Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SOME STRUCTURAL STUDIES ON THE GALACTAN FROM THE ALBUMEN GLANDS OF THE SNAIL, *Strophocheilus oblongus*

J. H. DUARTE* AND J. K. N. JONES
Department of Chemistry, Gordon Hall, Queen's University, Kingston, Ontario (Canada)
(Received April 23rd, 1970; accepted June 15th, 1970)

ABSTRACT

The galactan elaborated by the albumen glands of *Strophocheilus oblongus* is composed mainly, if not entirely, of β-linked D-galactopyranose residues. Acetolysis afforded D-galactose, 3-*O*-β-D-galactopyranosyl-D-galactose, and higher oligosaccharides. Borohydride reduction of the periodate-oxidized polysaccharide yielded a polyalcohol, which on hydrolysis with acid gave D-galactose and glycerol (Smith-type degradation). Controlled hydrolysis by acid of the polyalcohol (obtained after three Smith-type degradations) yielded a degraded polysaccharide (8%) and glycerol only. The fully methylated galactan after hydrolysis gave 2,3,4,6-tetra- (43 mole%), 2,4,6-tri- (11 mole%), 2,3,4-tri- (2.5 mole%), 2,4-di- (43 mole%), and trace amounts of 2,3,6-tri- (0.5 mole%)-*O*-methyl-D-galactose derivatives. These results indicate that the galactan of *S. oblongus* is a highly branched polysaccharide and that it has structural features which differentiate it from galactans of other molluscs that had been studied previously.

INTRODUCTION

Galactose-containing polysaccharides have been isolated from the albumen glands of several different species of mollusc. Although this type of polysaccharide was isolated[1] as early as 1885, structural studies were made only recently. Baldwin and Bell[2] investigated the galactan from *Helix pomatia*, which contains *ca.* 14% of L-galactose. They proposed alternative structures that showed a back-bone of (1→3), or (1→6)-linked D-galactose residues to which were attached single D- or L-galactose residues linked (1→6), or (1→3). On the other hand O'Colla[3], applying the Barry-degradation procedure[4] to the *H. pomatia* galactan, showed that the alternative structures proposed by Baldwin and Bell[2] were an oversimplification and that the polymer had a dichotomous structure. A highly branched structure for the galactan elaborated by *Biompholarid glabrata* was also proposed by Correa, Dmytraczenko, and Duarte[5]. Other studies have also been made on the galactans from albumen glands and eggs of the common gastropods[6–10].

*Address: Instituto de Bioquímica da Universidade Federal do Paraná Divisão de Bioquímica do Instituto de Biologia e Pesquisas Tecnológicas, P.O. Box 939, Curitibá, Paraná, Brazil.

TABLE I

DEGRADATION PRODUCTS FROM PERIODATE-OXIDIZED GALACTAN

Degradation number	*Periodate oxidation number*	*Non-dialyzable sample (g)*	*Dialyzable sample (g)*	*Moles of periodate per residue of galactose*	*Moles of formic acid per residue of galactose*	*Ratio of periodate to formic acid*
—	First	3.00 (100%) (100%)[b]	—	0.87 (0.82)[a]	0.40	2.1:1 (2.03)[a]
First	Second	1.58 (52%) (55%)[b]	0.66	0.78	0.78	2.1:1
Second	Third	0.72 (24%) (33%)[b]	0.20	0.49	0.23	2.1:1
Third	Fourth	0.25 (8.3%) (?2%)[b]	0.18			

[a]Values extrapolated to zero time. [b]Theoretical values for a polysaccharide having a (1→3)-linked, linear chain (11%).

The present communication describes structural studies of a polysaccharide isolated from the dissected albumen glands of *Strophocheilus oblongus*. A highly branched structure is verified as a characteristic feature of the galactans of gastropods.

RESULTS AND DISCUSSION

The glands, immediately after removal from the snails, were converted into an acetone-dried powder. Proteins were removed under mild conditions, first by treatment with proteolytic enzymes, and subsequently by the Sevag[11] procedure. Finally, the polysaccharide was precipitated as its cetyltrimethylammonium complex in the presence of a borate buffer (pH 8.5). Examination of the recovered polysaccharide by free-boundary electrophoresis indicated that the polysaccharide was homogeneous (or an inseparable mixture under the conditions used). It was judged suitable for structural investigation.

