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THE MOLECULAR STRUCTURES OF A GLUCAN AND A GALACTAN SYNTHESISED BY *Prototheca zopfii*

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ABSTRACT

When the unicellular organism *Prototheca zopfii* was grown on a malt–agar medium, a mixture of polysaccharides was synthesised which could be subsequently extracted from the dried cells with hot water and hot alkali. The major polysaccharide was a galactan which had a branched structure with main chains of (1→6)-linked D-galactopyranose residues, and ~10% of side chains containing terminal D-galactofuranose residues. A glycogen-type polysaccharide and a (1→4)-linked mannan were also produced.

INTRODUCTION

Prototheca zopfii is a colourless, unicellular, saprophytic organism which is usually assigned to the class Chlorophyceae and order Chlorococcales[1]. This order contains predominantly fresh-water and terrestrial green algae which possess a plant-like autotrophic nutrition, and the organisms normally store starch and oil as reserve food material and have cellulose-containing cell walls. The morphological characteristics of *P. zopfii* are very similar to those of *Chlorella*[2] except for the lack of chlorophyll and the "spore-like" reproductive mechanism. A further relationship between *Prototheca* and *Chlorella* was noted by Beijerinck[3] who showed the presence of polysaccharide which stained red–brown with iodine (glycogen) in the cell contents of *Prototheca* and achloric *Chlorella* strains.

Although the physiology and biochemistry of *P. zopfii* show many features similar to those for green and achloric *Chlorella* strains, there are important differences with respect to the requirement for carbon dioxide[4] and the nature of the cell walls[5]. In continuation of our comparative survey of protozoal polysaccharides, we have therefore isolated and characterised the reserve polysaccharides synthesised by *P. zopfii*. A preliminary account of this work has been published[6].

MATERIALS AND METHODS

Analytical methods. — Total, acid hydrolysis of polysaccharides was effected by heating with M sulphuric acid at 100° for 2.25 h. Hydrolysates were neutralised with barium carbonate and deionised by using mixed Amberlite IR-120 and IR-45 resins. For partial, acid hydrolysis, 50mM sulphuric acid at 100° for 20 min was used.

Reducing sugars (glucose, galactose, maltose, mannose) were estimated colorimetrically by using an alkaline copper reagent[7]. Glucose, galactose, and mannose, either free or glycosidically bound, were also estimated by a phenol–sulphuric acid method[8]. Qualitative paper chromatography was carried out in the following solvents: *A*, ethyl acetate–pyridine–water (10:4:3); *B*, butan-1-ol–ethanol–water (4:1:5); *C*, propan-1-ol–ethyl acetate–water (14:2:7); *D*, butanone–water–ammonia (sp. gr. 0.88) (200:17:1). Neutral sugars and methylated sugars were detected by using silver nitrate and aniline oxalate spray reagents, respectively. Protein was determined by a modified Lowry method[9], calibrated against bovine serum albumin.

The methods used for the structural analysis of the α-D-glucan, involving the use of alpha-, beta-, and iso-amylase, were those described previously[10]. The i.r. spectra of polysaccharides (1–2 mg) were determined for Nujol mulls, using a Perkin–Elmer Infracord spectrophotometer.

Methylation analysis. — The conditions used for the methylation of various polysaccharides are given in the Results section. The methoxyl content of methylated polysaccharides was determined by a semi-micro Zeisel method[11]. Methylated polysaccharides (2 mg) were methanolysed by heating with 3% methanolic hydrogen chloride (0.5 ml) in a sealed tube at 100° for 16 h. Qualitative g.l.c. of the resulting methyl glycosides was carried out as described by Aspinall[12] on a Pye–Argon Chromatograph, using columns containing the following liquid phases supported on acid-washed Celite 545 or Chromosorb W: (*a*) butane-1,4-diol succinate at 175°, (*b*) poly(ethylene glycol adipate) at 175°, (*c*) neopentyl glycol adipate at 125° and 150°, (*d*) XE-60 at 125°, (*e*) mixed Apiezon M-BS at 175°. A comprehensive range of methylated sugar glycosides of known retention times was available for comparative purposes. Methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside was used as the standard.

Periodate oxidation. — Polysaccharide solutions, whose concentrations had been determined by the phenol–sulphuric acid method[8], were oxidised in the dark at either 2° or room temperature (18±2°) with unbuffered sodium metaperiodate (initial pH, ~5). At least 100% excess of periodate was present. The reduction of periodate was measured spectrophotometrically[13], the production of formic acid by titration[14] with standard sodium hydroxide solution to an end-point of pH 5.8, and the liberation of formaldehyde by a chromotropic acid method[15]. The initial amounts of formic acid and formaldehyde released were obtained by extrapolation to zero time, thereby minimizing any effects due to "over-oxidation".

Growth of P. zopfii. — The organism was grown on a medium consisting of 3%

(w/v) of Remogen brand extract of malt (Jeffreys, Miller & Co. Ltd., Wigan) and 0.5% of Oxoid mycological peptone, which had a pH of 5.4.

