GLYCOGEN ACCUMULATION BY PLEOMORPHIC CELLS OF STREPTOCOCCUS SANGUIS

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SUMMARY: Cells of Streptococcus sanguis undergo gross morphological changes when cultured in the presence of oxygen. These cells accumulate large amounts of an intracellular polysaccharide. The chemical nature and location of this polysaccharide were investigated and we showed that it is an intracellular polymer composed primarily of glucose in a linkage of the glycogen-amylopectin type. Glycogen accumulation appeared to coincide temporally with the onset of pleomorphism while its disappearance coincided temporally with cell death.

Introduction: Streptococcus sanguis undergoes gross changes in morphology when grown in an atmosphere of oxygen (O cells) (1, 2). Normal growth and morphology require that the organism be grown in a nitrogen atmosphere (N cells). The changes in morphology that occur in the presence of oxygen are accompanied by alterations in the pattern of macromolecular synthesis. Previously we reported that RNA and protein synthesis are halted prematurely in O cells but that DNA synthesis continues at the same overall rate as in N cells (1). We now report that cells grown in the presence of oxygen accumulate significant amounts of an intracellular polysaccharide. Evidence is presented that this polysaccharide is of the glycogen-amylopectin type.

Materials and Methods: The isolation, characteristics and conditions of culture of S. sanguis strain G9B used in these studies have been described previously (1, 2).

Two methods of glycogen extraction were employed (see Table 1 for details). The first method involved lyophilization of washed cell suspensions, extraction of polysaccharide with 30% KOH at 100C for 3 hr followed by 60% ethanol precipitation according to the method of Herbert, et al. (3). The second method involved extensive sonication of cells, precipitation of protein by 10% trichloroacetic acid, followed by 60% ethanol precipitation of the trichloroacetic acid soluble material (polysaccharide) according to the method of Gibbons and Kapsimalis (4).

Total carbohydrate in the polysaccharide was determined by the anthrone method (3). The iodophilic nature of the compound was determined by measuring the absorption spectrum of a solution containing 0.01% polysaccharide, 0.02% I_2 and 0.2% KI in

a Gilford 240 spectrophotometer against an iodine-iodide solution of equal strength (5). To identify the monosaccharides, samples were hydrolyzed in 2N HCl at 100°C for 2 hr in sealed tubes. The material was dried in a vacuum dessicator over KOH pellets to remove HCl and resuspended in H_2O. The hydrolysate was analyzed for total carbohydrate (3) and for glucose using glucose oxidase (Glucostat, Worthington Biochemical Corp.). Portions of the hydrolysate were chromatographed on thin layer sheets of cellulose (Bakerflex) in butanol: pyridine: H_2O (6:4:3) and ethyl acetate: pyridine: H_2O (12:5:4). The sheets were sprayed for carbohydrates with aniline diphenylamine (Sigma). The hexosamine content of the hydrolysates were determined by the method of Rondle and Morgan (6). Rhamnose was determined by the method of Dische and Shettles (7).

To identify monosaccharides radioactively, cells were grown in medium containing 0.2 μ Ci/ml (Sp. Act. 0.027 mCi/mg) of uniformly labeled (^{14}C) glucose (New England Nuclear). After extraction and hydrolysis of the polysaccharide, samples were chromatographed as above. For autoradiography, sheets of Kodak RP54 Medical x-ray film were placed on top of the chromatogram and incubated in the dark for 2-3 weeks before developing. In addition, sections were cut out of the chromatograms, placed in vials with Liquifluor (New England Nuclear) and counted in an Intertechnique liquid scintillation spectrometer. Protein was determined by the method of Lowry et al. (8) using crystalline bovine serum albumin as a standard. Alpha amylase was purchased from Sigma Chemical Co. A modified thiosemicarbazide-osmium method was used to demonstrate polysaccharides in S. sanguis by electron microscopy (9).