The polymer was shown to be composed of D-galactose only, by hydrolysis with acid, followed by analysis of the sugar produced by chromatography, by the D-galactose-oxidase test[12], and by specific rotation ($[\alpha]_D$ +80°). No evidence was found for the presence of L-galactose. The isolated polymer contained no nitrogen, sulphur, or carbonyl groups. Its analysis corresponded to a hexosan and it can therefore be classed as a D-galactan. The low positive rotation (+21°) of the polymer, its i.r. absorption at 890 cm^{-1}, and the isolation after degradation of 3- and 6-*O*-β-D-galactopyranosyl-D-galactose suggest that the galactan is built up in the main, if not entirely, of β-linked D-galactopyranosyl residues. No evidence was found for the presence of galactofuranosyl residues in the galactan.

The periodate consumption of, and the formic acid produced from, the polysaccharide (see Table I) indicate that less than half of the D-galactose residues are periodate-oxidizable and, assuming that all of the residues are linked through (1→3) and (1→6) linkages, it indicates a preponderance of (1→3) over (1→6) linkages. This evidence, along with the methylation analysis (see below) shows that nearly all of the periodate-oxidizable residues of the galactan (*ca.* 41%) occur as non-reducing D-galactopyranose end-groups. The greater part of the galactan (*ca.* 59%) is resistant to periodate oxidation, indicating substitution of these residues at C-3. These results are consistent with the Smith degradation results, when D-galactose and glycerol were isolated in a molar ratio of 3 to 2 after acidic hydrolysis of the Smith-degraded polymer[13].

Hydrolysis of the methylated galactan (OMe, 44.4%) gave rise to 2,3,4,6-tetra- (43 mole%), 2,4,6-tri- (11 mole%), and 2,4-di-*O*-methyl-D-galactose (43 mole%), which were identified conclusively. The other methylated sugars, present in small amounts, could not be characterized by chemical tests. However quantitative analysis of their glycosides by g.l.c. led to the tentative identification of 2,3,4-tri- (2.5 mole%), and 2,3,6-tri-*O*-methyl-D-galactose (0.5 mole%). The significance of the presence of these two sugar derivatives is uncertain. These results are in satisfactory agreement with the data obtained from the periodate oxidation of the polysaccharide and its

degradation by the Smith procedure. However they do not settle whether the polysaccharide is of the type suggested by Baldwin and Bell[2] or whether there is multiple branching of the type suggested by O'Colla[3]. In order to determine the type of structure present in *S. oblongus* galactan, it was subjected to the sequence (*a*) periodate oxidation, (*b*) reduction by sodium borohydride, and (*c*) hydrolysis by cold, dilute acid (Smith degradation)[13] three times. A polysaccharide was isolated in 8% yield after the third degradation, indicating that the original galactan was multibranched. If the destruction of each D-galactose residue led to the exposure of another oxidizable D-galactose residue, the maximum yield obtainable after 3 degradations would be about 22%. The yield obtained (8%) is thus good evidence for a high degree of multiple branching.

It may be noted that 2,3,6-tri- and 2,3,4-tri-*O*-methyl-D-galactose were not identified. Although a tri-*O*-methyl fraction (2,4,6-tri-*O*-methyl-D-galactose) was isolated from the hydrolysis products of the methylated galactan of *H. pomatia*[2,3,40], it was believed to result from incomplete methylation of the polymer. The tetra-*O*-methyl fraction in the hydrolyzate was derived from D and L-galactose. The galactan from *B. glabrata* was not examined by the methylation procedure[5].

To summarize, the evidence indicates that the galactan of *S. oblongus* is highly branched and shows some features that differentiate it from the galactans of *H. pomatia* and *B. glabrata*.

