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TREATMENT OF RHAMNOGALACTURONAN I WITH LITHIUM IN ETHYLENEDIAMINE*

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ABSTRACT

Rhamnogalacturonan I is a pectic polysaccharide that is solubilized from the walls of suspension-cultured sycamore cells *(Acer pseudoplatanus)* by the action of a highly purified endo-1,4-α-polygalacturonanase. Rhamnogalacturonan I has a linear backbone consisting of the diglycosyl repeating unit, →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→. Approximately half of the α-L-rhamnosyl residues of the backbone are branched at O-4. Selective cleavage at the galactosyluronic acid residues of the backbone by treatment of rhamnogalacturonan I with lithium in ethylenediamine resulted in the release of the neutral glycosyl-residue sidechains that had been attached to the backbone. Various analytical techniques, including combined liquid chromatography–mass spectrometry, combined gas–liquid chromatography–mass spectrometry, and ^{1}H-nuclear magnetic resonance spectroscopy, were used to determine the structure of the side chains. The majority of the sidechains were isolated as oligoglycosylalditols, with rhamnitol at the "reducing" end. Terminal, 2-, 4-, or 6-linked galactosyl residues were found attached to O-4 of the rhamnitol residues. The 2-, 4-, and 6-linked galactosyl residues had terminal or 2-linked arabinosyl, or additional galactosyl, residues attached to them. Based on the results of fast-atom-bombardment mass spectrometry, the side chains were found to range in size from one to fourteen glycosyl residues. The side-chain structures suggest that there are four or more distinct families of side chains attached to the backbone of rhamnogalacturonan I.

INTRODUCTION

Rhamnogalacturonan I (RG-I) is a complex pectic polysaccharide[1] sol-

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ubilized from the primary cell-walls of suspension-cultured sycamore cells (*Acer pseudoplatanus*) by treatment with an endo-α-1,4-polygalacturonanase isolated from *Colletotrichum lindemuthianum*[2]. RG-I has a molecular weight of ~200,000, and is composed of L-arabinosyl, D-galactosyl, L-fucosyl, L-rhamnosyl, and D-galactosyluronic acid residues. The monosaccharide constituents of RG-I are interconnected by over 20 different types of glycosidic linkage[3].

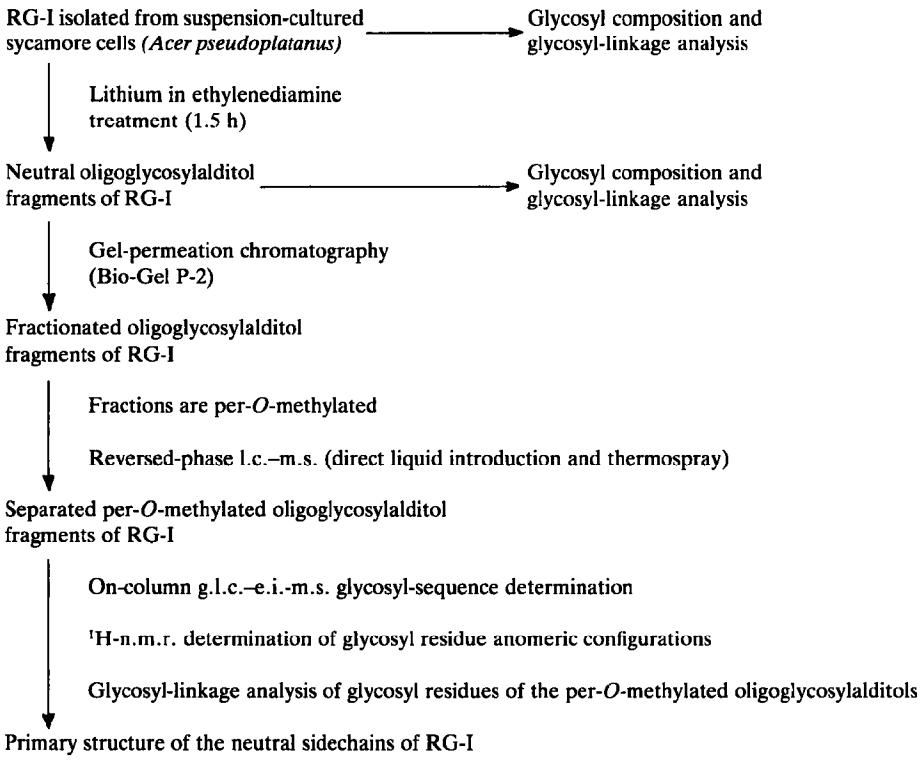
RG-I is constructed of a linear backbone having emanating side-chains[4]. The backbone consists[4] of the alternating diglycosyl sequence $\rightarrow$4)-α-D-GalA-(1$\rightarrow$2)-α-L-Rha-(1$\rightarrow$. About half of the L-rhamnosyl residues of the backbone are branched through O-4; these branch points are the points of attachment to the backbone of side chains that are primarily composed of neutral glycosyl residues[3,4].

A major question remaining to be answered about the structure of RG-I has been the nature of its side chains. It is not possible to study the side chains while they are attached to the backbone of RG-I due to the large size and structural complexity of the intact molecule. Random-cleavage techniques, such as partial hydrolysis of RG-I with acid, cannot be used because it would not be possible to determine which oligosaccharide fragments thus produced had originated from the same side-chains. Thus, a method was needed that would selectively cleave the backbone of RG-I, leaving the side chains relatively intact but separated from one another.

Treatment with lithium in ethylenediamine has been shown to degrade the glycosyluronic acid residues of complex carbohydrates, leaving intact the neutral glycosyl residues and their glycosidic linkages[5,6]. Thus, this procedure should cleave the backbone of RG-I between the side chains, leaving many or all of the side chains intact. We now describe the result of treating RG-I with lithium in ethylenediamine ("lithium treatment") and the structural characterization of the quantitatively preponderant side-chains recovered.

EXPERIMENTAL

Materials. — Lithium wire (45 mg/cm) was obtained from Aldrich Chemical Co. Sodium borohydride and sodium borodeuteride were from Alfa Products. Ethylenediamine was purchased from Sigma Chemical Co. Acetic anhydride, acetonitrile (h.p.l.c. grade), acetone, dimethyl sulfoxide, methyl iodide, pyridine, toluene, water (h.p.l.c. grade), and ammonium acetate were supplied by Fisher Scientific. Bio-Gels P-2 and P-6 were obtained from Bio-Rad. Acetone-d_6 was from KOR Biochemicals. Standard samples of partially *O*-methylated, partially *O*-acetylated alditol derivatives of arabinose, fucose, galactose, and rhamnose were gifts of T. T. Stevenson, J. R. Thomas, and L. D. Melton[7] of this laboratory.



Scheme 1. Procedure used in analyzing RG-I with lithium in ethylenediamine.

For RG-I, the side chains released by lithium treatment were analyzed by the methods outlined in Scheme 1. Each portion of the analysis was performed as described next.

Isolation of RG-I. — RG-I was isolated from primary cell-walls of suspension-cultured sycamore cells as described[4].

