batch cultures of *Escherichia coli* (Holme and Palmstierna, 1956). The marked iodophily of cells from the continuous culture indicates polysaccharide accumulation even at low dilution rates. From the cell N content, the polysaccharide in the continuous culture was 17% of the cell dry weight (Table 2).

It was hoped that evidence on utilization of reserve polysaccharide for growth might be obtained. Although the optical density (Table 1) fell from 0.102 to 0.037 in the interval between 28:45 and 44:50, the nitrogen content of the cells increased from 0.39 to 0.57 mg. At 28:45 the amount of cellobiose fermented was 32.8 mg, and at 44:50 it was 46.5 mg, an increase of 42%. The cell nitrogen increase in this period was 46%. The slightly greater increase in nitrogen suggests that the reserves were used in synthesis of nitrogenous materials, but the difference is so small that the evidence is not conclusive.

Accumulation of storage polysaccharide during growth in batch cultures is understandable on the basis that sugar is not limiting until later stages, and during excess supply is partially converted to polysaccharide. Storage of large amounts of starch in cells grown in continuous culture is more difficult to explain. Polymerization into reserves may require only the cellobiose concentrations indicated by the Cu-reducing materials in the continuous culture. Traces of cellobiose were not completely absorbed even at the slow feed rate. This suggests that a high concentration of cellobiose in the rumen would be required for rapid growth of *Ruminococcus*. Although this seems incompatible with the dense microbial population, S. R. Elsden (*personal communication*) has pointed out that concentration in the sense of the entire rumen may be misleading. Sugar at the site of cellulase activity may be very concentrated, even though the average concentration in the entire rumen is extremely small.

Polysaccharide may persist because it is an intermediate in utilization of cellobiose, possibly formed directly from cellulose.

Cell yields. In batch experiments, the cell yield was as much as 41% of the cellobiose used, on a dry weight basis, but much of this was storage polysaccharide. The cells from the batch culture of Table 1 at 44:50, showing no iodophily, constituted 11.7% of the fermented cellobiose.

In previous studies on the rumen cellulolytic cocci (Hungate, 1950), the recovered fermentation products accounted for only 70% of the sugar disappearing. The oxidation-reduction balance of the products suggested that the missing material was carbohydrate. In the light of the present results, the missing product appears to have been cell material and polysaccharide reserves.

In continuous culture, the average yield was more than 25% of the cellobiose available (Table 2). Some of this is accounted for by reserves, but even after correction to the maximal cell nitrogen observed in old batch cultures (10.5%) the average dry weight of cells amounted to 22% of the cellobiose utilized. Since clarified rumen fluid contains very little amino acids or other materials suitable for immediate polymerization into cells, and did not improve the growth yield in the batch experiments (Fig. 2), it probably cannot account for the high cell yield in the continuous culture.

These yields of *Ruminococcus* cells are higher than those usually obtained with pure cultures of anaerobic bacteria fermenting carbohydrates. The yields for *Propionibacterium pentosaceum* obtained by Bauchop and Elsden (1960) are somewhat lower, though results are not strictly comparable because they studied a batch culture and used a complex medium providing many of the monomers (Gunsalus and Shuster, 1961) for synthesis of cell substance.

The branched-chain acids in the medium presumably served for synthesis of valine, leucine, and isoleucine, and may have been assimilated into additional cell constituents. It is doubtful

that all amino acids would be preferentially formed from these materials, though the amounts required in the medium (2 mg per 10 ml) are greater than should be needed for synthesis of the corresponding amino acids. If a significant fraction of cell material was derived from the branched-chain acids, more cellobiose than that shown in column 5 of Table 2 would be available for adenosine triphosphate (ATP) production.

The average yield of cells (corrected to a 10.5% N content) in Table 2 is 102 mg per mole of cellobiose fermented. According to the estimates of Bauchop and Elsden (1960), this indicates almost 10 ATP per cellobiose molecule, or 5 per glucose equivalent. If the cell material was all derived from the branched-chain acids, this value would be reduced to a minimum of 7.3 ATP per molecule of fermented cellobiose. Such total synthesis is unlikely. Nine per cellobiose molecule is a reasonable estimate of available high-energy phosphates, based on the observed cell yield.

The chief fermentation waste products of *R. albus* are acetic and formic acids, hydrogen, carbon dioxide, and variable small amounts of lactic or succinic acid (Hungate, 1957). In the continuous culture, flushing with CO_2 prevented accumulation of hydrogen, and maintained a relatively low hydrogen tension in the medium. This may permit (Hungate, Science, *in press*) a production of four acetates from each cellobiose molecule, yielding a total of eight molecules of available ATP, assuming two obtained from each hexose via the Embden-Meyerhof scheme for pyruvate production. If the split of cellobiose to hexose is phosphorolytic, an additional ATP would be conserved, making a total of 9 ATP available per cellobiose fermented. This is efficient growth, particularly in view of the absence from the medium of amino acids and other prefabricated monomers.

Synthesis of nitrogenous cell materials other than "protoplasm" is a second explanation for the high yields of nitrogen and cell weight. Large capsules are formed by many strains of rumen cocci. Starved cells of the RAM strain were difficult to collect by centrifugation, 1 hr at 22,000 × *g* being required to sediment them. Even after this treatment, they were not well packed to a small volume, suggesting that the strain produced abundant capsular material, a common characteristic of *Ruminococcus* strains. Such material, if it contained nitrogen, as with *Bacillus anthracis*, might account in part for the large nitrogen assimilation. But also this product would form only with expenditure of some ATP.

Further study will be required to explain biochemically the high yields of *R. albus* cells. Regardless of the explanation, the results of the present study indicate that the quantity of microbial cells available to the ruminant may be considerably greater than previously realized.

Acknowledgments

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