Stock cultures were maintained in tubes containing 10-ml quantities of this medium. For bulk growth, 250-ml quantities, solidified by the addition of 1.5% (w/v) agar, were used in Roux bottles, each of which was inoculated with a tube of stock culture. After incubation at 24° for 7 or 8 days, the cells were washed off the agar surface with a little medium, centrifuged, washed in methanol, and stored in methanol at −20°. Microscopically, the organisms were mostly spherical, although some were ovoid, varying in size from 5 μm for a recently formed individual up to 20 μm for a cell in the process of division into eight daughter cells.

Disintegration of cells. — The cells were very resistant to many physical and chemical methods of disruption, and were unaffected by rapid freezing and thawing, ultrasonic vibrations, or by treatment with trichloroacetic acid, *N*,*N*-dimethylformamide, or detergents such as sodium lauryl sulphate or Teepol Eventually, the cells were washed with ether, dried *in vacuo* over phosphorus pentaoxide, ground with clean carborundum in a chilled mortar, and then stirred for 10 min with boiling water. After cooling and centrifuging, the supernatant solution gave a brown stain with iodine, similar to that of mammalian glycogens; after total, acid hydrolysis, paper chromatography (solvent *A*) showed the presence of galactose, glucose, and mannose. Attempts to isolate further polysaccharide material from the ground cells by stirring with cold water for longer periods were unsuccessful.

Extraction of polysaccharides. — Dried cells (60 g) were extracted in five separate batches. Each batch (12 g) was ground for 1–2 h with an equal weight of washed, fine-grade carborundum in a chilled mortar. The ground material was stirred for 30 min with 80 ml of water at 100°, cooled, and centrifuged. To the supernatant solution, ethanol (2 vol.) was added with stirring, and the suspension of polysaccharide was stored at 0°. The residue was extracted a further two or three times with hot water, the extracts then being treated as described above. The remaining residue was washed with ethanol and ether, dried *in vacuo*, and again ground with carborundum in an attempt to release further polysaccharide material. Very little additional polysaccharide was obtained on a further and final extraction with hot water.

The polysaccharide suspensions in ethanol, obtained from 60 g of cells, were combined and centrifuged. The precipitate was dissolved in 80 ml of cold water, and deproteinised by the addition of a solution of trichloroacetic acid (4 g) in water (20 ml) at 0°. After 6 h, the precipitated protein was removed by centrifugation. The polysaccharide was reprecipitated from aqueous solution three or four times using ethanol until, at the final stage, the addition of a few crystals of ammonium acetate was required to induce flocculation. The final, white precipitate (fraction A) was washed with ethanol and ether, and dried *in vacuo* over phosphorus pentaoxide; yield, 6.0 g.

The carborundum residues remaining after the hot-water extraction were extracted two or three times with 80 ml of hot, aqueous (40%) potassium hydroxide.

The extracts were combined and diluted with an equal volume of water, and ethanol (2 vol.) was added with stirring. The precipitate was collected by centrifugation, and suspended in cold water by stirring for 30 min. The insoluble material was collected by centrifugation and shown to be essentially free of carbohydrate by the phenol–sulphuric acid reagent[8]. The polysaccharide from the supernatant solution was precipitated several times with ethanol, and ammonium acetate was added on the final occasion. The alkali-extracted polysaccharides (fraction B) were washed with ethanol and ether, and then dried; yield, 2.2 g.

A sample of the dried carborundum residue was treated with M sulphuric acid at 100° for 2.5 h and, after cooling and neutralisation, the extract was analysed for carbohydrate content by the phenol–sulphuric acid reagent[8]. The carborundum residue contained only 0.9% of carbohydrate, estimated as galactose.

RESULTS

Preliminary examination of Fractions A and B

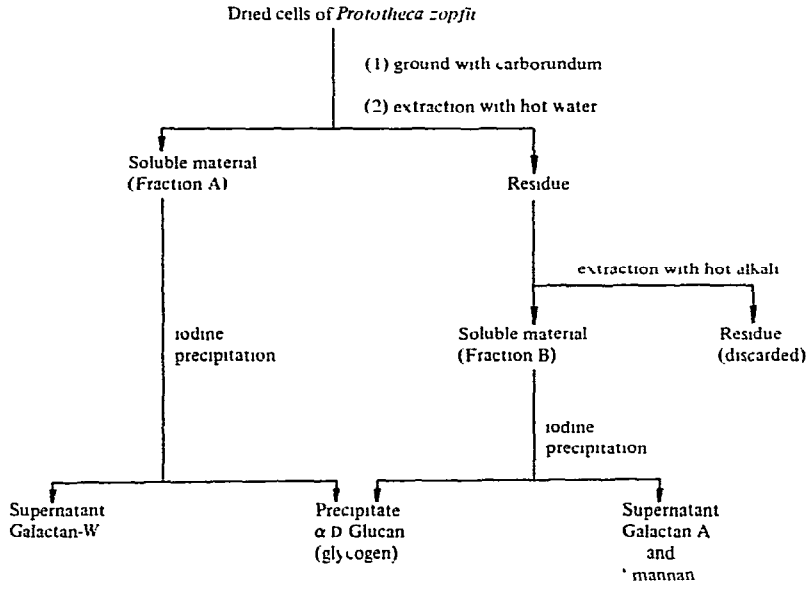
Fractions A and B were both soluble in water, giving solutions which gave brown colours with iodine. Paper chromatography (solvent *A*) of total acid hydrolysates showed that fraction A yielded galactose with much smaller amounts of glucose and mannose, whereas fraction B yielded approximately equal amounts of galactose and mannose with only a very small amount of glucose. Incubation of both fractions with salivary alpha-amylase resulted in the loss of iodine-staining power, and the production of reducing sugars, including glucose, maltose, and maltotriose, as shown by paper chromatography. These changes are similar to those which have been observed with mammalian glycogens. Fractions A and B therefore contained a mixture of polysaccharides.