Lithium treatment of RG-I. — RG-I was treated with lithium in ethylenediamine as follows. RG-I (22 mg) was dried overnight *in vacuo* at 40°, suspended in ethylenediamine (2 mL), and the mixture stirred until the carbohydrate had dissolved (30 min). Three small pieces of lithium wire (each 2–3 mm long) were added. The solution turned blue after stirring for 15 min; the blue color was maintained for 1 h by addition of two 2–3-mm pieces of lithium wire. The reaction was stopped by the introduction of distilled water (5 mL) while the solution was cooled in an ice bath. The water and ethylenediamine were evaporated from the mixture by three roto-evaporations with toluene (water and ethylenediamine form azeotropes with toluene). The resulting white powder was dissolved in water while being cooled in an ice bath, and the lithium ions were removed by passing the solution through a column of Dowex AG-50W X-12 (10 mL) eluted with distilled water. The eluate was lyophilized overnight, to yield lithium-treated RG-I. Exper-

iments describing determination of the optimal conditions for the lithium treatment of RG-I are outlined in the Results and Discussion section.

Gel-permeation chromatography of the oligoglycosylalditol products of lithium-treated RG-I. — The underivatized products of the lithium-treated RG-I were fractionated by gel-permeation chromatography on either a column (1.5 × 85 cm) of Bio-Gel P-6 (200–400 mesh) or a column (1.6 × 71 cm) of Bio-Gel P-2 (200–400 mesh) eluted with water. Fractions (1.5 mL) were collected from each column.

Glycosyl-composition analysis. — The glycosyl-residue compositions of RG-I (0.1 mg) and of RG-I after lithium treatment (0.1 mg) were determined by analyses of their per-*O*-acetylated derivatives by g.l.c. on a column (0.25 mm × 30 m) of Supelco SP-2330 in a Hewlett–Packard (HP) model 5880 gas–liquid chromatograph fitted with a flame-ionization detector (f.i.d.) and by gas–liquid chromatography–chemical ionization mass spectrometry (g.l.c.–c.i.-m.s.) on an HP model 5985 mass spectrometer using isobutane as the reagent gas. These derivatives were obtained by hydrolysis with 2M trifluoroacetic acid for 1 h at 120°, followed by reduction with sodium borodeuteride in M ammonium hydroxide (10 mg/mL) for 1 h at room temperature and per-*O*-acetylation with 1:1 (v/v) acetic anhydride–pyridine for 25 min at 120°.

Glycosyl-linkage composition analysis of RG-I and the oligoglycosylalditol products of lithium-treated RG-I. — RG-I (0.2 mg), or the mixture of oligoglycosylalditols produced by lithium treatment of RG-I (0.1 mg), or chromatography fractions of the oligoglycosylalditols (exact amounts not determined) produced by the lithium treatment of RG-I, were dried *in vacuo* overnight at 40°, and then stirred with dimethyl sulfoxide (1 mL) until the carbohydrate had dissolved (normally 15–30 min). Sodium dimethylsulfinylmethylide (0.325 mL, 4M) was added to the solution to yield a final concentration of M ylide. This solution was stirred for 15 min, and then an excess (0.15 mL) of methyl iodide was added[8]. The per-*O*-methylated compounds resulting were purified by chromatography on Sep-Pak C-18 cartridges (Waters and Associates) as described[9].

The glycosyl-linkage compositions of per-*O*-methylated samples were then determined by forming the partially *O*-acetylated, partially *O*-methylated alditol derivatives of the per-*O*-methylated samples by hydrolysis with 2M trifluoroacetic acid for 1 h at 120° reduction by sodium borodeuteride in 1:1 (v/v) ethanol–water (10 mg/mL) for 2 h at room temperature and *O*-acetylation with 1:1 (v/v) acetic anhydride–pyridine for 3 h at 120°. These were analyzed by g.l.c. on a column (0.25 mm × 30 m) of Supelco SP-2330 in an HP model 5880 gas–liquid chromatograph fitted with an f.i.d., and by g.l.c.–m.s. (HP model 5970 mass spectrometer) analyses.

Liquid chromatography–mass spectrometry (l.c.–m.s.) separation and analysis of the per-O-methylated oligoglycosylalditol products of lithium-treated RG-I. — The mixtures of partially fractionated oligoglycosylalditols obtained by gel-permeation chromatography were per-*O*-methylated and the ethers fractionated by reversed-phase l.c. on a column (4.5 mm × 25 cm) of ODS (5 μm particle size) from

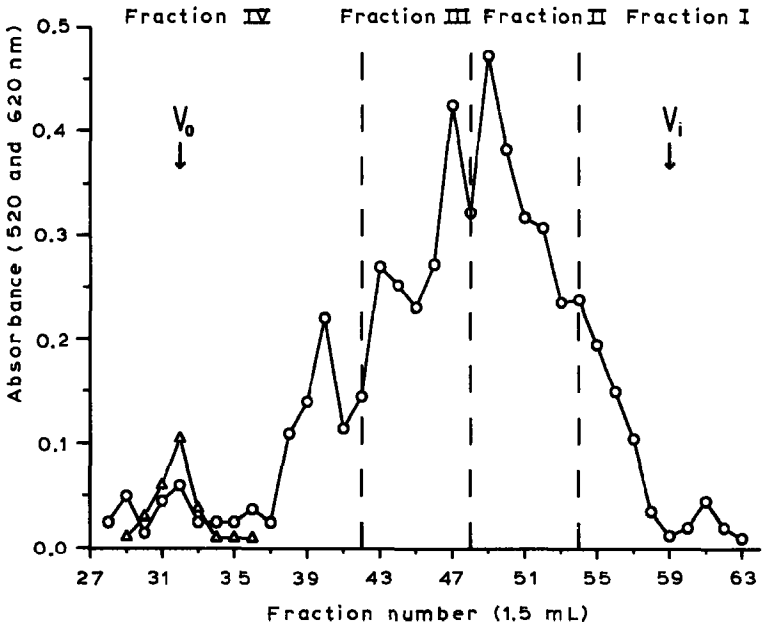


Fig. 1. Gel-permeation chromatography on P-2 of the products of lithium treatment of 22 mg of RG-I. The carbohydrate in each fraction was determined by the anthrone (circles) and Blumenkrantz–Asboe-Hansen (triangles) colorimetric methods. The fractions were combined as indicated by the dashed lines.

IBM Instruments Inc. The per-*O*-methylated oligosaccharides were eluted from the ODS column with a solvent gradient. The lower-molecular-weight, per-*O*-methylated oligoglycosylalditols derived from Fraction I (see Fig. 1) were eluted with a gradient (gradient I) that started at 1:3 (v/v) acetonitrile–water and increased to 7:13 at 30 min, 1:1 at 40 min, and 100:0 at 60 min. The intermediate-molecular-weight per-*O*-methylated oligoglycosylalditols derived from Fraction II (see Fig. 1) were eluted with a gradient (gradient II) that started at 3:7 (v/v) acetonitrile–water and increased to 2:3 at 30 min, 11:9 at 40 min, and 100:0 at 60 min. The higher-molecular-weight per-*O*-methylated oligoglycosylalditols (derived from Fractions III and IV: see Fig. 1) were eluted with a gradient (gradient III) that started at 3:7 (v/v) acetonitrile–water and increased linearly to 9:11 at 30 min, 3:2 at 40 min, and 100:0 at 60 min.

