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Cytoplasmic Reserve Polysaccharide of *Selenomonas ruminantium*

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Selenomonas ruminantium accumulated large quantities of intracellular polysaccharide when grown in simple defined medium in a chemostat, particularly at low dilution rate under NH_3 limitation when the carbohydrate content of the cells was >40% of the dry weight. This polysaccharide was used as a source of energy under conditions of energy starvation. Abundant, densely staining cytoplasmic granules were observed by electron microscopy in sections stained by the periodic acid-thiocarbohydrazide-osmium technique. The polysaccharide was extracted in 30% KOH followed by precipitation with 60% ethanol and was found to be a glucose homopolymer. Sepharose 4B gel filtration and iodine-complex spectroscopy showed that the polysaccharide was of the glycogen type with a molecular weight of 5×10^5 to $>20 \times 10^5$ and an average chain length of 12 glucose residues.

Cytoplasmic inclusions of reserve glucans containing predominantly α-(1→4) linkages frequently occur in bacteria, particularly under conditions of nitrogen limitation (25). Bacteria isolated from the rumen, including *Ruminococcus albus* (5, 14), *Megasphaera elsdenii* (3, 6), *Bacteroides amylogenes* (8), and streptococci (13), accumulate iodophilic reserve polysaccharides of the amylopectin-glycogen type, similar to those of nonrumen bacteria. *R. albus* accumulates large quantities of cytoplasmic polysaccharide granules during late log phase and early stationary phase and subsequently appears to use the material as an energy source, since after 4 h of stationary phase the granules virtually disappear (5). *M. elsdenii* shows similar accumulation of granules approaching stationary phase (6).

Hobson (11) found that the strict anaerobe *Selenomonas ruminantium,* a gram-negative, starch-digesting curved rod commonly found in the rumen, might accumulate considerable amounts of polysaccharide under conditions of excess glucose. This present study shows that *S. ruminantium* does indeed form large amounts of cytoplasmic polysaccharide of the glycogen type. Some properties of this material, and its role as an energy storage polymer, were investigated.

MATERIALS AND METHODS

Organism and culture conditions. *S. ruminantium* WPL 151/1 was isolated in this institute from the rumen of a barley-fed sheep by S. O. Mann. The organism was maintained and grown in continuous culture of a working volume of 300 ml as described previously (22). The culture medium contained (per liter, final volume): minerals solution a and b (12), 150 ml each; trace metals (7), 10 ml; 0.075% (wt/vol) resazurin, 4 ml; vitamins solution, a 10-fold concentration of the vitamin content of the complete medium of Scott and Dehority (18), 100 ml; glucose, 20 mmol; NH_4Cl, 10 mmol in glucose-limited culture or 1.2 mmol in ammonia-limited culture; reducing solution, 100 ml. Reducing solution contained 4% (wt/vol) $NaHCO_3$, 0.25% (wt/vol) Na_2S, and 0.25% (wt/vol) $Na_2S_2O_4$ and was sterilized by filtration. The rest of the medium was autoclaved at 109°C for 45 min.

Sampling of continuous culture. Samples were removed from the culture by a sampling hood similar to that described by Baker (2), except that anaerobic conditions were maintained. Samples (10 ml) were removed for the turbidimetric determination of dry weight (22), for the estimation of intracellular polysaccharide, and for fixing to obtain electron micrographs. Larger samples (100 or 200 ml) were removed for extraction of polysaccharide. When samples were not to be used immediately, the bacteria were harvested by centrifugation (3,000 × *g*, 4°C, 15 min for 10-ml samples; 11,600 × *g*, 4°C, 15 min for larger samples) and stored at −60°C. Otherwise, samples were stored on ice until used.

Total carbohydrate determination. Cell pellets were resuspended and washed twice in 40 mM KH_2PO_4, pH 7.0. The carbohydrate content of resuspended whole cells was determined by the anthrone method (19), with glucose as standard, and an incubation time of 7 min at 100°C. The phenol-H_2SO_4 method (17) was used to monitor the carbohydrate content of the various fractions obtained during extraction of polysaccharide from bacteria, again with glucose as standard.

Electron microscopy. Bacterial pellets were either fixed in the presence of ruthenium red and stained with uranyl formate and lead citrate (23) or stained by the PATO technique (9) in which sections are treated with periodic acid and then with thiocarbohydrazid and stained with osmium.