An attempt to isolate a homopolysaccharide by chromatography of fraction A on DEAE-cellulose was not successful. Elution with varying concentrations of sodium borate gave four peaks of polysaccharide material, each of which yielded galactose as the major sugar on acid hydrolysis, together with smaller and different amounts of glucose and mannose. Since complete separation had not been obtained, attempts were made to isolate pure glucan by iodine precipitation (*cf.* ref. 16).

Fraction A or B (0.1 g) dissolved in 25 ml of water was treated with 20% aqueous sodium chloride (10 ml) and concentrated iodine solution (1.25 ml); the latter contained 3 g of iodine and 5 g of potassium iodide in 25 ml. The resulting dark-brown solution was diluted to 45 ml with water and shaken for 5 min in a stoppered tube. After a further 5 min, the precipitated polysaccharide–iodine complex was collected by centrifugation (1500 *g* for 10 min), suspended in 20% aqueous sodium chloride (10 ml), and decomposed by the dropwise addition of 12.5% aqueous sodium thiosulphate. M Hydrochloric acid (5 ml), concentrated iodine solution (1.25 ml), and distilled water (25 ml) were added, and the solution was shaken and centrifuged as before. The iodine complex was suspended in ethanol (25 ml) and decomposed by the dropwise addition of aqueous sodium thiosulphate, and water

(11 ml) was then added to dissolve excess thiosulphate. The resulting white precipitate was centrifuged, washed successively with 70% ethanol (twice), ethanol, and ether, and then dried *in vacuo* over phosphorus pentaoxide. The polysaccharide so isolated gave only glucose on total, acid hydrolysis. The whole of the remaining fraction A (5.8 g) and B (2.0 g) was fractionated by this procedure, using the same relative volume of reagents, to give 0.33 and 0.15 g of glucan, respectively.

The supernatant solutions from the iodine precipitation were combined, titrated with sodium thiosulphate to remove excess of iodine, and dialysed against running water. Ethanol (2 vol.) was added, and the precipitates were centrifuged, washed, and dried. The products did not stain with iodine, and on total, acid hydrolysis gave galactose and mannose. Fraction A yielded 3.7 g of "hot-water" galactan, and fraction B yielded 1.3 g of "alkali" galactan. These will be referred to as galactan-W and galactan-A, respectively. The fractionation is summarised in Scheme 1.



Scheme 1. Fractionation of soluble polysaccharides from *Prototheca zopfii*.

Preliminary examination of galactan-A preparation

The polysaccharide preparation was soluble in cold water, $[\alpha]_D^{20}$ $-7°$ (*c* 1.65), giving a solution which did not stain with iodine. An acid hydrolysate contained approximately equal proportions of galactose and mannose.

Several methods for the fractionation of polysaccharides were investigated in an attempt to obtain a purified galactan and mannan. Selective precipitation with cetyltrimethylammonium bromide[17], iodine in the presence of calcium chloride[18], copper acetate[19], and Fehling's solution[20] failed to give a complete separation,

although polysaccharide fractions containing different ratios of galactose to mannose were obtained. DEAE-cellulose chromatography was also unsuccessful. Since the galactan-W preparation was shown in later experiments to be essentially a homopolysaccharide (containing 96% of galactose), it seems probable that the galactan-A preparation is a mixture of this galactan, together with other polysaccharide material containing mannose residues. For convenience, this latter will be referred to as "mannan", although the use of this term does not necessarily imply that it is a homopolysaccharide. The infrared spectrum showed peaks in the region of 880–900 cm^{-1} characteristic[21] of β-D-galactopyranose and β-D-mannopyranose residues.

Examination of galactan-W

Since further purification could not be effected by dialysis or reprecipitation, structural studies were carried out at this stage. The galactan had $[\alpha]_D^{20}$ +41° (*c* 0.1, water) and contained 6% of protein, which was presumably an impurity.