Each Fraction (I–IV) that contained per-*O*-methylated oligoglycosylalditols was divided into two portions. The first, consisting of ~80% of the fraction, was separated by the appropriate l.c. solvent gradient and analyzed by l.c.–m.s. In this procedure, the l.c. effluent was analyzed by introducing ~3% of the effluent into the m.s. source *via* a direct-liquid-introduction (d.l.i.) interface (HP model 5985). The rest of the l.c. effluent was collected in fractions for other analyses.

The remaining 20% of each per-*O*-methylated oligoglycosylalditol fraction was separated by the appropriate l.c. solvent gradient and analyzed by thermospray

m.s. using a Vestec thermospray interface. In these analyses, the effluent from the l.c. column (0.5 mL/min) was introduced into a mixing tee (Kratos) and 0.14M ammonium acetate (0.7 mL/min) was added to increase the proportions of pseudomolecular ions in the resulting mass spectra. This mixture (1.2 mL/min) was vaporized and introduced into the source of the mass spectrometer (HP model 5987).

G.l.c.–m.s. analysis of per-O-methylated oligoglycosylalditols. — The per-*O*-methylated oligoglycosylalditols in the l.c. fractions of the d.l.i. l.c.–m.s. experiments were also analyzed by g.l.c.–m.s. (electron impact, e.i.) in a capillary column (25 m × 0.32 mm i.d., or 15 m × 0.25 mm i.d.) of DB-1 (J and W Scientific Co.) in an HP model 5985 or model 5987 mass spectrometer system. The temperature program used in both mass spectrometers was 2 min at 50°, 30°/min to 220°, and 6°/min to 340°.

^{1}H-N.m.r. analysis of per-O-methylated oligoglycosylalditols. — ^{1}H-N.m.r. spectra of the per-*O*-methylated oligoglycosylalditols in the diverted l.c. fractions of the d.l.i. l.c.–m.s. experiments were recorded with a Bruker WM-250 Fourier-transform n.m.r. spectrometer operated at 250 MHz. Samples were dissolved in hexadeuterioacetone (99.997%). Chemical shifts are reported relative to pentadeuterioacetone (δ 2.04).

Fast-atom-bombardment-mass spectrometry (f.a.b.-m.s.). — The oligoglycosylalditols produced by lithium treatment of RG-I were analyzed by f.a.b.-m.s. after conversion into their per-*O*-acetyl and per-*O*-methyl derivatives, as described[10].

RESULTS AND DISCUSSION

Production of oligoglycosylalditols from RG-I side chains

Determination of conditions for lithium treatment of RG-I. — The following experiments were performed in order to determine conditions wherein lithium degraded most of the galactosyluronic acid residues in RG-I and simultaneously permitted the recovery of most of the neutral glycosyl residues of the polysaccharide. Six samples (0.5 mg) of dry RG-I were dissolved in ethylenediamine (0.5 mL) by stirring for ~15 min. Lithium wire (2–3 mm) was added to each solution, and with additional stirring the solutions turned a deep blue. The blue color was maintained in the six samples for 5, 10, 20, 40, 60, or 90 min. Additional pieces of lithium wire were added when necessary to maintain the blue color. By treating the polysaccharide with an excess of reagent (the blue color served as an indicator) for a specified length of time, we were better able to reproduce the extent of degradation observed, regardless of the amount of polysaccharide treated. The reactions were quenched at the specified times by addition of 5 mL of water. The reaction products were isolated as described in the Experimental section.

The Blumenkrantz–Asboe-Hansen colorimetric assay[11] for glycosyluronic acid residues was used to determine the content of galactosyluronic acid residue in

a 0.25-mL aliquot (out of 5 mL) of each 0.5-mg sample of lithium-treated RG-I. This value was compared to the content of galactosyluronic acid residue in a 0.25-mL aliquot of a 0.5-mg sample of RG-I that had been dissolved in ethylenediamine (5 mL) without lithium and isolated by the procedure used for the lithium-treated samples. The proportion of galactosyluronic acid residues degraded in each sample is presented in Fig. 2. Of the galactosyluronic acid residues, ~10% remained after lithium treatment for 60–90 min (see Fig. 2).

Previous experiments[6] had established that lithium treatment of glycosyluronic acid-containing polysaccharides produces neutral oligoglycosyl and oligoglycosylalditol products; a mixture of glycoses and alditols is formed from the residues that were glycosidically attached to the glycosyluronic acid residues. The glycoses produced, but not reduced by the lithium treatment, were converted into their corresponding alditols by reduction with sodium borohydride. After this reduction, each sample was acid-hydrolyzed and the products reduced with sodium borodeuteride in order to convert into an alditol any glycosyl residue cleaved by the acid hydrolysis, and at the same time to label the alditols thus formed with a deuterium atom on C-1. The samples were then per-*O*-acetylated and analyzed by g.l.c. and g.l.c.–c.i.-m.s. Comparison of these analyses of the lithium-treated RG-I samples with those of the control RG-I sample identified the neutral glycosyl residues whose glycosidic bonds had been cleaved by the lithium treatment (see

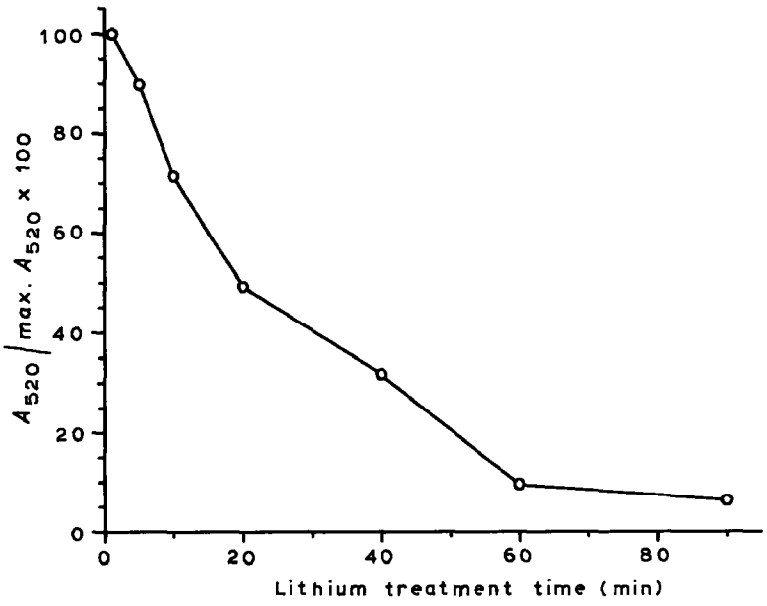


Fig. 2. Recovery of galactosyluronic acid residues from lithium-treated RG-I after various reaction times. The uronic acid content of the samples was determined by the Blumenkrantz–Asboe-Hansen colorimetric method. The data are plotted as the absorbance of each sample divided by the maximum absorbance for the sample to which no lithium was added.