Extraction of polysaccharide. The method of extraction of the cytoplasmic polysaccharide followed

a general procedure outlined by Herbert et al. (10). The cell pellet was washed twice in distilled water, and ca. 25 mg (dry weight) of bacteria was resuspended in 2 ml of 30% (wt/vol) KOH. This was incubated at 100°C for 3 h and centrifuged (3,000 × *g*, 15 min, 4°C), and 6 ml of distilled water and 16 ml of ethanol were added to the supernatant fluid to precipitate glycogen-like material. This was centrifuged (11,600 × *g*, 15 min, 4°C), and washed twice with 60% (vol/vol) ethanol.

Iodine-complex spectroscopy. The final pellet of extracted polysaccharide was suspended in a small volume of distilled water, and the carbohydrate content was determined. The visible spectrum of a solution containing 0.01% (wt/vol) polysaccharide, 0.02% (wt/vol) iodine, and 0.2% (wt/vol) potassium iodide was taken using a solution of 0.02% (wt/vol) iodine and 0.2% (wt/vol) potassium iodide as a reference (1). Solid $(NH_4)_2SO_4$ was added to each solution to give 50% saturation, and the measurements were repeated. A double-beam recording spectrophotometer (CE 595; Cecil Instruments, Cambridge, England) was used to determine spectra.

Chemical analysis of polysaccharide. The pellet from the KOH and ethanol extraction was dried in a rotary evaporator at 30°C. This material was hydrolyzed and analyzed for sugars by the method of Sloneker (20, 21). Gel filtration, based on the method of Brown et al. (3), employed a Sepharose 4B column (2.2 by 30 cm) eluted with water at 5 ml/h. Standard dextrans were obtained from Pharmacia. Protein was measured with the Folin reagent (16).

RESULTS AND DISCUSSION

Carbohydrate content of *S. ruminantium* in continuous culture. The carbohydrate content of *S. ruminantium* varied with dilution rate (*D*) and with limiting nutrient. Under glucose limitation, the supply of energy limits bacterial growth (22), and the quantity of carbohydrate found in bacteria was least, particularly at low *D* (Fig. 1). In contrast, at low *D* under NH_3 limitation, where glucose (and hence energy) is in excess, the carbohydrate content of cells was greatest (Fig. 1). These properties suggested that much of the carbohydrate content of *S. ruminantium* was likely to be an energy storage polysaccharide. A further experiment, in which the supply of fresh medium to a glucose-limited chemostat at $D = 0.38\ h^{-1}$ was switched off, and the carbohydrate content of the culture fell by 50% after 3 h, supported this hypothesis.

Energy reserve polysaccharides have been found in several anaerobic bacteria (4, 15, 24), including rumen bacteria (5, 6, 8, 14). The extent to which polysaccharide is accumulated by bacteria depends upon the growth conditions (25), and this is reflected in the different carbohydrate contents found by different workers with, for example, *R. albus* growing under different conditions (5, 14). The polysaccharide content of *S. ruminantium* falls within a range (15.4 to 48.8%, Fig. 1) similar to that of *R. albus* (17 to 35%,

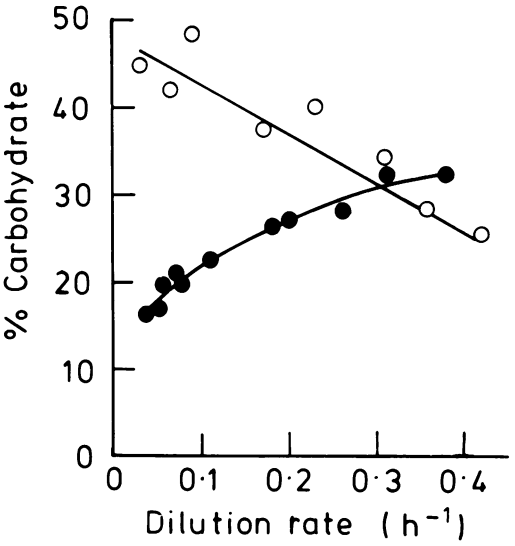

FIG. 1. *Carbohydrate content of S. ruminantium grown in continuous culture in simple defined medium. The total carbohydrate content of washed bacteria was determined by an anthrone method. Symbols: ●, glucose-limited cultures; ○, NH_3-limited cultures.*

reference 14) and is consistent with the original findings of Hobson (11).

Electron microscopy. Sections of *S. ruminantium* from NH_3-limited culture were stained with uranyl formate and lead citrate, but despite the high carbohydrate content of the cells (Fig. 1), no identifiable electron-light inclusions of the type seen in *R. albus* (5) and *M. elsdenii* (6) were found (Fig. 2). Granules were only observed in lysed cells which had lost much of their cytoplasmic contents (Fig. 2). Staining by the PATO technique, however, in which the staining of polysaccharide is accentuated, showed distinct granules of polysaccharide of diameter ca. 0.04 μm dispersed evenly throughout the cytoplasm (Fig. 3).