An acid hydrolysate was fractionated by paper chromatography (solvent *A*), and the component sugars were eluted with water and their concentrations determined by using the alkaline copper reagent. Galactose and mannose, in the molecular proportions of 26:1, were present. The major sugar was characterised as D-galactose on the basis of the $[\alpha]_D^{20}$ value (+82.1°; lit. 80.2→83.5°) and the formation of D-galactose phenylosazone, (m.p. 188–192°; lit. m.p. 190–194°). The minor sugar was characterised as mannose since the derived *p*-nitroaniline derivative had m.p. 216–217°; lit. m.p. 218–219°.

Galactan-W was subjected to partial hydrolysis with acid. The polysaccharide (10.3 mg) was treated at 100° with 10mM sulphuric acid (10 ml); portions (1 ml) were withdrawn at intervals and neutralised, and the concentration of reducing sugar (expressed as galactose) was determined with the alkaline copper reagent[7]. The results (Fig. 1) show that ~11% of galactan-W was relatively rapidly hydrolysed. In a separate experiment, paper chromatography of a partial, acid hydrolysate showed that galactose and traces of an oligosaccharide with the R_{GLC} value of 6-*O*-β-D-galacto-

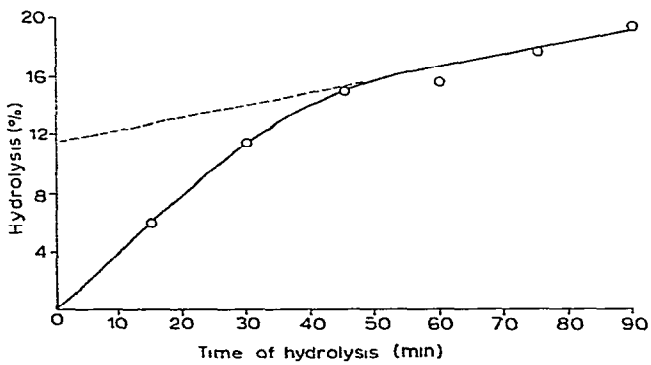


Fig. 1. Rate of hydrolysis of galactan-W by 10mM sulphuric acid at 100°.

pyranosyl-D-galactopyranose were present. Hydrolysis under stronger conditions liberated mannose and a larger amount of galactose, but did not increase the amount of oligosaccharide material.

Periodate-oxidation analysis of galactan-W

Galactan-W (16.2–17.8 mg; 100–110 μmoles as "anhydrogalactose") was oxidised by sodium metaperiodate (750 μmoles) in the dark at 20°, and at 2°. Samples were removed at intervals for the spectrophotometric measurement of the amount of periodate reduced[13]. In both experiments, an initial, rapid oxidation was followed by a slower oxidation, which resulted in a linear relation between the amount of periodate reduced and the period of oxidation. Extrapolation of this latter oxidation to zero time indicated an initial reduction of 1.63 and 1.42 molecular proportions of periodate per monosaccharide residue at 20° and 2°, respectively.

The amount of formaldehyde which was liberated when galactan-W (9.9–16.2 mg; 61–100 μmoles) was oxidised in the dark at 2° by using (*a*) 30mM and (*b*) 0.8mM sodium metaperiodate was measured by the chromotropic acid method[15]. The rates of release of formaldehyde are shown in Fig. 2(*a*) and *b*), and correspond to initial values of 0.12 and 0.11 mol., respectively. The rate of release of formic acid during the oxidation of galactan (21.7 mg; 134 μmoles) with 0.1M sodium metaperiodate at 20° is also shown in Fig. 2(*c*). Extrapolation to zero time gave an initial release of 88.7 μmoles of formic acid, equivalent to the production of 0.66 mol.

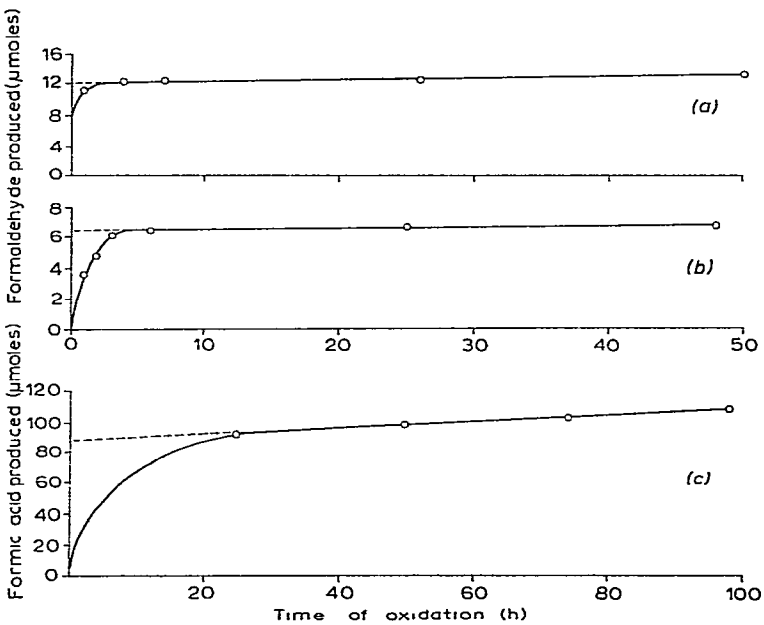


Fig. 2. Periodate oxidation of galactan-W: (*a*) release of formaldehyde on oxidation with 30mM periodate at 2°, (*b*) release of formaldehyde on oxidation with 0.8mM periodate at 2°, (*c*) release of formic acid on oxidation with 0.1M periodate at 20°; all results are expressed as μmoles.