Results: To insure that any observed difference in glycogen content of N and O cells was not an artefact of extraction, two methods of glycogen isolation were compared (Table I). Sonication yielded approximately the same amount of protein from O cells as did KOH extraction. The amount of protein recovered from N cells was usually somewhat greater when sonication was employed. With both extraction procedures, considerably more carbohydrate was routinely extracted from O cells than from N cells. The KOH procedure was utilized in the next series of experiments for two reasons. First, greater amounts of polysaccharide were isolated from both N and O cells by KOH extraction; furthermore, chromatography of hydrolyzed samples indicated that polysaccharide obtained by sonication-TCA treatment contained rhamnose and hexosamines (indicative of cell wall components) whereas KOH extracted polysaccharides did not.

We isolated polysaccharide from N and O cells after various periods of growth (Table 2). No significant change was found in the amount of anthrone positive

Table I

Comparison of methods of carbohydrate extraction of S. sanguis N and O cells

	Method 1*		Method 2**	
	N cells	O cells	N cells	O cells
Protein:				
mg/g dry wt cells	597	765	751	711
Carbohydrate:				
mg/g dry wt cells	3.5	30	0.96	12.0
mg/g protein	5.8	39	1.28	17

*Six liter batches of O cells or 3 liter batches of N cells were grown for 5 hr in BHI broth at 37°C, centrifuged at 5000 xg for 15 min, washed 2 times with phosphate buffered saline (PBS), 2 times with distilled H_2O and lyophilized. To 50 mg cells, 1 ml 30% KOH was added and the suspension incubated in an oven at 100°C for 3 hr. To the cooled mixture, 3 ml of H_2O and 8 ml of 95% ethanol were added. After being incubated overnight at 4° C, the suspension was centrifuged at 15,000 xg for 15 min. The precipitate was washed twice with 60% ethanol, once with absolute ethanol and once with ether. The precipitate was dried in vacuo over warm H_2O resuspended in H_2O and dialyzed overnight against 100 volumes of distilled H_2O.

**Six liter batches of O cells or 3 liter batches of N cells were grown for 5 hr in BHI broth at 37°C, centrifuged at 5000 xg for 15 min, washed 2 times with PBS, 2 times with distilled H_2O. The cells were suspended in H_2O (lg cells/ml), chilled in an ice-salt bath and treated for 90 min in a LabLine Labsonic System. An equal volume of 50% $CaCl_2$ was added and the mixture was heated at 80°C for 10 min. After the mixture was cooled to 0°C, trichloroacetic acid was added to a final concentration of 10%. The precipitate was removed by centrifugation and the supernatant was treated with ethanol as in method 1.

material extracted from N cells at various times during a 24 hr growth period. However, there was significantly more polysaccharide present in O cells as early as 2 hrs after inoculation. By 5 hr, O cells contained approximately 10 to 20 times more polysaccharide than N cells of the same age. This increase appears to be temporally related to the onset of pleomorphism reported previously (1). It is noteworthy that the synthesis of other cellular macromolecules (DNA, RNA and protein) by N and O cells did not differ significantly at 2 hr (1). Thus polysaccharide accumulation precedes other physiological and morphological changes reported previously (1). The maximum amount of polysaccharide was found in