TABLE I

COMPARISON OF NEUTRAL GLYCOSYL-RESIDUE COMPOSITION ANALYSIS OF RG-I AND LITHIUM TREATED RG-I

Glycosyl residue	*Lithium-treatment time (min)*					
	0	*1*	*5*	*20*	*40*	*60*
	(mol %)					
Rhamnosyl	14	15	16	14	14	13
Fucosyl	3	3	3	5	3	5
Arabinosyl	39	38	37	42	43	44
Galactosyl	40	40	40	34	36	34
	Relative recution of residue during lithium treatment (mol %)					
Rhamnosyl	0	4	11	53	78	86
Fucosyl	0	1	0	4	3	3
Arabinosyl	0	2	5	4	5	9
Galactosyl	0	3	0	4	8	7

Table I). Of the rhamnosyl residues, 86% were, as expected, released by the 60-min lithium treatment, that is, by degradation of the galactosyluronic acid residues. This value was consistent with recovery of ~10% of the galactosyluronic acid residues after a 60-min lithium treatment. The same treatment released 10% or less of the arabinosyl and galactosyl residues. These values represented acceptable levels of recovery, and in all subsequent experiments the RG-I samples were treated with lithium for 1 h.

Large-scale lithium treatment of RG-I. — RG-I (22 mg) was dried *in vacuo* overnight at 40°, and then dissolved in ethylenediamine (2 mL) with stirring for 30 min and sonication for 5 min. Lithium wire (~6 mm total) was added, and the solution turned blue with additional stirring. The blue color was maintained for 1 h, after which the reaction was quenched and the products isolated as described in the Experimental section. The yield (by weight) of the carbohydrate recovered after lyophilization was 11.8 mg (72%); had all of the neutral glycosyl residues of RG-I been recovered, the theoretical yield would have been 16.5 mg. It was determined by the Blumenkrantz–Asboe-Hansen colorimetric assay that ~90% of the galactosyluronic acid residues were decomposed.

Glycosyl-residue and glycosyl-linkage composition analyses. — The glycosyl-residue composition analysis of 1% of the lithium-treated RG-I was determined by g.l.c.–m.s. analysis of the alditol acetates (see Experimental section). The glycosyl-residue composition was the same as that obtained for the 60-min lithium treatment presented in Table I.

The glycosyl-linkage composition of an aliquot (0.1 mL/10 mL) of lithium-treated RG-I was determined by g.l.c.–m.s. analysis of the partially methylated, partially acetylated alditols (see Table II). The glycosyl-linkage compositions of

TABLE II

GLYCOSYL-LINKAGE COMPOSITION OF RG-I AND LITHIUM–ETHYLENEDIAMENE TREATED RG-I

Glycosyl residue[a]	*RG-I (mol %)*	*Lithium–ethylenediamine-treated RG-I (mol %)*
T-Rha	1.6	0.6
2-Rha	9.9	1.1
2,4-Rha	9.7	1.3
2,3,4-Rha	1.5	0.1
T-Gal	9.3	12.0
2-Gal	1.5	1.9
3-Gal	4.5	5.7
4-Gal	10.4	12.8
6-Gal	11.0	11.5
2,4-Gal	0.6	0.3
2,6-Gal	0.9	0.6
3,6-Gal	0.9	0.6
4,6-Gal	2.1	2.3
T-Ara	11.9	14.1
2-Ara	2.7	3.4
3-Ara	2.7	3.3
5-Ara	9.7	12.4
2,5-Ara	1.7	1.9
3,5-Ara	4.1	4.5
T-Fuc	2.3	2.9
Pre-red rhamnitol	—	trace
Pre-red 4-rhamnitol	—	5.1
Pre-red galactitol	—	0.1
Pre-red 4-galactitol	—	0.4
Pre-red 6-galactitol	—	0.1
Pre-red 2-arabinitol	—	trace
Pre-red 5-arabinitol	—	0.4
Pre-red 3,5-arabinitol	—	0.2

[a]T = nonreducing terminal. Numbers preceding residues indicates points of attachment of other glycosyl residues in the intact polysaccharide. Pre-red = glycosyl residue that was reduced either by the lithium treatment or by the sodium borohydride reduction that followed the lithium treatment.

the arabinosyl, fucosyl, and galactosyl residues of lithium-treated RG-I were similar to those of untreated RG-I, except that after lithium treatment, these residues constitute more of the total (88 *vs.* 74 mole percent). Lithium treatment lowered the proportion of rhamnosyl residues detected during glycosyl-linkage analysis; the 2- and 2,4-linked rhamnosyl residues accounted for ~20% of the neutral residues of untreated RG-I but only ~2% of the treated sample.

The loss of rhamnosyl residues after lithium treatment is consistent with the expected effect of lithium on the known structure of the RG-I backbone and the measured loss of galactosyluronic acid residues. Thus, lithium fragmentation of the galactosyluronic acid residues of the RG-I backbone followed by reduction with sodium borohydride would produce rhamnitol from unbranched rhamnosyl residues and 4-linked rhamnitol from branched rhamnosyl residues. Only a trace of

rhamnitol and 5.1 mole percent of 4-linked rhamnitol were recovered (theoretical recovery, 8.8 and 8.4 mole percent, respectively). The low yields of these derivatives were expected, as per-*O*-methylated rhamnitol is extremely volatile and would be lost in the synthesis and purification of the partially methylated alditol acetates. Even the mono-acetylated derivative of 4-linked pre-reduced rhamnitol is sufficiantly volatile that 60% recovery of this derivative was at least as good as expected based on previous experience[7].

Fractionation of the oligoglycosylalditol fragments of RG-I. — The mixture of oligoglycosylalditol fragments produced by lithium treatment of RG-I (11.3 mg) was dissolved in water and chromatographed on a column of Bio-Gel P-2 eluted with water. The carbohydrate, located by the Blumenkrantz–Asboe-Hansen and anthrone colorimetric assays, was eluted in a broad band that extended from the void to the included volumes of the column (see Fig. 1). The fractions that contained carbohydrate were pooled, as indicated, into Fractions I–IV (see Fig. 1). Aliquots (0.15 mL/2 mL) of Fractions I–IV were removed for f.a.b.-m.s. analysis. The rest of each Fraction was reduced with sodium borohydride and the product per-*O*-methylated. Aliquots (0.10 mL/1 mL) of the per-*O*-methylated carbohydrates in each sample were also saved for f.a.b.-m.s. analysis.

L.c.–m.s. and mass-spectrometric analysis of the per-O-methylated oligoglycosylalditols in Fractions I–IV. — Fractions I–IV (see Fig. 1) were each divided into two portions (8:2, v/v) and each portion was subjected to l.c. on an ODS column as described in the Experimental section.

The l.c. effluent from the separation of ~20% of each fraction was analyzed by thermospray m.s. The ionization in this procedure was "true thermospray", in that the ammonium acetate present in the l.c. effluent assisted in the formation of pseudomolecular ions as the solvent ions were stripped away from the carbohydrate molecules[12]. In order to "soften" the ionization process, and thereby increase the relative abundance of the pseudomolecular ions, the ionization was performed without the use of an ionizing medium (such as an electron beam). Monitoring the effluent in this way provided information on the composition of the per-*O*-methylated oligoglycosylalditols as they were eluted from the column. For example, the deoxyhexitol, deoxyhexose, hexose, and pentose content of each oligoglycosylalditol eluted could be calculated from knowledge of their molecular weights, which were obtained from the thermospray mass spectra.