Galactan-W (1.9 g) was oxidised at 2° with 40mM sodium metaperiodate (750 ml) for 136 h. Ethylene glycol (1.65 ml) was then added to reduce the remaining periodate, and the mixture was dialysed at 2° against eight changes of distilled water. The galactan polyaldehyde solution (~1 litre) was then treated with sodium borohydride (2 g) at 20° for 10 h. Excess borohydride was destroyed by the dropwise addition of M hydrochloric acid, until the pH of the solution was 7. After concentration to ~250 ml, and further dialysis at 2° against distilled water, the galactan polyalcohol was isolated by freeze-drying.

Galactan polyalcohol (0.8 g) was hydrolysed with 0.25M sulphuric acid (100 ml) at 20° for 10 h. After neutralisation with barium carbonate, the hydrolysate was deionised with mixed Amberlite IR-200 and IR-400 resins, and concentrated. Paper chromatography indicated the presence of glycerol (+++), erythritol (++), and traces of three oligosaccharides. The hydrolysate was fractionated by preparative paper chromatography (solvent *A*). The presence of glycerol was confirmed by preparation of the *p*-nitrobenzoate (m.p. 192–194°; lit. m.p. 194–196°). The oligosaccharides, which had $R_{GLYCEROL}$ values of 0.16, 0.40, and 0.47 in solvent *A*, produced different amounts of galactose, mannose, and erythritol on complete hydrolysis with acid. No structural implications are drawn from these observations, since the amount of material available was inadequate for rigorous chemical characterisation.

Methylation analysis of galactan-W

The galactan (150 mg) was methylated by several treatments with methyl sulphate and 30% (w/v) aqueous sodium hydroxide[22] at 0° under nitrogen, followed by treatment with methyl iodide and dry silver oxide[23]. The methylated polysaccharide was extracted with hot chloroform, the extract was evaporated to a small volume, and the product was precipitated with light petroleum (b.p. 40–60°). Final yield, 44 mg (Found: OMe, 42 2%).

TABLE I

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED GALACTAN-W AND GALACTAN-A BY G.L.C.

Sugar	*Methyl glycoside*	*Ring form*	*Relative amounts*[a]	
			Galactan-W	*Galactan-A*
Galactose	Tetra-*O*-methyl-*β*	Furanose	++	++
	Tetra-*O*-methyl-*α*	Furanose	±	±
	2,3,4,6-Tetra-*O*-methyl	Pyranose	±	±
	2,3,4-Tri-*O*-methyl	Pyranose	+++	+++
	2,4,6-Tri-*O*-methyl	Pyranose	±	±
	2,4-Di-*O*-methyl	Pyranose	++	++
Mannose	2,3,6-Tri-*O*-methyl	Pyranose	±	+++

[a]Visual estimation of peak areas; large, medium, small, and very small peaks are indicated by +++, ++, +, and ±, respectively. The results are derived from chromatograms from all five columns (see Experimental), most of the runs being carried out on columns (*a*) and (*b*).

Methylated galactan (2 mg) was heated for 16 h at 100° with 3% methanolic hydrogen chloride (0.5 ml), and, after removal of the hydrogen chloride, the mixture of methyl glycosides was dissolved in a few drops of chloroform and analysed by g.l.c. The methyl glycosides of seven methylated monosaccharides were detected (Table I), the relative amounts being estimated from the areas of the peaks on the recorder charts.

Methylated galactan (35 mg) was hydrolysed by successive treatments at 100° with 90% (v/v) formic acid (2 ml) and M sulphuric acid (4 ml). After neutralization (barium carbonate), the hydrolysate was analysed by paper chromatography. The results of qualitative experiments are shown in Table II. By quantitative chromatography on Whatman 3MM paper (solvent *D*), followed by hypoiodite estimation, the following ratios were obtained: di-*O*-methylgalactose, 16.3%; tri-*O*-methyl sugar, 73.3%; tetra-*O*-methylgalactose, 10.4%. It should be noted that the furanoid and pyranoid forms of the last sugar were not separated by paper chromatography.

