Identification of Intracellular Polysaccharide Granules in Thin Sections of *Nocardia asteroides*

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SUMMARY

Nocardia asteroides WVU strain 3672 accumulates storage polysaccharides when grown in a glucose-supplemented medium containing a growth-limiting concentration of nitrogen. Structural analysis of an isolated and purified sample identified the material as a glycogen-like polysaccharide possessing an average chain length of 11.6 glucosyl units and iodine absorption maximum at 460 nm. Cytochemical staining of thin sections of whole organisms revealed granules ranging in size from 60 to 100 nm in diameter distributed throughout the cytoplasm. Glycogen granules could easily be differentiated from lipid storage vacuoles after treatment of thin sections with a glycogen-specific staining procedure. Negatively stained preparations of glycogen granules isolated with cold water revealed α -particles composed of indistinct sub-units.

INTRODUCTION

A number of bacterial species synthesize glycogen-like polysaccharides under various environmental growth conditions. Although these compounds have been analysed chemically, ultrastructural methods have only occasionally been used to investigate glycogen storage in bacteria. A large number of cytoplasmic 'holes' ranging from 50 to 100 nm in diameter was found in thin sections of *Escherichia coli* grown under conditions favouring glycogen synthesis (Cedergren & Holme, 1959; Holme & Cedergren, 1961). No cytoplasmic 'holes' were seen in bacteria grown under conditions which restricted glycogen synthesis. Similar results were obtained with a strain of *Streptococcus mitis* which synthesized large amounts of glycogen (Berman, Gibbons & Nalbandian, 1967). Cytoplasmic granules, approximately 60 nm in diameter, were observed. No such granules were formed in a mutant strain of this organism which was unable to synthesize glycogen. Glycogen granules have also been reported in thin sections of *Myxococcus xanthus* (Voelz, Voelz & Ortigoza, 1966), *Arthrobacter crystallopoietes* (Boylan & Pate, 1973) and certain ammonia- and nitrate-oxidizing marine bacteria (Watson, Graham, Remsen & Valoise, 1971; Watson & Waterbury, 1971).

The purpose of the present work was to investigate the structure of storage polysaccharide isolated from *Nocardia asteroides* and to study the ultrastructural aspects of its accumulation.

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METHODS

Bacterium. *Nocardia asteroides* wvu strain 3672 was used throughout this investigation. The organism was isolated in 1957 from a sputum sample of a human case of pulmonary nocardiosis at the Hopemont Sanitarium, Hopemont, West Virginia, U.S.A. Stock cultures were maintained on Sabouraud's dextrose agar slants at 4 °C with periodic passage on sheep-blood agar plates. Starter cultures were prepared by inoculating liquid media with several colonies from an agar slant. A 1 % (v/v) inoculum from an actively growing culture was then used to seed the growth medium.

Defined medium. The synthetic growth medium used in all experiments was prepared by adding the following ingredients to approximately 800 ml distilled water: N_2HPO_4 , 2.2 g; KH_2PO_4 , 1.32 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{Fe}_2(\text{SO}_4)_3$, 0.5 mg; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.2 mg; CaCl_2 , 2.0 mg; glycine, 0.45 g; malic acid, 3.3 g; glucose, 10 g; and ammonium hydroxide, 0.21 g. The pH was adjusted to 6.8 with 10 N-NaOH and the volume made to 950 ml with distilled water. A 20 % (w/v) aqueous solution of glucose was autoclaved separately and 50 ml added aseptically to the sterilized medium. The organism was grown at 37 °C in 2 l Erlenmeyer flasks containing 1 l medium on a New Brunswick Model G-10 rotary shaker (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey, U.S.A.), at 180 rev./min, to ensure adequate aeration. To measure growth 10 ml portions were dispersed with a glass tissue homogenizer (Bellco Glass, Vineland, New Jersey, U.S.A.) and read in a Klett-Summerson colorimeter at 420 nm (filter 42). Cell suspensions were diluted, if necessary, to maintain readings in the linear range, i.e. less than 175. A Klett reading of 100 was equivalent to approximately 0.5 mg/ml.