The l.c. effluent from the separation of the remaining 80% of Fractions I–IV was monitored by d.l.i.-m.s. In this method, a small portion of the l.c. column effluent (0.01–0.02 mL/min out of 0.5 mL/min) was introduced as a fine jet directly into the source of the mass spectrometer. The material entering the mass spectrometer was analyzed by c.i.-m.s. using the l.c. solvent as the reagent gas. The rest of the effluent was collected in fractions so that the oligoglycosylalditols could be analyzed by other techniques.

The relative abundance of pseudomolecular ions in d.l.i.-m.s. was not as great as in thermospray-m.s. However, d.l.i.-m.s. did provide more structural in-

formation about the per-*O*-methylated oligoglycosylalditols, by producing diagnostic chemical-ionization rearrangement-ions[13] and fragmentation-ions[14]. Together, the two l.c.–m.s. techniques provided the molecular weight and composition as well as a great deal of structural information about the per-*O*-methylated oligoglycosylalditols in Fractions I–IV. Furthermore, d.l.i.-m.s. made possible the collection of pure and partially purified per-*O*-methylated oligoglycosylalditols for further analysis.

Characterization of the oligoglycosylalditols derived from RG-I side chains

Methods used to determine the structures of the per-O-methylated oligoglycosylalditols. — The structures of the per-*O*-methylated oligoglycosylalditols in Bio-Gel P-2 Fractions I–IV were determined from f.a.b., thermospray, c.i., and e.i. mass spectra, from ^{1}H-n.m.r. spectra, and from glycosyl and glycosyl-linkage compositions. The thermospray and c.i. mass spectra (obtained during l.c.–m.s. analyses) usually provided the pseudomolecular ion $[(M + NH_4)^+$ or $(M + H)^+]$ that gave the number and types (hexose, pentose, or deoxyhexose, *etc.*) of residues present in each per-*O*-methylated oligoglycosylalditol. The thermospray and c.i.-mass spectra also provided fragment ions of the A and J series[14] (A-series fragment-ions result from charge retention on the nonreducing end of oligoglycosylalditols, and J-series fragment-ions result from charge retention on the reducing end of the molecules). Analysis of A- and J-series fragment-ions identified the terminal and alditol residues of the per-*O*-methylated oligoglycosylalditols.

Rearrangement (elimination) ions[13] were commonly observed in the c.i.-mass spectra of the per-*O*-methylated oligoglycosylalditols. These fragment ions result from elimination of the glycosyl residue attached to the alditol of oligoglycosylalditols. By successive eliminative loss of the residue attached to the alditol, the sequence of the internal glycosyl residues of the oligoglycosylalditol could be deduced. Caution must be used when interpreting these rearrangement ions because they have the *m/z* value of potentially valid, pseudomolecular ions.

E.i.-mass spectra were normally obtained during g.l.c.–m.s. analysis of the per-*O*-methylated oligoglycosylalditols. These spectra contained fragment ions of the A, J, and alditol-cleavage series[15,16] that defined the glycosyl-residue sequence of the per-*O*-methylated oligoglycosylalditols. The J_2, J_1, and J_0 fragment-ions also provided information about the sequence and the points of attachment between the residues[16]. Glycosyl-linkage composition analysis of a purified per-*O*-methylated oligoglycosylalditol provided the points of attachment of its glycosyl residues and, by comparison of g.l.c. retention times of the derived partially methylated alditol acetates to those of known compounds, identified the glycosyl residues themselves. ^{1}H-n.m.r. spectroscopy was used to determine the anomeric configuration of the glycosyl linkages of the per-*O*-methylated oligoglycosylalditols.

The oligoglycosylalditols identified in Fractions I–IV. — The structures, or partial structures, of the per-*O*-methylated oligoglycosylalditols identified in Bio-Gel Fractions I–IV are listed in Table III. The ions from mass-spectrometric

TABLE III

LIST OF STRUCTURES AND PARTIAL STRUCTURES OF THE CHARACTERIZED OLIGOGLYCOSYLALDITOLS DERIVED FROM THE SIDE CHAINS OF RG-I

Numerical designation herein	*Per-O-methylated oligoglycosylalditol*
1	β-D-Gal*p*-(1→4)-L-Rhaol
2[a]	Ara-(1→5)-Araol
3[a]	Gal→Galol
4	Ara-(1→4)-Rhaol
5	α-L-Ara*f*-(1→3)-β-D-Gal*p*-(1→4)-L-Rhaol
6	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-L-Rhaol
7	β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol
8[a]	Ara→Ara→Araol
9[a]	Gal→Gal→Galol
10	Ara→Ara→Rhaol
11	Fuc→Ara→Rhaol
12	α-L-Ara*f*-(1→2)-α-L-Ara*f*-(1→3)-β-D-Gal*p*-(1→4)-L-Rhaol
13	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol
14	α-L-Fuc*p*(1→2)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol
15[a]	Ara→Ara→Ara→Araol
16[a]	Gal→Gal→Gal→Galol
17[b]	L-Ara*f*-(1→5)-L-Ara*f*-(1→2)-L-Ara*f*-(1→3)-D-Gal*p*-(1→4)-L-Rhaol
18	Gal→Gal→Gal→Gal→Rhaol
19	Ara→Ara→Ara→Ara→Araol

[a]Each of these per-*O*-methylated oligoglycosylalditols was eluted with more than one retention time, indicating the existence of more than one form of each (*i.e.*, possessing different anomeric configurations or linkage points). [b]The glycosyl sequence of the 2-linked Ara and 5-linked Ara was not determined for this per-*O*-methylated oligoglycosylalditol; the sequence displayed is for presentation purposes only.

analysis of the per-*O*-methylated oligoglycosylalditols that allowed their structures to be elucidated are listed in Tables IV and V. The results of the ^{1}H-n.m.r. analyses of these per-*O*-methylated oligoglycosylalditols are given in Table VI, and the glycosyl-linkage compositions in Table VII.

Examples of how these results were used to determine the structures of the per-*O*-methylated oligoglycosylalditols shown in Table III are described in the following sections.

