Some Properties of the Polysaccharide from Cell Cultures Infected with TRIC Agent (*Chlamydia trachomatis*)

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SUMMARY

The polysaccharide, elaborated in trachoma-inclusion conjunctivitis (TRIC) agent inclusions, was isolated from baby hamster kidney (BHK) cells grown and infected in suspension cultures. It was characterized by physical, chemical and enzymic methods as a glycogen with an average chain length of 14 to 16 glucose units.

INTRODUCTION

Chlamydiae are obligate intracellular micro-organisms that are classified into two main subgroups, A and B (Gordon & Quan, 1965). Members of group A, which includes trachoma-inclusion conjunctivitis (TRIC) agents, develop in host cells in well-defined cytoplasmic inclusions that fill with a glucose polymer as infective particles form. Inclusion material stains brown with iodine, is degraded by salivary amylase (Rice, 1936) and co-precipitates with shellfish glycogen (Fan & Jenkin, 1970). It is therefore frequently referred to as glycogen. The difficulties of obtaining sufficient quantities of the polymer for more detailed analyses were overcome by the growth of a TRIC agent in suspended cell cultures (Garrett & Harrison, 1973), and the structure of the isolated polysaccharide has now been investigated.

METHODS

TRIC agent. The fast-killing variant (Taverne, Blyth & Reeve, 1964) of strain TRIC/2/GB/MRC-4/ON (Jones, 1961) was grown in baby hamster kidney (BHK) cells in suspension (Garrett & Harrison, 1973).

Enzymes. Amyloglucosidase (Sigma) from *Rhizopus* sp. was purified by the method of Johnson & Fusaro (1966). Purified samples of *Cytophaga* isoamylase and sweet potato β -amylase were kindly given by Dr J. J. Marshall. Glucose oxidase (Glucostat Regular, Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.) was reconstituted in 0.5 M-tris-HCl buffer which effectively inhibits possible carbohydrase contaminants but does not interfere with glucose oxidase activity (Dahlqvist, 1964).

Reagents were obtained as follows: shellfish glycogen (Mann Research Laboratories, New York, U.S.A.); rabbit liver glycogen and maltose monohydrate (BDH); bovine plasma albumin (Armour Pharmaceutical Co. Eastbourne); anthrone (BDH) was recrystallized from acetic acid.

Analytical methods. Polysaccharide was measured by the method of Mokrasch (1954) after free glucose had been destroyed, where necessary, by boiling in 5 M-KOH (van der Vies, 1954); occasionally it was also measured by digestion with amyloglucosidase and

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determination of the glucose released by glucose oxidase (Johnson & Fusaro, 1966). Reducing sugars were measured against a maltose standard by the method of Nelson (1944) modified to give a final volume of 4.6 ml. Protein was measured against a bovine plasma albumin standard by the method of Lowry *et al.* (1951) modified to give a final volume of 1.1 ml.

The reaction of iodine with polysaccharide was studied by adding 0.1 ml of 0.08 % (w/v) I_2 in 0.8 % (w/v) KI and 0.2 M-HCl to 0.3 ml of an aqueous solution containing about 150 mg polysaccharide. The absorbance of the iodine-polysaccharide complex was read at 490 nm.

Acid hydrolysis of polysaccharide. Samples were hydrolysed with 1 M- H_2SO_4 or 2 M-HCl in sealed tubes at 100 °C for 2 h. The H_2SO_4 was neutralized with $BaCO_3$; the HCl was removed by evaporating *in vacuo* over $CaCl_2$ and NaOH.

Thin-layer and paper chromatography of hydrolysates and digests was done on pre-coated cellulose plates (Merck) and Whatman No. 4 paper in *n*-butanol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951). Carbohydrates were detected with $AgNO_3$ -NaOH (Partridge, 1948); background colour was bleached with 20 % (w/v) $Na_2S_2O_3 \cdot 5H_2O$.

Infrared spectrometry. Freeze-dried samples were analysed in a Perkin-Elmer spectrometer.

Isolation of polysaccharide. The polysaccharide associated with TRIC-infected BHK cells is degraded enzymically by cell homogenates at neutral or slightly acid pH (Evans, 1972; Garrett & Harrison, 1973). To avoid this, cells infected 48 h earlier were therefore harvested by centrifugation at 600 *g* for 10 min and extracted with 5 % (w/v) trichloroacetic acid (TCA) for 1 h at 0 °C; preliminary experiments showed that TCA extracted all the polysaccharide from infected cells. The suspension was centrifuged at 600 *g* for 10 min, the precipitate washed twice with 5 % (w/v) TCA, and the extract and washings were combined and dialysed against distilled water. The non-diffusible fraction was adjusted to pH 8 with NaOH and concentrated by freeze-drying. Some purification of this material was achieved by passage through an Amberlite MB-6 resin (22 × 1.5 cm) at 4 °C, and the polysaccharide was recovered in the void volume as an opalescent solution; samples of each fraction were analysed for polysaccharide by the method of Mokrasch (1954).