Harvesting cultures and carbohydrate extraction. Bacteria were harvested by centrifuging in a Sorvall RC-2B refrigerated centrifuge at 13000 g for 30 min, followed by washing with 4 °C distilled water. Alkali-soluble carbohydrate was extracted from approximately 8 g (wet wt) of bacteria by heating at 100 °C in 30 % (w/v) KOH for 45 min. Debris removed by centrifuging at 5000 g for 10 min was discarded and storage polysaccharide was precipitated from the supernatant by adding 1.2 vol. of 95 % (v/v) ethanol. Storage polysaccharide was collected by centrifuging at 15000 g for 15 min and further purified by treatment with 95 % (v/v) ethanol and 10 % (w/v) trichloroacetic acid (Cowgill & Pardee, 1958). Carbohydrate was estimated by the anthrone method (Seifter, Dayton, Novic & Muntwyler, 1950), using D-glucose as a standard.

Acid hydrolysis. A purified sample of *Nocardia* glycogen (10 mg) was hydrolysed for 2 h in 1.0 M-HCl at 100 °C in a sealed tube. The hydrolysate was neutralized, evaporated in a rotary flash evaporator and re-dissolved in distilled water. A 10 μl portion (approximately 10 μg of sugar) was applied to silica gel-impregnated glass-fibre paper, ITLC-SA (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.) which had been pretreated with 0.1 M- KH_2PO_4 and activated for 1 h at 105 °C. The components were separated by ascending one-dimensional chromatography in a closed chamber with a solvent consisting of chloroform:acetic acid:water (55:30:5). The chromatogram was developed three successive times to distances of 10, 13 and 15 cm, respectively, before spraying with a solution of paranisidine to locate sugars (Block, Durrum & Zweig, 1958). Ten μl of a mixture of five sugars, 10 μg each, was chromatographed simultaneously with the hydrolysate. The sugars and their respective R_F values were: galactose, 0.22; glucose, 0.26; mannose, 0.31; fructose, 0.38; ribose, 0.67.

Iodine absorption. The absorption spectrum of the polysaccharide-iodine complex was measured in a Gilford 240 recording spectrophotometer after addition of *Nocardia* storage

polysaccharide to a solution of I_2 -KI (Krisman, 1962). The spectrum produced was compared with that of soluble starch (Fisher Scientific, Pittsburgh, Pennsylvania, U.S.A.), rabbit-liver glycogen type III, and oyster glycogen type III (Sigma).

Enzyme hydrolysis. Glucose released from glycogen in the form of glucose 1-phosphate by treatment with phosphorylase *a* (Sigma) was measured spectrophotometrically by following the reduction of NAD in the presence of commercial phosphohexose isomerase and glucose 6-phosphate dehydrogenase (Boehringer Mannheim Corporation, New York, N.Y., U.S.A.) (Passonneau, Gatfield, Schulz & Lowry, 1967). Commercial rabbit-liver glycogen type III served as a control.

End-group determination. The average chain length of *Nocardia* storage polysaccharide was determined on a 10 mg sample by oxidation with sodium metaperiodate with methyl α -D-glucoside as a standard (Fales, 1959).

Electron microscopy. Bacteria collected from mid-exponential-phase cultures (2 days) by centrifuging for 30 min at 13 700 g at 4 °C and then washed with distilled water were prefixed in 1 % (v/v) glutaraldehyde for 30 min, fixed overnight in 1 % (w/v) OsO_4 and embedded in Epon-812 (Kellenberger, Ryter & Séchaud, 1958). Thin sections were cut on a Model MT-2 Porter-Blum ultramicrotome (Ivan Sorvall, Inc., Norwalk, Connecticut, U.S.A.) with a Dupont diamond knife, placed on copper grids previously covered with Formvar and carbon films, poststained with 2 % (w/v) aqueous uranyl acetate and 2 % (w/v) lead citrate (Reynolds, 1963) for 5 min each, and observed under an RCA electron microscope, Model EMU-3G, at 100 kV.