TABLE II

EXAMINATION OF ACID HYDROLYSATES OF METHYLATED GALACTAN-W AND GALACTAN-A BY PAPER CHROMATOGRAPHY

R_{GAL}[a]	*Methylated sugar*	*Relative amounts*[b]	
		Galactan-W	*Galactan-A*
0·15	Di-*O*-methylgalactose	+	+
0 48	2,3,4-Tri-*O*-methylgalactose	+ + +	+ + +
0·53	2,4,6-Tri-*O*-methylgalactose	±	±
1·00	2,3,4,6-Tetra-*O*-methylgalactose	+	+
0 64	2,3,6-Tri-*O*-methylmannose	±	+ +

[a]Relative to 2,3,4,6,-tetra-*O*-methyl-D-galactose; solvent *D*; authentic samples of the methylated sugars were available as reference compounds; aniline oxalate was used as the spray reagent. [b]Visual estimation, as in Table I.

Methylation analysis of galactan-A

The results obtained by g.l.c. and paper chromatography indicated that 2,3,4-tri-*O*-methyl-D-galactose was the major methylated sugar from galactan-W. It was therefore desirable to characterise this sugar unambiguously. Although it was appreciated that galactan-A was a mixture, methylation analysis was carried out as follows. Galactan-A (1.0 g), partially methylated by the Haworth procedure[22], was further methylated, using methyl sulphate and solid sodium hydroxide in tetrahydrofuran[24]. The product (250 mg) had OMe 42.4%.

Analysis of a methanolysate by g l.c. and of an acid hydrolysate by qualitative paper chromatography gave results which were identical with those from the hot-water galactan, except that the relative proportion of 2,3,6-tri-*O*-methyl D-mannose was greatly increased (see Tables I and II). It should be noted that other methyl ethers of D-mannose were not detected. Quantitative estimation of the acid hydrolysate gave

the following results: di-*O*-methylgalactose, 12.3%; tri-*O*-methylgalactose, 49.5%; tri-*O*-methylmannose, 29.4%; tetra-*O*-methylgalactose, 8.8%. The relative amounts of the methylated galactose residues are therefore di-*O*-methyl, 17.4%; tri-*O*-methyl, 70.1%; tetra-*O*-methyl, 12.4%.

The remainder of the acid hydrolysate was fractionated by preparative paper chromatography (solvent *D*), the area containing 2,3,4-tri-*O*-methylgalactose isolated, the sugar eluted with water, the eluate evaporated to dryness, and the residue converted into the aniline derivative[25]. The crystalline product had m.p. 163–165°; lit. m.p. 164–169°.

Properties of the glucan

The polysaccharide was readily soluble in water, giving an iodophilic solution. In aqueous solution, the iodine complex showed λ_{max} at 485 nm; in the presence of 50% saturated ammonium sulphate, it had λ_{max} 510 nm. The polysaccharide was characterized as a glucan since a partial, acid hydrolysate contained glucose, maltose, and higher maltosaccharides (paper chromatography in solvent *A*).

Evidence that the glucan had a glycogen-type structure was obtained by enzymic degradation studies, by methylation analysis, and from the i.r. spectrum, which showed bands at 760, 850, and 932 cm^{-1}, identical with those of an authentic sample of glycogen from *Cardium tuberculatum*.

Incubation of the glucan with beta-amylase at pH 4·6 gave 49% conversion into maltose. When the glucan was incubated successively with yeast isoamylase at pH 5·8, and then beta-amylase at pH 4·6, the percentage conversion into maltose was 73. The combined action of the two enzymes at pH 5·8 gave 93% conversion into maltose. These results show that the glucan has α-(1→4)-D-glucosidic inter-residue linkages, and α-(1→6)-D-glucosidic inter-chain linkages. Treatment with salivary alpha-amylase gave 77% apparent conversion into maltose after 24 h. Since the percentage of inter-chain linkages (P_R) is related[10] to the extent of alpha-amylolysis (P_M) by the equation $P_R = 23{\cdot}5–0{\cdot}211\ (P_M)$, this result indicates the presence of 7·3% of inter-chain linkages, corresponding to an average chain-length of 14 D-glucose residues.

The glucan (32 mg) was methylated first with methyl sulphate and barium hydroxide, in suspension in methyl sulphoxide[26], and then with methyl iodide and silver oxide in *N*,*N*-dimethylformamide[27]. The methylated glucan (6 mg) was analysed qualitatively. A portion (2 mg) was hydrolysed at 100° by successive treatment with 90% formic acid followed by M sulphuric acid. Paper-chromatographic analysis of the neutralized (barium carbonate) hydrolysate in solvents *B* and *D* showed the presence of sugars with the R_{GLC} values of 2,3,6-tri-*O*-methyl-D-glucose, together with much smaller amounts of 2,3,4,6-tetra-*O*-methyl- and di-*O*-methyl-D-glucose. The methylated glucan (2 mg) was also methanolysed, and analysed by g.l.c. using a column containing neopentyl glycol sebacate (10% w/w) supported on Chromosorb W, operating at 185°. The methyl glycosides of the following methylated monosaccharides were detected: major component, 2,3,6-tri-*O*-methyl-D-glucopyranose;

minor components, 2,3,4,6-tetra- and 2,3- di-*O*-methyl-D-glucopyranose. These results confirm the nature of the inter-residue and inter-chain linkages as (1→4)- and (1→6)-D-glucosidic, respectively.