Determination of average chain lengths of polysaccharide. The average unit chain length ($\overline{CL}$) and the average outer chain length ($\overline{OCL}$) were determined by the method of Gunja-Smith, Marshall & Smith (1971) which is based on the enzymic degradation of branched α -1,4 and α -1,6 glucose polymers. Isoamylase hydrolyses α -1,6 bonds and liberates unbranched chains with reducing sugar ends; β -amylase hydrolyses alternate α -1,4 bonds of unbranched chains and leaves stubs of maltose and maltotriose at branch points (Fig. 1). Polysaccharide (0.5 to 1 mg/ml) in 20 mM-sodium acetate-acetic acid, pH 5.5, was incubated with *Cytophaga* isoamylase (0.06 units/ml) at 37 °C. The degree of β -amylolysis was determined by digestion of polysaccharide (0.25 to 0.5 mg/ml) with β -amylase (30 units/ml) in 10 mM-sodium acetate-acetic acid buffer, pH 4.8. Duplicate samples from both digests were taken at intervals, inactivated by heating at 100 °C for 20 min and analysed for reducing sugar. Average chain lengths (expressed in glucose units) were calculated from the formulae:

$$\text{Average chain length } (\overline{CL}) = \frac{2 \times \text{total polysaccharide in digest (maltose equiv.)}}{\text{Reducing sugar released (maltose equiv.)}}$$

$$\text{Average outer chain length } (\overline{OCL}) = \overline{CL} \times (\% \beta\text{-amylolysis}) + 2.5$$

$$\text{Average interior chain length } (\overline{ICL}) = \overline{CL} - \overline{OCL} - 1.$$

Fig. 1. Glycogen structure proposed by Gunja-Smith *et al.* (1970). ϕ , Reducing end group; —, α -1,4 glucan chains; $\rightarrow$, α -1,6 glycoside branch points.

Fig. 2.

Fig. 2. Ultraviolet spectrum of polysaccharide from TRIC agent before (A) and after (B) passage through Amberlite MB-6 resin. The concentration of the preparation used in B was 20 times that used in A.

Fig. 3.

Fig. 3. Effects of enzymic degradation of polysaccharides on iodine adsorption. Δ , TRIC agent polysaccharide control; $\blacktriangle$, TRIC agent polysaccharide + isoamylase; $\circ$, shellfish glycogen control; $\bullet$, shellfish glycogen + isoamylase.

RESULTS

A 1 l culture containing 10^9 infected BHK cells yielded about 20 mg polysaccharide (expressed as glucose equivalents). Passage of the extract through a column of Amberlite MB-6 resin reduced 260 nm-absorbing contaminants to negligible amounts (Fig. 2) and the protein content from 10 % to 1.5 % of the glucose content; at least 95 % of the polysaccharide applied to the column was recovered in the void volume.

Chemical composition. Glucose was the only sugar detectable by thin-layer and paper chromatography of acid hydrolysates of the polysaccharide. However, measurements of sugar by the anthrone reaction and by sequential digestion with amyloglucosidase and glucose oxidase showed that 5 % of the polymer was either another sugar or composed of glucose groups bound differently from that in a glycogen.

Measurement of average chain lengths. Separate samples of polysaccharide were degraded with isoamylase, with β -amylase, or with isoamylase followed by β -amylase. Average chain

Table 1. *Chain lengths of TRIC agent polysaccharide*

	$\overline{\text{CL}}$	$\overline{\text{OCL}}$	$\overline{\text{ICL}}$	β -Amylolysis (%)	
				Before debranching	After debranching
TRIC agent polysaccharide	14-16	11-12	2-3	60	95
Shellfish glycogen					
(This study)	12	7	3	38	95
(Gunja-Smith <i>et al.</i> 1971)	10	6	3	39	101
(Walker & Whelan, 1960)	11	7	2	39	ND

ND, Not done.

Fig. 4. Infrared spectra of TRIC agent polysaccharide (1), shellfish glycogen (2), and rabbit liver glycogen (3).

lengths were calculated from the amounts of reducing sugar released after completion of these digestions (Table 1). Complete digestion of the polysaccharide with isoamylase and then β -amylase revealed glucose, maltose and a very small amount of material that reduced AgNO_3 and ran on thin-layer chromatograms with an R_{maltose} of 0.69.