The structures of per-O-methylated alditols in Fraction I. — Fraction I (see Fig. 3) was shown by thermospray l.c.–m.s. to be largely composed of a mixture of per-*O*-methylated deoxyhexitol, per-*O*-methylated hexitol, and per-*O*-methylated pentitol. Glycosyl-linkage analysis of RG-I and of lithium-treated RG-I (see Table II) established that the only deoxyhexosyl residues in RG-I were rhamnosyl and fucosyl residues; only the rhamnosyl residues were reduced by the lithium treatment. Because the only pentosyl residues in RG-I were arabinosyl residues and the only hexosyl residues in RG-I were galactosyl residues, the per-*O*-methylated alditols in Fraction I were derived from rhamnosyl, galactosyl and arabinosyl residues. The rhamnosyl residues present in RG-I were probably glycosidically

TABLE IV

DIAGNOSTIC THERMOSPRAY AND D.L.I. L.C.–M.S. IONS OF THE PER-*O*-METHYLATED OLIGOGLYCOSYLALDITOLS ISOLATED FOLLOWING LITHIUM TREATMENT OF RG-I

Per-O-*alkylated oligoglycosyl-alditol*	*R.t.*[a]	*TSP-m.s. ions*[b]		*C.i.-m.s. ions*[c]				
		$(M+NH_4)^+$	aJ_2+18	$(M+H)^+$	$aJ2+H_2O$	*Elim1*	*Elim2*	A_1
1	27.85[d]	458	223	441	223	—	—	219
		(83)	(9)	(100)	(1)			(4)
2	25.99[d]	400	209	383	209	—	—	175
		(65)	(100)	(100)	(45)			(5)
3	23.61[d]	488	253	471	253	—	—	219
		(34)	(100)	(100)	(36)			(42)
4	30.39[d]	414	223	397	223	—	—	175
		(10)	(5)	(4)	(74)			(33)
5	43.20[e]	618	223	601	223	397	—	175
		(100)	(5)	(82)	(1)	(8)		(37)
6	24.96[e]	662	223	645	223	441	—	219
		(100)	(12)	(56)	(19)	(43)		(70)
7	27.43[e]	662	223	645	223	441	—	219
		(100)	(1)	(50)	(10)	(39)		(61)
8	43.39[d]	560	209	543	209	383	—	175
		(12)	(20)	(100)	(6)	(10)		(51)
9	23.45[e]	692	253	675	253	471	—	219
		(85)	(21)	(27)	(34)	(54)		(62)
10	29.15[e]	574	223	557	223	397	—	175
		(20)	(18)	(0.9)	(41)	(4)		(39)
11	43.37[e]	588	223	571	223	411	—	189
		(100)	(60)	(31)	(9)	(21)		(60)
12	50.21[e]	778	223	761	223	557	—	175
		(100)	(3)	(22)	(1)	(5)		(62)
13	31.41[e]	866	223	849	223	645	441	219
		(100)	(11)	—	(7)	(21)	(18)	(43)
14	37.15[e]	836	223	819	223	615	411	189
		(100)	(17)	(0.5)	(1)	(3)	—	(5)
15	45.00[e]	720	209	703	209	543	383	175
		(100)	(16)	(3)	(64)	(0.7)	(8)	(100)
16	28.25[e]	896	253	879	253	675	471	219
		(100)	(4)	—	(100)	(0.9)	(14)	(43)
17	51.49[f]	938	223	921	223	717	557	175
		(100)	(40)	—	(40)	(2)	(5)	(100)
18	32.15[f]	1070	223	—	223	849	645	219
		(100)	(4)	—	(100)	—	(2.3)	(21)
19	55.10[e]	880	209	863	209	703	543	175
		(37)	(4)	—	(57)	(0.9)	(2)	(27)

[a]Retention time (min) on the IBM ODS l.c. column. [b]Thermospray-mass spectrometry ions; the figures in parentheses show peak intensity relative to base peak (100). [c]Chemical ionization-mass spectrometry ions obtained during direct liquid introduction-mass spectrometry; the figures in parentheses show peak intensity relative to base peak (100). [d]Retention time during l.c.–m.s. using gradient system I, described in the Experimental section. [e]Retention time during l.c.–m.s. using gradient system II, described in the Experimental section. [f]Retention time during l.c.–m.s. using gradient system III, described in the Experimental section.

TABLE V

DIAGNOSTIC E.I. M.S. IONS FOR THE PER-*O*-METHYLATED MONO-, DI-, TRI-, AND TETRA-GLYCOSYL ALDITOLS DERIVED FOLLOWING LITHIUM TREATMENT OF RG-I

Per-O-methylated monoglycosyl-alditol	*R.t.*[a]	*Electron impact-m.s. ions*[b]					*Alditol*			
		aJ_2	aJ_1	aJ_0	bA_1	bA_2				
4	7.30[c]	205	265		175	143				
		(41)	(1)		(26)	(20)				
1	9.72[d]	205	265		219	187	351, 89			
		(42)	(12)		(7)	(21)	(0.1), (18)			
2	8.65[d]	191	251		175	143	249, 133			
		(70)	(0.1)		(73)	(100)	(1) (26)			
3	11.98[d]	235	295		219	187	133, 177			
		(39)	—		(4)	(21)	(12) (5)			
Per-O-methylated diglycosyl-alditol					abJ_2	abJ_1	cA_1	cA_2	cbA_1	cbA_2
5	17.47[d]	205		251	409	469	175	143	379	347
		(21)		(3)	(0.2)	—	(62)	(100)	(2)	(0.1)
6	13.79[c]	205	265		409	469	219	187	423	391
		(97)	(47)		(2)	(2.4)	(41)	(100)	(1.7)	(0.9)
7	14.57[c]	205	265		409	469	219	187	423	391
		(100)	(47)		(8)	(0.1)	(26)	(95)	(0.9)	(0.1)
8	14.22[c]	191	251		351	411	175	143	335	303
		(33)	(8)		(0.1)	(5)	(57)	(83)	(0.2)	(0.3)
9	18.20[d]	235	295		439	499	219	187	423	391
		(35)	(0.4)		(10)	(1)	(55)	(68)	(11)	(0.1)
10	14.45[c]	205	265		365	425	175	143	335	303
		(24)	(5)		(1)	(0.1)	(100)	(77)	(9)	(6)
11	15.10[d]	205	265		365	425	189	157	349	303
		(39)	(2)		(2)	(1.6)	(23)	(9)	(17)	(1)

Per-O-methylated triglycosyl-alditol					abJ_2	abJ_1	$abcJ_2$	$abcJ_1$	dA_1	dA_2	dcA_1	dcA_2	$dcbA_1$	$dcbA_2$
12	21.31[c]	205		251	409	469	569	629	175	143	335	303	539	507
		(33)		(7)	(1)	(0.1)	—	(0.1)	(100)	—	(39)	(27)	(0.4)	(0.1)
13	21.87[c]	205	265		409	469	613	673	219	187	423	391	627	595
		(68)	(23)	(2)	(3)	(0.2)	—	(58)	(100)	(4)	—	—	—	
14	19.83[c]	205	265		409	469	613	673	189	157	393	361	597	565
		(40)	(17)		(0.6)	(1)	—	(0.5)	(19)	(5)	(6)	(0.2)	(0.8)	(0.1)
15	17.22[c]	191	251		351	411	511	571	175	143	335	303	495	463
		(33)	(8)		(0.1)	(5)	—	(0.3)	(57)	(83)	(0.2)	(0.3)	(1)	(0.7)
16	18.20[d]	235	295		439	499	639	699	219	187	423	391	627	595
		(35)	(0.4)		(10)	(1)	—	—	(55)	(68)	(11)	(0.1)	(0.4)	(0.1)