EXPERIMENTAL

General. — Free-boundary electrophoretic analysis was made in a Perkin–Elmer 38A apparatus with 0.05M sodium borate buffer (pH 9.2) at 20 mA and 150 V. I.r. absorption spectra were determined in KBr discs or Nujol mulls and were recorded on a Beckman IR-5A spectrophotometer. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter at 23 ±3°. Gas-liquid partition chromatography (F and M chromatograph model 402) of sugar derivatives was carried out on (*a*) 15% w/w of butan-1,4-diol succinate polyester on 80–100 mesh acid-washed Celite, column 120 × 0.5 cm at 175°; (*b*) 10% w/w polyphenyl ether [*m*-bis(*m*-phenoxyphenoxy)benzene], column 120 × 0.5 cm at 200°; (*c*) 14% w/w ethylene glycol succinate polyester on 80–100 mesh Chromosorb W, column 100 × 0.6 cm at 155° (in all three cases helium was the carrier gas used, with a flow rate of 50 ml.min^{-1}); and (*d*) 3% w/w of ECNSS-M on 100–120 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa. U.S.A.) with a column 120 × 0.6 cm at 175° and with helium as the carrier gas, flow rate 80 ml.min^{-1}. The retention time (T) of methylated sugars in g.l.c. are quoted relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucoside. Column (*c*) was used for quantitative analysis of the methylated sugars by the triangulation procedure[14]. Column (*d*) was used to determine the sugars as their alditol acetates[15].

Paper chromatography was performed by the descending method with Whatman No. 1 filter paper and the following solvent systems (all v/v); (*e*) 1:5:3:3 (upper

layer) benzene–butyl alcohol–pyridine–water; (*f*) 200:17:1 butanone–water–ammonia (*d*. 0.88). The sugars were detected with either alkaline silver nitrate[16] or *p*-anisidine hydrochloride[17] spray reagent. T.l.c. was carried out with silica gel-coated plates and the following solvent systems (all v/v) (*g*) 9:1 benzene–methanol; (*h*) 5:1 chloroform–methanol; or (*j*) 200:47:15:1 benzene–ethanol–water–acetic acid. The sugars were located by spraying the air-dried plates with 5% ethanolic sulphuric acid followed by heating them on a hot plate. The rate of movement of free sugars, R_{Gal}, is expressed relative to that of D-galactose, and the rate of movement of methylated sugars, R_G, is relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose, and R_{Mg} relative to that of its methyl β-glucoside. Acetylation of the sugars was performed in 2:1(*v/v*) pyridine–acetic anhydride at 100° and was monitored by t.l.c. (solvents *g* and *h*).

Isolation and purification of the galactan. — Snails, *S. oblongus*, were collected in Rio Grande do Sul, Brazil. Albumen glands (250) were dissected and homogenized in a blender with acetone (3 × 2 liters). The solids were collected by filtration and exhaustively extracted with hot (*a*) 2:1 (v/v) chloroform–methanol for 24 h, (*b*) butyl alcohol saturated with water for 12 h, and then (*c*) with acetone for 6 h. The dry, extracted powder (58 g) was suspended in 0.1M phosphate buffer (1 liter), pH 8.5, and crystalline subtilisin (300 mg, Type VII, Sigma Cnemical Co.) was added. The mixture was kept for 96 h at 35° and toluene was used as preservative. The polysaccharide solution was then boiled for 6 h under reflux, cooled, and dialyzed against tap water. The solution was centrifuged, and deproteinized by the method of Sevag (8 times). The aqueous phase was then lyophilized. The dry residue (30 g) was dissolved in water (400 ml) and fractionated by the addition of cetyltrimethylammonium bromide (10%, 400 ml) at pH 7. The precipitate, mainly nucleic acids[18,19,20] (by their u.v. spectra), was discarded. Borate buffer (1%, 400 ml) was then added, to pH 8.5, and the resultant precipitate collected. It was dissolved in 2M acetic acid, and ethanol (4 vols) was added. The precipitate was collected, washed with ethanol (3 × 250 ml), acetone (4 × 250 ml), and then dried *in vacuo* over phosphoric oxide. The product (23 g, 35%) had $[\alpha]_D$ +21.2° (*c* 0.55, water) and contained 9% of water that was very difficult to remove.

Anal. Found: C, 44.4; H, 6.6. Calc. for $C_6H_{10}O_5$ + 9% H_2O; C, 44.4; H, 6.6%. N and S were absent.

Hydrolysis of the polysaccharide gave crystalline D-galactose, m.p. 166–167°, $[\alpha]_D$ +116° → +80° (*c* 1.7, water), which afforded D-*lyxo*-hexulose phenylosazone[21], m.p. 195–196°. No other sugar could be detected by paper chromatography and the sugar was oxidized completely by D-galactose oxidase*.