Extraction of crude polysaccharide. A preliminary fractionation of polysaccharides was performed by extraction of washed whole cells with 30% KOH at 100°C, and subsequent precipitation of KOH-soluble glycogen and mannans in 60% ethanol. A total of 0.9% of recovered carbohydrate was found in the KOH-insoluble material, and this material had a λ_{max} of 488 nm in phenol-H_2SO_4 characteristic of hexose polymers (10). The soluble material remaining after ethanol precipitation comprised 2.3% of the carbohydrate recovered. From the λ_{max} of 479 nm in phenol-H_2SO_4, it was concluded that this material consisted of pentoses (10). The remainder, precipitated by 60% ethanol, was a white powder which was only partially soluble in water. This

powder was composed of 41.3% carbohydrate and 0.7% protein, with the remaining material, which could be removed by centrifugation, resistant to acid digestion (2 N trifluoracetic acid, 100°C, 5 h) and probably derived from the polycarbonate centrifuge tubes used during centrifugation of the KOH hydrolyzate. Removal of most of this material from an aqueous suspension of the crude extract by centrifugation did not remove either carbohydrate or protein from the suspension. The phenol-H_2SO_4 λ_{max} of the extracted polysaccharide was 488 nm, an indication of a glucose polymer (10). In an extraction of 23.4 mg of bacteria from an NH_3-limited culture at $D = 0.03\ h^{-1}$, 8.7 mg of KOH-soluble, ethanol-insoluble polysaccharide was obtained. This compared with a direct estimation of 11.9 mg as the carbohydrate content of whole bacteria.

This extraction therefore indicated that the polysaccharide accumulated by *S. ruminantium* during growth in continuous culture was a glucan- or mannan-like polymer and that this material constituted almost all of the cellular carbohydrate.

Chemical analysis of crude polysaccharide. Duplicate analyses of the material obtained by the above extraction procedure showed that the polysaccharide was composed of molar proportions 98.7% glucose, 0.5% rhamnose, 0.6% arabinose, and 0.2% xylose, and so it was concluded that the reserve polysaccharide of *S. ruminantium* was a glucan homopolymer.

Iodine-complex spectroscopy. Archibald et al. (1) found that spectroscopic examination of the reaction between iodine and polymers of the glycogen-amylopectin type can yield information about the nature of the polysaccharide. The λ_{max} of the iodine complex, the shape of the spectrum, and the absorbance at λ_{max} can distinguish glycogens from the less-branched amylopectins. A spectrum of the iodine complex of the polysaccharide from *S. ruminantium* was made with the crude extract derived from KOH extraction and ethanol precipitation. No attempt was made to remove the acid-resistant material. The powder was suspended in the I_2-KI solution to a concentration of 0.01% polysaccharide, the polysaccharide content of the powder having been previously determined by the phenol-H_2SO_4 method. The iodine complex had a broad smooth spectrum, a λ_{max} at 484 nm, and an absorbance of 0.159 for this solution, all typical of glycogens from a range of sources (1). These properties were very similar to those of *M. elsdenii* glycogen, which had a λ_{max} of 490 to 495 nm and 0.57 maximum absorbance of a 0.03% solution (6).

λ_{max} was shortened slightly, to 465 nm, by addition of $(NH_4)_2SO_4$ to half saturation. However, the main spectroscopic change was a large increase in absorbance at λ_{max} to 0.719. This again is typical of a highly branched glycogen (1). The λ_{max} indicates that the average chain length of *S. ruminantium* glycogen is 12.0 ± 1.6 glucose residues (1).

Molecular weight determination. The crude glycogen preparation from KOH-ethanol extraction was loaded on to a Sepharose 4B column and was found to elute in a single sym-

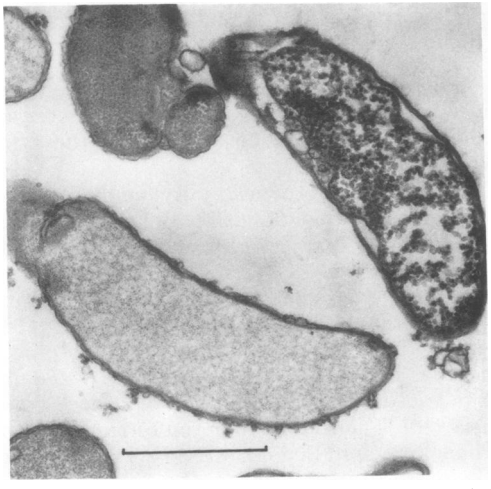

FIG. 2. *Electron micrograph of S. ruminantium from an NH_3-limited culture, $D = 0.03\ h^{-1}$ fixed in the presence of ruthenium red and stained with uranyl formate and lead citrate. Scale bar, 1.0 μm.*