Reaction with iodine. Further information on the nature of the polysaccharide was obtained by examining the complex formed with I_2 -KI in the presence and absence of $(\text{NH}_4)_2\text{SO}_4$. The addition of an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ increased the extinction of the complex twofold and altered the λ_{max} from 465 to 505 nm. These reactions are typical for glycogens, and an approximate value of 16 ± 2 for $\overline{\text{CL}}$ is derived from λ in 50 % saturated $(\text{NH}_4)_2\text{SO}_4$ and the formula $\overline{\text{CL}} = 16 \pm 0.114 (\lambda_{\text{max}} = 500)$ (Archibald *et al.* 1961).

Debranching enzymes, such as isoamylase, hydrolyse α -1,6 bonds and convert highly branched glucans to a mixture of straight chain glucans which may then be complexed with I_2 -KI. Isoamylase treatment increased the iodine-staining powers of shellfish glycogen and polysaccharide from TRIC-infected cells (Fig. 3).

Infrared spectrometry. The infrared spectrum of TRIC agent polysaccharide was identical with that of glycogen from shellfish or from rabbit liver (Fig. 4).

DISCUSSION

Polysaccharide from inclusions formed by a TRIC agent was separated from BHK host cells and from the chlamydiae that induced its formation, and the results presented here substantiate the long-held belief that it is a glycogen. Production of a brown complex with iodine is not, by itself, sufficient evidence to identify the inclusion material as a glycogen; other polysaccharides and protein also stain weakly with iodine. However, the large changes in absorbance and $\lambda_{\max}$ that accompanied the addition of $(\text{NH}_4)_2\text{SO}_4$ to polysaccharide-iodine mixtures are characteristic of glycogens, and, most important, highly purified and specific enzyme preparations of isoamylase and β -amylase (Gunja-Smith *et al.* 1971; Mercier, Frantz & Whelan, 1972) digested the polymer almost completely. The average chain length (16 ± 2 glucose units) calculated from the $\lambda_{\max}$ of the iodine complex agrees well with that (14 to 16) from the much more reliable enzymic method. The increase in iodine-staining power that accompanied digestion of the polysaccharide with isoamylase (Fig. 3) confirmed that the polymer was an α -1,4 glucan joined by α -1,6 branch points. The small residue of polysaccharide resistant to enzyme action may be a core in which glucose is held by bonds other than α -1,4 and α -1,6. The β -amylolysis limit, the average chain lengths and the infrared spectrum are all similar to the values reported for other glycogens.

Recent findings suggest that chlamydiae produce an enzyme (or enzymes) that directs polysaccharide synthesis, although the host cell controls the amount of polymer formed in the inclusion (Jenkin & Fan, 1971; Evans, 1972).

Although the function of the glycogen is still unknown, the fact that it is elaborated late in the growth cycle (Reeve & Taverne, 1967) and deposited outside the elementary bodies makes it unlikely that it serves as an energy reserve similar to the storage glycogens of some other bacteria. Under certain conditions the polysaccharide is so closely associated with elementary bodies as to resemble a capsule, and inclusion material containing elementary bodies and retaining the shape and staining characteristics of the original inclusion has been isolated free from host cells (Blyth, Taverne & Garrett, 1971). Similar aggregates have also been observed in TRIC-infected yolk sacs (Sowa & Collier, 1960). The polysaccharide may therefore be a structural component involved in some way with the development or release of infective particles. Its presence must alter the osmotic pressure within the inclusion, and could thus affect transport of nutrients or metabolites through the inclusion membrane.

Enzyme deficiencies in certain glycogen storage diseases lead to the production of various abnormal liver glycogens (Manners, 1962; Edstrom, 1972), some of which resemble amylopectins more closely than glycogens. It is tempting, therefore, to speculate that trimethoprim, which inhibits TRIC elementary body formation (Reeve, Taverne & Bushby, 1968) and which can induce the formation of inclusions that stain blue or black with iodine (P. Reeve and J. Taverne, unpublished observations), may alter the normal synthesis of polysaccharide by inhibiting branching enzymes. Trimethoprim at ten times the median inhibitory concentration for TRIC agent is required to elicit toxic effects in host cells (Reeve *et al.* 1968), and examination of the structures of polysaccharides formed in the presence of antibacterial drugs may identify the role played by TRIC agent in polysaccharide synthesis.

The polysaccharide may have some serological activity; non-particulate matter in inclusions stains with fluorescent antibody against TRIC antigen (A. E. Mogg, unpublished observations), and rabbit antisera prepared against crude polysaccharide extracts of TRIC-infected BHK cells neutralize TRIC agent infectivity (A. Evans, unpublished

observations). However, the role of polysaccharide in these reactions has not been established.