DISCUSSION

Structural studies of protozoal and algal polysaccharides are frequently hindered by the resistance of the cell walls to disruption, which makes subsequent extraction of the polysaccharides inefficient. This situation has already been observed with *Haematococcus pluvialis*[10] and was a difficulty in the present investigation. The resistance to disintegration may be attributable in part to the effect of storage of the cells in cold methanol, which appears to increase the insolubility of polysaccharide mixtures. This change may resemble the denaturation of proteins by organic solvents.

Disruption was finally achieved by grinding the dried cells with carborundum, and a substantial amount of polysaccharide could then be extracted with either hot water or alkali. From 60 g of cells, 8·2 g of a mixture of polysaccharides was obtained. Insoluble cell-wall polysaccharides, such as β-(1→3)-D-glucan or cellulose, would remain in the residue, and would be largely unaffected by the hydrolysis conditions used to determine the amount of non-extracted carbohydrate (~0.9%). The observed yield is therefore not necessarily an indication of the total polysaccharide content of the cells.

Approximately 75% of the polysaccharide was soluble in hot water, and consisted mainly of a galactan with smaller amounts of a glucan which could be selectively precipitated with iodine, and was subsequently characterized as having a glycogen-type structure. Although many algae and related unicellular organisms accumulate polysaccharides which resemble plant starches or amylopectins, the production of glycogen-type polysaccharides is of rarer occurrence. For *Prototheca* to be considered as an achloric mutant of *Chlorella*, which produces starch as the reserve carbohydrate, the transformation from an autotrophic to a heterotrophic mode of life must have been accompanied by certain alterations in the polysaccharide-synthesizing enzyme systems.

The water-soluble galactan (galactan-W) yielded D-galactose (96·3%) and D-mannose (3·7%) on hydrolysis. In view of the similar structure of the related alkali-soluble galactan (galactan-A), it seems probable that the small amount of mannose arises from a mannan impurity, rather than from a mannogalactan type of polysaccharide.

Treatment of galactan-W with dilute acid gave significant amounts (~11%) of galactose initially, with only trace amounts of oligosaccharides, suggesting the presence of some acid-labile galactosidic linkages. Under similar conditions, polysaccharides containing only pyranosidic linkages yield oligosaccharides rather than monosaccharide(s) during the initial stages of depolymerisation.

Definitive information on the structure of the galactans was obtained by methylation analysis. It should be emphasised that galactan-W and galactan-A both

gave the same mixture of methylated derivatives of D-galactose in the same relative proportions (Tables I and II), so that only one type of galactan structure need be considered. The presence of 2,3,4-tri-*O*-methyl-D-galactose as the major sugar shows the presence of a high proportion of (1→6)-linked-D-galactopyranose residues. In addition, the presence of 2,4-di-*O*-methyl-D-galactose indicates that many of these residues are also substituted at C-3. However, a novel feature was the presence of the two methyl glycosides of tetra-*O*-methyl-D-galactofuranose which were detected by g.l.c. using a wide variety of columns and operating temperatures. Since only trace amounts of tetra-*O*-methyl-D-galactopyranose were present, it follows that the majority of the non-reducing, terminal groups in the galactan are D-galactofuranose residues. With both galactans, these amount to 10–12% of the total residues, and there are at least equivalent amounts of galactose residues triply-linked at C-1, C-3, and C-6. It is possible that some of the di-*O*-methylgalactose was due to undermethylation, and was not structurally significant. The presence of some (1→3)-linked-D-galactopyranose residues was shown by the small amount of 2,4,6-tri-*O*-methyl-D-galactose.

The above conclusions were supported by periodate-oxidation analysis. The rapid release of 0·66 mol. of formic acid shows the presence of a high proportion of (1→6)-linked D-galactopyranose residues. Since galactofuranose end-groups do not generate formic acid, virtually all of this product must have arisen from the (1→6)-linked residues, which therefore account for two-thirds of the total. The presence of galactofuranoses residues was confirmed by the rapid release of 0·11–0·12 mol. of formaldehyde. It is unlikely that any of the latter arose from reducing end-groups, since these are very probably (1→6)-linked. Hence, this result indicates the presence of 11–12% of galactofuranose end-groups, in good agreement with the value of 10% obtained by methylation analysis.