Thin sections were cytochemically stained for storage polysaccharide by using the glycogen-specific procedure of J. Revel and M. Karnovsky (personal communication). Sections prepared as previously described were mounted on Formvar and carbon-coated nickel grids and treated with solutions of 3 % (w/v) H_2O_2 , 1 % (w/v) periodic acid and finally 2.5 % (w/v) sodium chlorite in 10 M-acetic acid, with rinsing in distilled water between treatments. Following the final rinse, grids were stained with 2 % (w/v) uranyl acetate (5 min) and 2 % (w/v) lead citrate (2 min).

Negative staining. Glycogen isolated and purified from *N. asteroides* by cold water extraction (Bueding & Orrell, 1964) was negatively stained according to the methods of Gordon (1972). A dilute aqueous solution containing 0.5 mg/ml was applied to a Formvar and carbon-support film and treated with an 0.3 % (w/v) solution of potassium phosphotungstate (pH 7.0). Commercial cold water-extracted rabbit-liver glycogen, type V (Sigma), was also negatively stained.

RESULTS

Structural analysis

Development of thin-layer chromatographs of the acid-hydrolysed storage polysaccharide revealed a single compound with R_f 0.27, indicating that glucose was the only monomeric sugar unit present. Treatment of the polysaccharide with commercial phosphorylase *a* rapidly released glucose 1-phosphate, indicating the presence of α -1,4-linked glucose residues in the structure (Fig. 1). The rate of degradation equalled that obtained using commercial rabbit-liver glycogen. According to Passonneau *et al.* (1967) the small increase in extinction observed during the secondary phase of glycogen degradation is due to the presence of α -1,6 branched linkages in the structure.

The wavelength of maximum absorption of the polysaccharide-iodine complex was 450 to 460 nm. When compared with several well-characterized commercial preparations of glycogens (Fig. 2) the spectrum was most similar to that produced by rabbit-liver glycogen.

Fig. 1. The effect of phosphorylase *a* on (●) commercial rabbit-liver glycogen (0.3 mM) and (○) *Nocardia asteroides* storage polysaccharide.

Fig. 2. The iodine absorption spectra of rabbit-liver glycogen (●), oyster glycogen (▲), *Nocardia asteroides* storage polysaccharide (○), and soluble starch (Δ).

Table 1. *Structural properties of glycogens isolated from various sources*

Glycogen sources	Average chain length*	Maximum absorption (nm)
<i>Nocardia asteroides</i>	11·6†	450–460
Rabbit liver	17·0	460
Oyster	12·0	410
<i>Escherichia coli</i> ‡§	10·0–13·0	420–455
<i>Aerobacter aerogenes</i> §	13·0	485
<i>Arthrobacter</i> sp.	9·0	420–440

* Measured as glucosyl units.

† Average value of two separate tests each containing triplicate determinations.

‡ Range of five strains tested.

§ Sigal, Cattaneo & Segel (1964).

|| Ghosh & Preiss (1965).

Glycogen isolated from bacteria during peak accumulation (5 days) had an average chain length of 11·6 glucosyl units as determined by end-group analysis. This value was compared with commercial rabbit-liver and oyster glycogens and with values obtained by other investigators by using the same periodate oxidation procedure of Fales (1959) to analyse glycogens from other bacteria (Table 1).

Electron microscopy

Thin sections of *N. asteroides* harvested in mid-exponential phase of growth (2 days) showed large cytoplasmic vacuoles (Fig. 3), believed to be composed of lipid material. In addition, many bacteria also contained smaller vacuoles, 60 to 100 nm in diameter (Fig. 4), which were similar in size and shape to the glycogen storage granules observed in thin sections of *Streptococcus mitis* (Berman *et al.* 1967) and *Arthrobacter crystallopoietes* (Boylen & Pate, 1973). To distinguish glycogen granules from lipid-containing vacuoles more readily, thin sections were also treated with a glycogen-specific stain (Figs. 5 and 6). The bacteria now revealed numerous darkly stained bodies, 60 to 100 nm in diameter, distributed throughout the cytoplasm. Lipid vacuoles remained unstained and were clearly distinguishable from glycogen granules. When the periodate oxidation step was omitted from the glycogen-staining procedure, glycogen granules did not stain and only lipid vacuoles were visible in the sections (Fig. 7).