Periodate oxidation of the galactan. — A sample of the polysaccharide was oxidized in sodium metaperiodate solution (0.02M) in the dark for 150 h at 0°. Portions of solution were withdrawn at intervals and analyzed for periodate uptake[22] and yield of formic acid (potentiometrically[23]). At the same time a sample of methyl α-D-glucopyranoside was oxidized as a check on the procedure. Table I shows that after

*Worthington Biochemical Corporation, Freehold, N. J., U. S. A.

TABLE II

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED GALACTAN

O-*Methylated methyl* D-*galactosides*	*Relative retention times* (T) *of methyl glycosides*				*Mole percentage*
	Column (*a*)	*Column* (*b*)		*Column* (*c*)	
2,3,4,6-Tetra-	1.82	1.50 sh	1.60	2.00	43.0
2,3,6-Tri-	3.40	1.69		3.92	0.5
2,4,6-Tri-	4.20 m 4.77 s	2.09 m	2.37 s	5.15 m 6.00 s	11.0
2,3,4-Tri-	7.44		2.90	9.80	2.5
2,4-Di-		3.74 m	4.40 s	25.50 m 30.50 s	43.0

The relative intensities of peaks are indicated by s = strong, m = moderate, sh = shoulder.

150 h one mole of the polysaccharide consumed 0.82 mole of periodate with the formation of 0.40 (3) mole of formic acid.

In a second experiment the polysaccharide (20 mg) was oxidized as already described and, after 150 h, ethylene glycol (0.5 ml) was added and the mixture was dialyzed for 24 h against tap water. The non-dialyzable "polyaldehyde" was then reduced to the polyalcohol by sodium borohydride (20 mg). The product was isolated after dialysis and lyophilization; it had $[\alpha]_D$ +20.3° (*c* 0.4, water). This product was hydrolyzed in 0.5M sulphuric acid (2 ml) for 6 h at 100°. The solution was neutralized (barium carbonate) filtered, and the filtrate deionized (Rexyn 101-H^+) and concentrated to a small volume. The products were treated with sodium borohydride and the resulting alditols were acetylated and analyzed by g.l.c. (column *d*). Galactitol acetate and glycerol acetate were found present in a molar ratio of 1.5:1.

Sequential Smith-degradation of the galactan[13,24]. — The polysaccharide (3 g) was oxidized with sodium metaperiodate solution (1 liter, 0.05M) for 150 h at 0.2°. Ethylene glycol (5 ml) was then added, and the solution was dialyzed against running water for 48 h. Sodium borohydride (1.5 g) was added, followed by, after 24 h, 2M acetic acid to destroy excess of borohydride. The solution was dialyzed and then lyophilized.

The residue was hydrolyzed with sulphuric acid (0.05M, 1 liter) for 30 h at 26° and the hydrolyzate dialyzed against distilled water. The neutral, organic material that passed through the membrane after treatment with sodium borohydride was shown to be glycerol only, identified as its triacetate by g.l.c. and as its tris(*p*-nitrobenzoate), m.p. 197–198°, identical with an authentic specimen[25,26]. The periodate oxidation–borohydride reduction followed by treatment with dilute acid (Smith procedure) was repeated a further two times (see Table II) and the degraded polysaccharide was isolated in 8% yield.

Acetolysis of the galactan. — A sample of the acetylated polysaccharide (2.5 g) was acetolyzed by the procedure of Goldstein and Whelan[27]. The acetolyzate was *O*-deacetylated by the Zemplén procedure and the product (2.25 g, 90%) was isolated in the usual way. Paper-chromatographic examination of the resultant syrup showed the presence of D-galactose, 3-*O*-*β*-D-galactopyranosyl-D-galactose (R_{Gal} 0.49), 6-*O*-*β*-D-galactopyranosyl-D-galactose (R_{Gal} 0.30)[28], and higher oligosaccharides. The R_{Gal} values of the disaccharides are in good agreement with those obtained by Stoddart and Jones[28]. The mixture of sugars was fractionated on a charcoal–Celite[29] column yielding D-galactose (1.68 g, 77.4%), disaccharides (0.29 g, 13.4%), trisaccharides (0.04 g, 1.9%), and higher oligosaccharides (0.16 g, 7.4%). These results will be amplified and discussed in a forthcoming publication.