The presence of (1→6)-linked, pyranoid residues was also confirmed by the Smith-degradation method[28]. The final hydrolysate contained glycerol which was characterised as the *p*-nitrobenzoate. However, oligosaccharides such as galactosyl-glycerol were not isolated. These should arise from galactopyranose branch-points, but may have been lost by absorption on the ion-exchange resin used during deionisation of the hydrolysate. Similar losses have been noted with Smith-degradation analyses of other polysaccharides, such as the xylan from *Rhodymenia palmata*[29]. The low specific rotation of galactan-W, the detection of an oligosaccharide with the R_{GLC} value of 6-*O*-*β*-D-galactopyranosyl-D-galactopyranose, and the i.r. spectrum of galactan-A all suggest that the anomeric configuration of the inter-residue linkage is probably *β*-D. Hence, the simplest structure which can be proposed for the galactan is a main chain of *β*-(1→6)-linked D-galactopyranose residues, a proportion of which are substituted at C-3 with side chains which are terminated by acid-labile D-galactofuranose residues (Fig. 3). The molecular size of the galactan and the location of the small proportion of (1→3)-linked D-galactopyranose residues is not yet known. Moreover, methylation analysis gave no information as to whether the furanose end-groups were directly or indirectly attached to the main chain. Attempts to prepare an oxidised polysaccharide containing D-galactofuranosiduronic residues, which on

partial acid hydrolysis should yield a relatively acid-stable aldobiouronic acid, have not yet been successful[29].

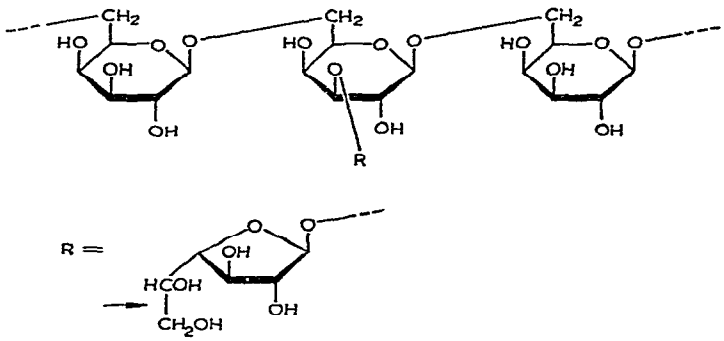


Fig. 3. Basic structure of *Prototheca* galactan. The arrow shows the position of periodate oxidation which readily release formaldehyde.

Previous studies from these and other laboratories[30] have shown that most protozoa synthesise α-D-glucans (starch, amylopectin, or glycogen) or β-(1→3)-D-glucans (paramylon, leucosin, astasian) as reserve carbohydrate. The current investigation has shown that *P. zopfii* synthesises two types of polysaccharide, namely, a glycogen and a galactan. The latter is of a hitherto unknown type. Apart from the reported presence of a galactan from the trypanosomid flagellate *Crithidia fasciculata*[31], polymers of D-galactose do not appear to be widely distributed amongst the protozoa.

Some biological implications of these essentially chemical results should be considered. Firstly, the organism was grown on agar, and it might be postulated that this was the source of the D-galactose. However, more-recent studies[29] have shown that growth on a liquid medium containing acetate as carbon source also leads to the formation of a galactan. Secondly, many carbohydrates containing D-galactose are synthesised from precursors derived from UDP-glucose and the enzyme UDP-glucose 4-epimerase. Extracts of *P. zopfii* have recently been shown[29] to contain this activity. Thirdly, at the time of completion of the present work (1967), galactofuranose residues had been demonstrated in only a few fungal and bacterial polysaccharides[32] and, although additional examples have been reported since then (*e.g.* refs. 33 and 34), they still represent a relatively rare carbohydrate in Nature.

The mode of biosynthesis of galactofuranosyl residues has been the subject of some speculation. In *Pencillium charlesii*, galactocarolose, a polymer of galactofuranose, is synthesised from UDP-glucose without fission of the hexose carbon chain, and UDP-α-D-galactofuranose has been shown to be an intermediate[35]. The nucleoside diphosphate sugar content of extracts of *P. zopfii* is currently being examined.

The cell walls of *P. zopfii* have been examined by Turner and Lloyd[5] who showed that an acid hydrolysate contained only glucose and mannose. It is therefore probable that the small amount of mannose-containing polysaccharide in the

galactan-W preparation, and the larger amount in the galactan-A preparation, which is relatively insoluble in hot water but soluble in hot alkali, represents cell-wall material. The methylation results indicate the presence of a high proportion of (1→4)-linked D-mannopyranose residues. Most fungal mannans are composed of (1→2)- and (1→6)-linked D-mannopyranose residues[32], but the cell walls of many higher plants contain (1→4)-linked mannan.

The overall results from the present investigation would not support the view that *Prototheca* is simply the colourless counterpart of *Chlorella*. The cell walls of *Chlorella pyrenoidosa* contain α-cellulose, a hemicellulose composed of galactose, mannose, arabinose, xylose, and rhamnose residues, and some associated glycoproteins[36], whereas a mixture of hemicelluloses and starch is obtained by extraction of the cells with hot dilute alkali[37]. The chemical composition and structure of these polysaccharides is therefore significantly different from those synthesized by *P. zopfii*.

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