Per-O-methylated tetraglycosyl-alditol					abJ_2	abJ_1	$abcJ_2$	$abcJ_1$	eA_1	eA_2	edA_1	edA_2	$edcA_1$	$edcA_2$
17	14.45[c]	205		251	409	469	569	629	175	143	335	303	539	507
		(24)		(5)	(1)	(0.1)	(1)	(2)	(100)	(64)	(9)	(6)	(1)	—
18	24.39[c]	205	265		409	469	613	673	219	187	423	391	627	595
		(100)	(39)		(3)	(0.2)	(1)	—	(43)	(92)	(0.5)	(0.1)	(0.1)	(0.1)
19	20.43[c]	191	251		351	411	511	571	175	143	335	303	495	463
		(28)	(3)		—	(6)	(0.9)	—	(100)	(21)	(3)	(0.2)	(4)	—

[a]Retention time (min) on the fused silica DB-1 capillary column; g.l.c. conditions were as described in the Experimental section. [b]Figures in parentheses show peak intensity relative to base peak (100). [c]G.l.c. retention time on the DB-1 capillary column (15 m × 0.25 mm i.d.). [d]G.l.c. retention time on the DB-1 capillary column (30 m × 0.32 mm i.d.).

TABLE VI

^{1}H-N.M.R. CHEMICAL SHIFTS AND COUPLING CONSTANTS OF THE ANOMERIC PROTONS OF THE PER-*O*-METHYLATED OLIGOGLYCOSYLALDITOLS ISOLATED FROM RG-I

Per-O-methylated oligoglycosylalditol	*Chemical shift*	$J_{1,2}$ *(Hz)*	*Assignment*
1	4.28	7.06	β-D-Gal*p*
5	5.18	br. s	α-L-Ara*f*
	4.35	7.60	β-D-Gal*p*
6	4.61	6.93	β-D-Gal*p*
	4.33	6.93	β-D-Gal*p*
7	4.62	7.00	β-D-Gal*p*
	4.33	6.57	β-D-Gal*p*
12	5.32	br. s	α-L-Ara*f*
	5.16	br. s	α-L-Ara*f*
	4.31	7.92	β-D-Gal*p*
13	4.64	6.93	β-D-Gal*p*
	4.32	6.60	β-D-Gal*p*
	4.32	6.60	β-D-Gal*p*
14	5.47	2.45	α-L-Fuc*p*
	4.27	6.60	β-D-Gal*p*
	4.84	7.92	β-D-Gal*p*

linked to galactosyluronic acid residues in the RG-I backbone. The origin of the arabinosyl and galactosyl residues is not yet known; they could have been attached to galactosyluronic acid residues or to neutral glycosyl residues degraded by lithium. These per-*O*-methylated alditols probably represent a larger proportion of the products of lithium-degraded RG-I than is made evident by Fraction I, as a large proportion of these per-*O*-methylated alditols were probably lost during purification due to their volatility.

Fraction I also contained small amounts of monoglycosylalditols and disaccharide methyl glycosides **1**–**3**; these were present in greater amounts in Fraction II and therefore are described.

The structures of per-O-methylated oligoglycosylalditols in Fraction II. — Thermospray l.c.–m.s. analysis of Fraction II (see Fig. 4) showed that per-*O*-methylated monoglycosylalditol **1** was a major component, and that per-*O*-methylated mono- and di-glycosylalditols **2**–**4**, **7**–**9**, and **11**; (see Table III) were relatively minor components of the sample.

The thermospray mass spectrum of per-*O*-methylated monoglycosylalditol **1** established the presence of a hexosyl (that is, galactosyl) residue and a deoxyhexitol (that is, rhamnitol) residue by the pseudomolecular $(M + NH_4)^+$ ion at *m/z* 458.

TABLE VII

GLYCOSYL-LINKAGE COMPOSITION OF THE PER-*O*-METHYLATED OLIGOGLYCOSYLALDITOLS ISOLATED FROM RG-I

Per-O-methylated oligoglycosylalditol	*Glycosyl or alditol residue*	*Point of attachment of O-methyl substituents*	*Linkage deduced*
1	rhamnitol	1,2,3,5	pre-reduced 4-linked
	galactopyranosyl	2,3,4,6	terminal
5	rhamnitol	1,2,3,5	pre-reduced 4-linked
	galactopyranosyl	2,4,6	3-linked
	arabinofuranosyl	2,3,5	terminal
6	rhamnitol	1,2,3,5	pre-reduced 4-linked
	galactopyranosyl	2,3,6	4-linked
	galactopyranosyl	2,3,4,6	terminal
7	rhamnitol	1,2,3,5	pre-reduced 4-linked
	galactopyranosyl	2,3,4	6-linked
	galactopyranosyl	2,3,4,6	terminal
12	rhamnitol	1,2,3,5	pre-reduced 4-linked
	galactopyranosyl	2,4,6	3-linked
	arabinofuranosyl	3,5	2-linked
	arabinofuranosyl	2,3,5	terminal
13	rhamnitol	1,2,3,5	pre-reduced 4-linked
	galactopyranosyl	2,3,6	4-linked
	galactopyranosyl	2,3,4	6-linked
	galactopyranosyl	2,3,4,6	terminal
14	rhamnitol	1,2,3,5	pre-reduced 4-linked
	galactopyranosyl	2,3,6	4-linked
	galactopyranosyl	3,4,6	2-linked
	fucopyranosyl	2,3,4	terminal
17	rhamnitol	1,2,3,5	pre-reduced 4-linked
	galactopyranosyl	2,4,6	3-linked
	arabinofuranosyl	3,5	2-linked
	arabinofuranosyl	2,3	5-linked
	arabinofuranosyl	2,3,5	terminal

The aJ_2 + 18 fragment-ion at *m/z* 223 indicated that the deoxyhexitol residue was at the reducing end of the molecule; the bA_1 fragment ion at *m/z* 219 indicated that the hexosyl residue was the nonreducing terminus. The c.i. mass spectrum (obtained by d.l.i.-m.s.) of **1** confirmed the assignments made on the basis of the thermospray mass spectrum [$(M + H)^+$ at *m/z* 441, aJ_2 + 18 at *m/z* 223, and bA_1 at *m/z* 219]. The presence of **1** in the l.c. effluent, as indicated by the thermospray and d.l.i.-m.s. experiments, was confirmed by g.l.c.–e.i.-m.s. The g.l.c. retention time was consistent with that of a monoglycosylalditol. The e.i.-mass-spectral fragment-ions (aJ_1 fragment ion at *m/z* 265, aJ_2 fragment ion at *m/z* 205, bA_1