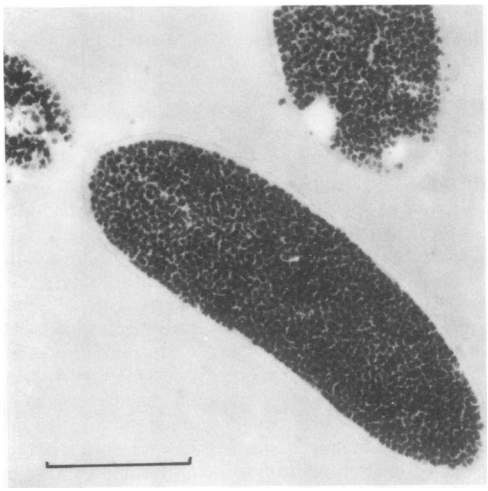

FIG. 3. *As Fig. 2, except stained by the PATO technique. Granules of heavily stained polysaccharide material are evenly dispersed throughout the cytoplasm. Scale bar, 1.0 μm.*

metrical peak (Fig. 4) only slightly behind blue dextran (average molecular weight = 2×10^6). The column was calibrated with a series of commercial dextrans, and from these it was calculated that *S. ruminantium* glycogen had a molecular weight in the range 5×10^5 to more than 20×10^5, larger than that of *M. elsdenii* (3). Extracts from NH_3-limited and glucose-limited cultures of *S. ruminantium* eluted in the same way.

It is concluded, therefore, that *S. ruminantium* contains glycogen similar in structure to that obtained from many other sources (1, 25). This polymer satisfied the three criteria for energy storage (26), in that it is accumulated under conditions of energy excess, it is metabolized under conditions of energy starvation, and hydrolysis yields a compound (glucose) which may be utilized for energy production. Hydrolysis of storage glycogen can clearly supply bacterial energy requirements for endogenous metabolism for several hours in the absence of other energy sources. This property may be of particular importance to starch hydrolyzers, whose energy source appears abundantly but only transiently in the rumen and who may require to survive for considerable periods in the absence of an exogenous source of energy.

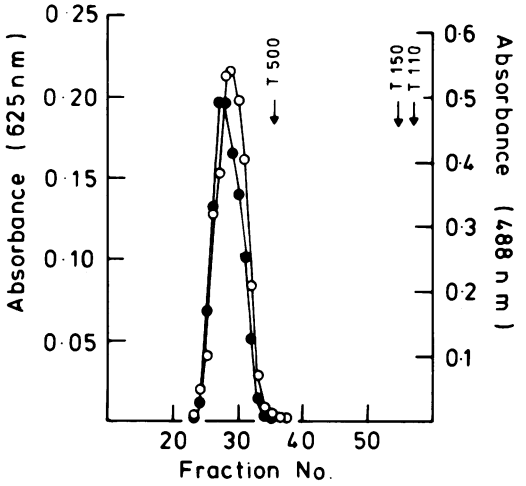

FIG. 4. *Gel filtration of S. ruminantium polysaccharide. A 2-mg amount of crude polysaccharide and 2 mg of blue dextran 2000 were applied to a column (2.2 by 30 cm) of Sepharose 4B. Fractions (1.5 ml) were collected from a flow rate of eluant (distilled water) of 5 ml/h. Fractions were analyzed for the absorbance of blue dextran 2000 at 625 nm (●), and 0.2 ml of each fraction was analyzed by the phenol-H_2SO_4 method. The absorbance at 488 nm (○) is of a reaction mixture containing 0.2 ml of sample, 0.8 ml of distilled water, 1.0 ml of 5% (wt/vol) phenol and 5 ml of H_2SO_4 (specific gravity, 1.84) and is the difference between the total absorbance and the absorbance due to reaction of blue dextran in the phenol-H_2SO_4 assay. The absorbance due to blue dextran in this assay was absorbance at 488 nm = 2.0 × absorbance at 625 nm. The peaks for elution of dextrans T110, T150, and T500 (Pharmacia) are indicated by arrows.*

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