Negatively stained preparations of commercial rabbit-liver glycogen extracted with cold water (Fig. 8) revealed α -particles as large as 200 nm in diameter, composed of numerous subunits (β -particles). Similarly stained preparations of nocardial glycogen, extracted also with cold water (Fig. 9), revealed α -particles 60 to 100 nm in diameter which lacked a definite subunit structure. The comparable size of isolated α -particles and intracellular glycogen granules indicated that the α -particles were little degraded during the cold water extraction.

DISCUSSION

In bacteria the common reserve compounds, which include lipid, poly- β -hydroxybutyrate, glycogen and polyphosphate, are stored in various types of cytoplasmic vacuoles, granules or solid inclusions. These storage units, which generally are not membrane-limited, can often be detected cytochemically or in thin sections viewed under the electron microscope. According to Kellenberger & Ryter (1964), glycogen particles usually appear as small, round granules of rather diffuse outline. Their empty appearance reflects the low electron-scattering power common to all polysaccharides. This observation adequately describes the

Fig. 3. Thin section of 2-day-old *N. asteroides* poststained with uranyl acetate and lead citrate. L, lipid-containing vacuoles.

Fig. 4. Thin section of 2-day-old *N. asteroides* poststained with uranyl acetate and lead citrate. G, glycogen granules.

Figs. 5 and 6. Thin sections of 2-day-old *Nocardia asteroides* treated with a glycogen-specific stain. L, lipid-containing vacuoles; arrow, glycogen granules.

Fig. 7. Thin section of 2-day-old *Nocardia asteroides* treated with a glycogen-specific stain with the periodic acid-oxidation step omitted. L, lipid-containing vacuoles.

appearance of cytoplasmic granules produced by cells of *N. asteroides* grown in a nitrogen-limiting, glucose-supplemented medium.

Nocardia asteroides when grown under nitrogen-limiting conditions (McFarland, 1967) accumulates up to 40 % of its dry weight as neutral lipids, which include fatty acids, glycerides and waxes. Poly- β -hydroxybutyrate, however, is not produced. In the present investigation

Fig. 8. Negatively stained commercial cold water-extracted rabbit-liver glycogen granules.

Fig. 9. Negatively stained, cold water-extracted *Nocardia* glycogen granules.

large vacuoles were observed in thin sections, in addition to glycogen granules, and probably function as depots for neutral lipid storage.

Glycogen granules could not be seen in early stationary-phase cells (5 days) without the use of the glycogen stain, even though glycogen accumulation is known to reach a maximum during this portion of the growth cycle. Although lipid vacuoles were still observable, the extreme disorganization of cytoplasmic constituents made differentiation of the smaller glycogen granules difficult in unstained sections (Fig. 10). Similar results were obtained by Boylen & Pate (1973), who found that glycogen storage granules could not be seen in thin sections of starved *Arthrobacter crystallopoietes*, even though the bacteria contained as much as 50 % of their original anthrone-positive carbohydrate. With the use of the glycogen stain it became evident that *N. asteroides* continued to produce glycogen granules well into the stationary phase of growth (Fig. 11).

Although nocardial glycogen shows a chemical similarity to glycogens isolated from other organisms, the structure of *Nocardia* α -particles, extracted with cold water, shows a marked difference from the structure of rabbit-liver glycogen. The indistinct nature of the nocardial α -particles was similar to the appearance of glycogens isolated with cold water from species of *Mycobacterium* (Antoine & Tepper, 1969) which also lack a clearly defined subunit-type structure.

Fig. 10. Thin section of 5-day-old *N. asteroides* poststained with uranyl acetate and lead citrate. L, lipid-containing vacuoles.

Fig. 11. Thin sections of 5-day-old *N. asteroides* treated with a glycogen-specific stain. L, lipid-containing vacuoles; arrow, glycogen granules.

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