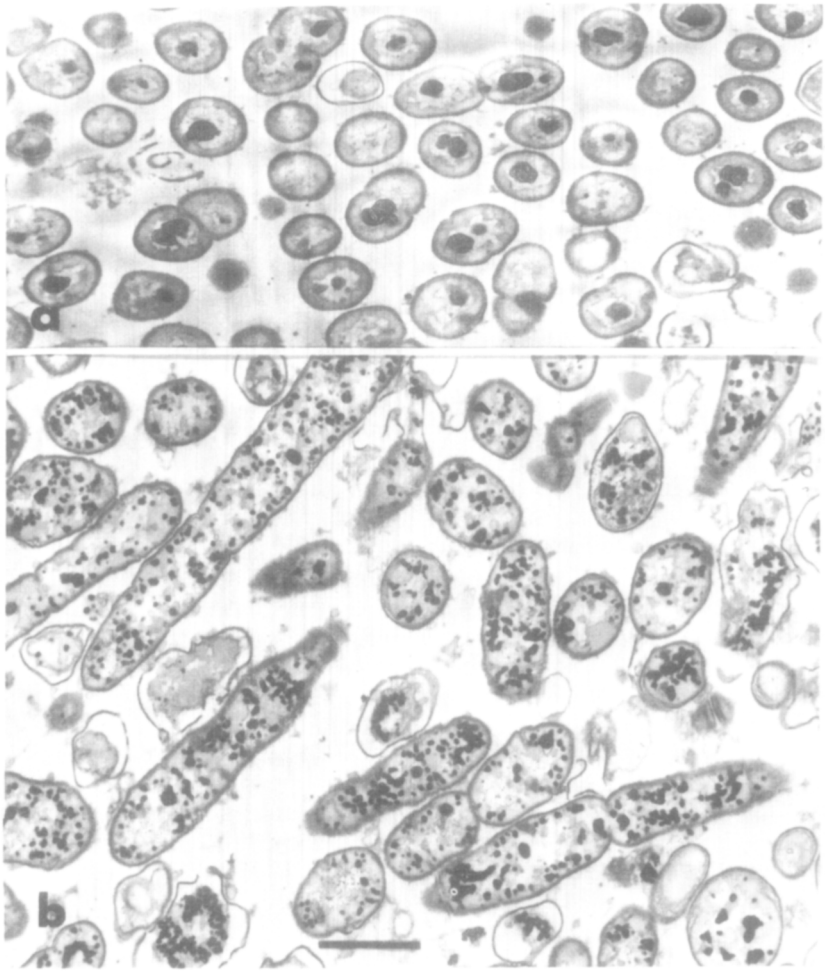

Fig. 1. Electron micrographs of N and O cells of Streptococcus sanguis stained for polysaccharide (9). S. sanguis cells (100 ml) were centrifuged and resuspended in 3% glutaraldehyde, oxidized in 1% periodic acid and subsequently treated with 2.5% thiosemicarbazide in acetate buffer, pH 4.5 at 50° C. Excess thiosemicarbazide was removed and the cells were exposed to 2% osmic acid for 3 hr, then dehydrated in graded hexylene glycol solution. Specimens were embedded in a low viscosity medium according to the method of Spurr (10). Sections (0.1 μm) were examined without additional staining in a Philips EM-300 electron microscope. a) N cells b) O cells. Marker bar in Fig. 1b indicates 1 μm.

early stationary phase (5 hr) O cells whereas older cultures (24 hr) contained significantly less polysaccharide. This loss of polysaccharide coincides temporally with the loss of viability that occurs in O cultures between 5 and 24 hr (1, 2).

Table 2

Polysaccharide extracted* from N and O cells of Streptococcus sanguis

Time after inoculation (hr)	mg polysaccharide/ g dry wt cells** O cells	N cells
0	2.8	2.8
2	11.5	3.6
4	25.4	4.3
5	30.0	3.5
24	0.63	2.8

* Using method 1 described in Table 1.
** For this experiment, 6l of N cells and 12l of O cells were grown in BHI broth. At the times indicated, 1l of N cells and 2l of O cells were chilled to O°C and harvested according to method 1 of Table 1.

The intracellular location of the accumulated polysaccharide was demonstrated by electron microscopy of thin sections of N and O cells specifically stained for polysaccharide after 5 hr of growth (Fig. 1). The O cells which showed the gross pleomorphism reported previously (1, 2) were filled with densely stained polysaccharide granules (Fig. 1b). In contrast, N cells contained very few of these granules (Fig. 1a). The time of appearance of polysaccharide granules was studied by staining and fixing samples of N and O cells after 0, 2, 4, 5, 6 and 24 hr of growth. The appearance of these cells in the electron microscope corroborated the biochemical data, i.e., many O cells contained significant quantities of polysaccharide, even after 2 hr of growth. Moreover, the number of granules per O cell as well as the number of cells containing granules increased with the age of the culture up to 6 hr, then fell by 24 hr. On the other hand, N cells contained few granules at any time during growth.