Methylation of the galactan. — The polysaccharide (2.0 g) was methylated four times by the Haworth[30] procedure. The reaction mixture was neutralized (dilute sulfuric acid), salts were removed by dialysis, and the product (1.9 g) isolated after each methylation. As the product still contained OH groups (i.r. evidence) it was methylated twice more in tetrahydrofuran solution with dimethyl sulphate and powdered sodium hydroxide[31,32]. The product (1.32 g), $[\alpha]_D$ −63° (*c* 2.45, chloro-

form), precipitated from chloroform solution by addition of light petroleum, showed no hydroxyl-group absorption in the i.r. spectrum. After treatment with pyridine–acetic anhydride it showed no carbonyl absorption.

Anal. Calc. for $C_9H_{16}O_5$: OMe, 45.5. Found: OMe, 44.4%.

Methanolysis of the methylated galactan. — The methylated polymer (0.11 g) in 7% methanolic hydrogen chloride (5 ml) solution was heated for 6 h at 100° in a sealed tube. The cooled solution was neutralized with silver carbonate and the syrupy mixture of methyl glycosides (0.09 g) was isolated in the usual way. Examination (t.l.c.) showed the product to be a mixture of at least three components, having R_{Mg} 0.998, 0.916, and 0.730, close to those of methyl 2,3,4,6-tetra- (1.000), methyl 2,4,6-tri- (0.918), and methyl 2,4-di-*O*-methyl-D-galactosides (0.732). Analysis by g.l.c. (Columns *a*, *b*, and *c*) (see Table II) showed that retention times (T)[14,33] of the methyl galactosides corresponded to the presence of methyl 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, 2,3,6-tri-, and 2,4-di-*O*-methyl-D-galactosides.

Chemical identification of the methylated sugars. — The methylated galactan (1 g) was dissolved in 90% aqueous formic acid (40 ml) and the solution was heated for 6 h at 100°. The tube and contents were cooled and the solution evaporated to a syrup. The latter was dissolved in 0.25M sulphuric acid (50 ml) and heated in a sealed tube for 14 h at 100°. The solution was cooled, neutralized (barium carbonate) and the syrupy product (0.95 g) isolated in the usual way. The product was then fractionated on a column of silica gel, 60–120 mesh, (50 × 4.5 cm) (The British Drug Houses Ltd.) with 5:1 (v/v) chloroform–methanol (5 liters) as eluent. Fractions (10 ml) were collected and the fractionation was monitored by t.l.c. Where necessary, purification of the fractions was achieved by preparative t.l.c. with layers (20 × 20 cm) of silica gel on aluminium (solvent *e*). *Fraction I* (0.40 g, 40%) was identified as 2,3,4,6-tetra-*O*-methyl-D-galactose; $[\alpha]_D$ +108° (*c* 1.47, water). The derived *N*-phenyl-D-galactosylamine[34,35] had m.p. 194.5–195.5°, $[\alpha]_D$ −139° (*c* 0.90, pyridine) and $[\alpha]_D$ −80°→ −82.3° (after 20 h, *c*, 0.66, acetone). *Fraction II* (0.11 g, 12%) was 2,4,6-tri-*O*-methyl-D-galactose hydrate[36], m.p. 86–90°, $[\alpha]_D$ +83° (*c* 1.12, water). Its *N*-phenylglycosylamine[37] derivative had m.p. 170–171°, $[\alpha]_D$ +25° (*c* 0.60, methanol). *Fraction III* was 2,4-di-*O*-methyl-D-galactose (0.4 g, 44%) and had m.p. 101°, $[\alpha]_D$ +112°→+85° (*c* 0.92, water[38,39]). Its *N*-phenylglycosylamine derivative had m.p. 215–216°, $[\alpha]_D$ −180° (*c* 1.10, pyridine)[40].

The traces of other sugars present were identified tentatively by g.l.c. as 2,3,4-tri-*O*-methyl and 2,3,6-tri-*O*-methyl-D-galactose. The amounts present were estimated from the g.l.c. graphs by triangulation[14] and by weighing or measuring the area of the graph paper under the peaks corresponding to the various sugars. (See Table II.)

ACKNOWLEDGMENT

The authors thank the National Research Council of Canada for a grant (A0019) that made this work possible.

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