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REFERENCES

- ARCHIBALD, A. R., FLEMING, I. D., LIDDLE, A. M., MANNERS, D. J., MERCER, G. A. & WRIGHT, A. (1961). α -1,4 Glucosans. XI. The absorption spectra of glycogen- and amylopectin-iodine complexes. *Journal of the Chemical Society*, 1183-1190.
- BLYTH, W. A., TAVERNE, J. & GARRETT, A. J. (1971). Trachoma inclusions separated from cells. In *Trachoma and Related Disorders*, pp. 79-87. Edited by R. L. Nichols. Amsterdam: Excerpta Medica.
- DAHLQVIST, A. (1964). Method for assay of intestinal disaccharidases. *Analytical Biochemistry* **7**, 18-25.
- EDSTROM, R. D. (1972). Structure of a low molecular weight form of glycogen isolated from the liver in a case of glycogen storage disease. *Journal of Biological Chemistry* **247**, 1360-1367.
- EVANS, A. (1972). The development of TRIC organisms in cell cultures during multiple infection. *Journal of Hygiene* **70**, 39-48.
- FAN, V. S. C. & JENKIN, H. M. (1970). Glycogen metabolism in chlamydia-infected HeLa cells. *Journal of Bacteriology* **104**, 608-609.
- GARRETT, A. J. & HARRISON, M. J. (1973). The development of a TRIC agent (*Chlamydia trachomatis*) and its associated polysaccharide in suspended cell cultures. *Journal of General Microbiology* **78**, 297-303.
- GORDON, F. B. & QUAN, A. L. (1965). Occurrence of glycogen in inclusions of the psittacosis-lymphogranuloma venereum-trachoma agents. *Journal of Infectious Diseases* **116**, 186-196.
- GUNJA-SMITH, Z., MARSHALL, J. J., MERCIER, C., SMITH, E. E. & WHELAN, W. J. (1970). A revision of the Meyer-Bernfeld model of glycogen and amylopectin. *FEBS Letters* **12**, 101-104.
- GUNJA-SMITH, Z., MARSHALL, J. J. & SMITH, E. E. (1971). Enzymatic determination of the unit chain length of glycogen and related polysaccharides. *FEBS Letters* **13**, 309-311.
- JEANES, A., WISE, C. S. R. & DIMLER, R. J. (1951). Improved techniques in paper chromatography of carbohydrates. *Analytical Chemistry* **23**, 415-420.
- JENKIN, H. M. & FAN, V. S. C. (1971). Contrast of glycogenesis of *Chlamydia trachomatis* and *Chlamydia psittaci* strains in HeLa cells. In *Trachoma and Related Disorders*, pp. 52-59. Edited by R. L. Nichols. Amsterdam: Excerpta Medica.
- JOHNSON, J. A. & FUSARO, R. M. (1966). The quantitative enzymic determination of animal liver glycogen. *Analytical Biochemistry* **15**, 140-149.
- JONES, B. R. (1961). TRIC virus infections in London. *Transactions of the Ophthalmological Society of the United Kingdom* **81**, 367-378.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MANNERS, D. J. (1962). Enzymic synthesis and degradation of starch and glycogen. *Advances in Carbohydrate Chemistry* **17**, 371-430.
- MERCIER, C., FRANTZ, B. M. & WHELAN, W. J. (1972). An improved purification of cell-bound pullulanase from *Aerobacter aerogenes*. *European Journal of Biochemistry* **26**, 1-9.
- MOKRASCH, L. C. (1954). Analysis of hexose phosphates and sugar mixtures with the anthrone reagent. *Journal of Biological Chemistry* **208**, 55-59.
- NELSON, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry* **153**, 375-380.
- PARTRIDGE, S. M. (1948). Filter paper partition chromatography of sugars. *Biochemical Journal* **42**, 238-248.
- REEVE, P. & TAVERNE, J. (1967). Strain differences in the behaviour of TRIC agents in cell cultures. *American Journal of Ophthalmology* **63**, 1167-1173.
- REEVE, P., TAVERNE, J. & BUSHBY, S. R. M. (1968). Inhibition by pyrimidine analogues of the synthesis of folic acid by trachoma agents. *Journal of Hygiene* **66**, 295-306.
- RICE, C. E. (1936). The carbohydrate matrix of the epithelial-cell inclusion in trachoma. *American Journal of Ophthalmology* **19**, 1-8.
- SOWA, J. & COLLIER, L. H. (1960). Isolation of trachoma virus from patients in West Africa. *Journal of Hygiene* **58**, 99-108.

- TAVERNE, J., BLYTH, W. A. & REEVE, P. (1964). Toxicity of the agents of trachoma and inclusion conjunctivitis. *Journal of General Microbiology* **37**, 271–275.
- VAN DER VIES, J. (1954). Two methods for the determination of glycogen in liver. *Biochemical Journal* **57**, 410–416.
- WALKER, G. J. & WHELAN, W. J. (1960). The mechanism of carbohydrase action. 8. Structures of the muscle-phosphorylase limit dextrins of glycogen and amylopectin. *Biochemical Journal* **76**, 264–268.