It has been shown that the intracellular polysaccharide accumulated by many oral streptococci grown in the presence of excess glucose is of the glycogen-amylopectin type (4, 11, 12). Tests were performed to determine the chemical nature of the internal polysaccharide made by O cells of S. sanguis (Table 3). Several lines of evidence suggest that the polysaccharide is of the glycogen-amylopectin type.

First, we found that the polysaccharide was iodophilic. The absorption maximum of the polysaccharide-iodine complex was 500 mμ, close to that found for rabbit muscle glycogen (490 mμ). Moreover, we found that the polysaccharide was sensitive to the enzyme α-amylase, i.e., in the presence of α-amylase, the polysaccharide was no longer iodophilic. These observations suggested that the polysaccharide probably contained repeating units of glucose. To confirm this, the polysaccharide made by O cells of S. sanguis was hydrolyzed with 2N HCl and analyzed qualitatively for monosaccharides by thin layer chromatography. In two solvent systems, one major spot was found which had the same relative mobility (Rg) as authentic glucose. Using the aniline diphenylamine spray (Sigma Chemical Co.) the color of this spot was the same as that produced by glucose. No traces of rhamnose or other wall associated sugars were detected. When one of the chromatograms was sprayed with the glucose oxidase reagent, the pink color that developed with the chromogen as well as the position of the spot corresponded to that of glucose. Finally, when O cells were grown in medium containing uniformly labeled glucose, the hydrolysed polysaccharide chromatographed as a single radioactively labeled area (detected by autoradiography and by scintillation counting of chromatogram slices) which co-chromatographed with glucose.

Discussion: The results indicate that O cells of S. sanguis accumulate significant amounts of an intracellular polysaccharide of the glycogen-amylopectin type. Glycogen accumulation coincides temporally with the development of pleomorphism

and glycogen disappearance coincides temporally with cell death (1). However, these temporal relationships do not necessarily imply causal relationships. S. sanguis, like many other oral streptococci, accumulates large amounts of glycogen when the glucose concentration of the growth medium is increased to 2% (4,11,12 and R. Eisenberg, unpublished results). Since these cells do not become pleomorphic, it is apparent that glycogen accumulation can be stimulated under conditions that have no effect on morphology. It is quite possible that the state of unbalanced growth, previously shown to accompany pleomorphism in S. sanguis (1) is the cause of glycogen accumulation by O cells. Numerous studies have shown that many environmental conditions affect glycogen production in a wide variety of bacteria. These conditions include nitrogen starvation, low pH, sulfur or phosphate deficiency and inhibition of protein synthesis by agents such as chloramphenical (4,14,15). It is reasonable that the inhibition of protein synthesis that occurs in O cells is related to glycogen accumulation in S. sanguis. The observation that increased glycogen accumulation precedes inhibition of protein synthesis suggests that a change in the level of a glycogen intermediate such as ADP glucose (13) may be the controlling factor. Alternatively, inhibition of synthesis of a key protein rather than proteins in general may be responsible for triggering glycogen accumulation.

It is puzzling that no noticeable changes either in the O cell wall or its polysaccharide content (Fig. 1b) were noted in our studies. Pooley and Shockman (16) found that chloramphenical treatment of S. faecalis or starvation of cells for an essential amino acid led to dramatic increases in wall constituents and an increase in wall thickness. Treatment of Staphylococcus aureus with tetracycline also was reported to lead to thickened cell walls (17). Preliminary experiments in our laboratory indicate that N cells of S. sanguis exhibit the same phenomenon of wall thickening when tetracycline is added to log phase cultures. We are examining

these cells to determine whether they also accumulate glycogen. It is possible that certain conditions of unbalanced growth lead to glycogen accumulation but not increased cell wall synthesis and vice versa. This idea is reasonable since different intermediates and different enzymes are required for glycogen synthesis than for cell wall polysaccharide synthesis.

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