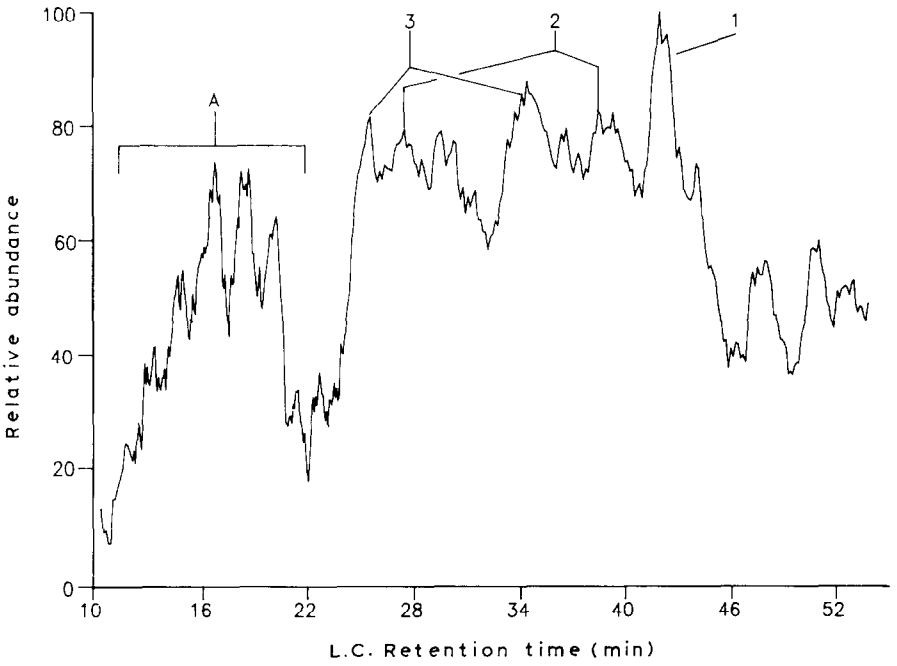


Fig. 3. Reversed-phase h.p.l.c.–m.s. elution profile of the per-*O*-methylated oligoglycosylalditols of Fraction I. The total ion chromatogram is plotted *vs.* the retention time. The numbers above the total ion chromatogram indicate the location of the individual oligoglycosylalditols listed in Table III. A is the location of several unresolved per-*O*-methylated alditols and methyl glycosides of rhamnose, galactose, and arabinose. The rise in the baseline at retention time ~25 min was due to an increase in the multiplier voltage.

fragment ion at *m/z* 219, and bA_2 fragment ion at *m/z* 187) confirmed the structure of **1**. The alditol series of fragment-ions at *m/z* 351 and 89 indicated that the alditol from **1** was a 4-linked deoxyhexitol residue.

The ^{1}H-n.m.r. spectrum of **1** contained a doublet with a coupling constant of 7 Hz at a chemical shift of 4.28 p.p.m., which corresponded to the β-anomeric proton of the galactopyranosyl residues. Glycosyl-linkage analysis of **1** confirmed the presence of a terminal galactopyranosyl residue (determined by the e.i.-mass spectrum of its partially *O*-methylated alditol acetate and by comparison of its retention time with that of the synthesized galactosyl derivative). The glycosyl-linkage analysis also confirmed the presence of a partially *O*-methylated 4-linked rhamnitol residue (determined by its e.i.-mass spectrum and by comparison of its retention time with that of the synthesized derivative). The structure of **1** is shown in Table III.

The complete structures of **2**–**4**, and **11** were not determined due to the small proportion of each in Fraction II. Their partial structures are shown in Table III. Per-*O*-methylated monoglycosylalditols having partial structures **2** and **3** were eluted at more than one retention time for each structure (21.7 min and 26.0 min

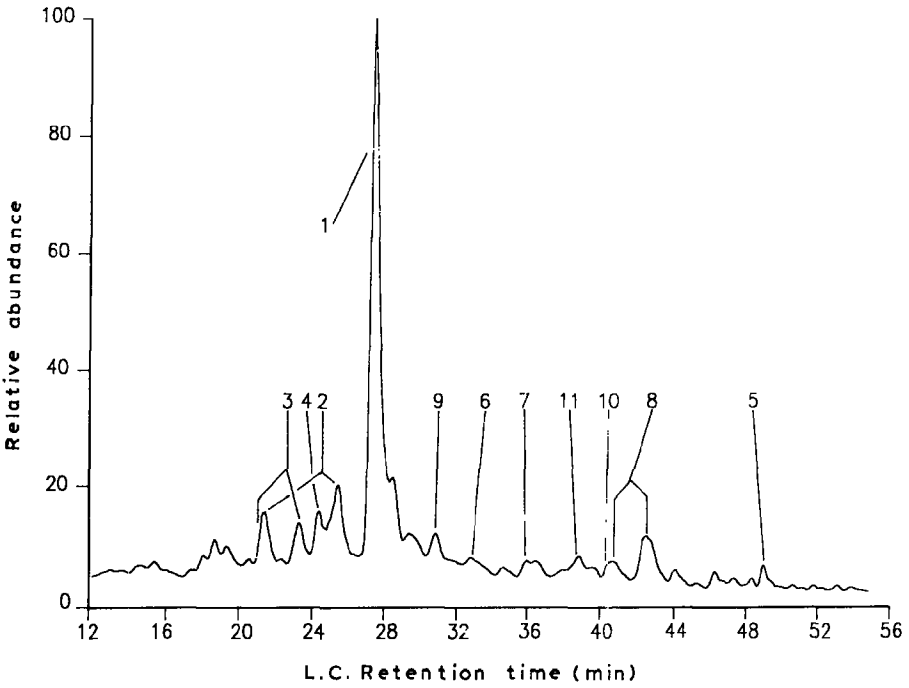


Fig. 4. Reversed-phase h.p.l.c.–m.s. elution profile of the per-*O*-methylated oligoglycosylalditols of Fraction II. The total ion chromatogram is plotted *vs.* the retention time. The numbers above the total ion chromatogram indicate the location of the individual oligoglycosylalditols listed in Table III.

for **2** and 21.5 min and 23.6 min for **3**). This is evidence for the existence of more than one per-*O*-methylated oligoglycosylalditol having the same partial structures as **2** and **3**, but having different anomeric configurations or linkage points, or both.

*The structures of per-*O*-methylated oligoglycosylalditols in Fraction III.* — Thermospray l.c.–m.s. analysis of Fraction III (see Fig. 5) established the presence of per-*O*-methylated diglycosyl-, triglycosyl-, and tetraglycosyl-alditols **5–9**, and **12–16** (see Table III).

The pseudomolecular $(M + NH_4)^+$ ion at *m/z* 618 in the thermospray mass spectrum of per-*O*-methylated diglycosylalditol **5** indicated that it was composed of a pentosyl (arabinosyl), a hexosyl (galactosyl), and a deoxyhexitol (rhamnitol) residue. The aJ_2 + 18 fragment-ion at *m/z* 223 indicated that rhamnitol was at the reducing end. The cA_1 fragment ion at *m/z* 175 established that an arabinosyl residue was the nonreducing-terminal residue. The c.i.-mass spectrum confirmed these assignments [pseudomolecular $(M + H)^+$ ion at *m/z* 601 and the same J- and A-series fragment-ions as in the thermospray mass spectrum]. In addition, the informative c.i. rearrangement ion at *m/z* 497 (see Table IV) confirmed the glycosyl sequence of **5** that had been derived from the J- and A-series of fragment ions.

G.l.c.–m.s. analysis of **5** helped to define its structure. The e.i.-mass spectrum contained a J_0 fragment ion at *m/z* 251 that indicated that the hexosyl residue

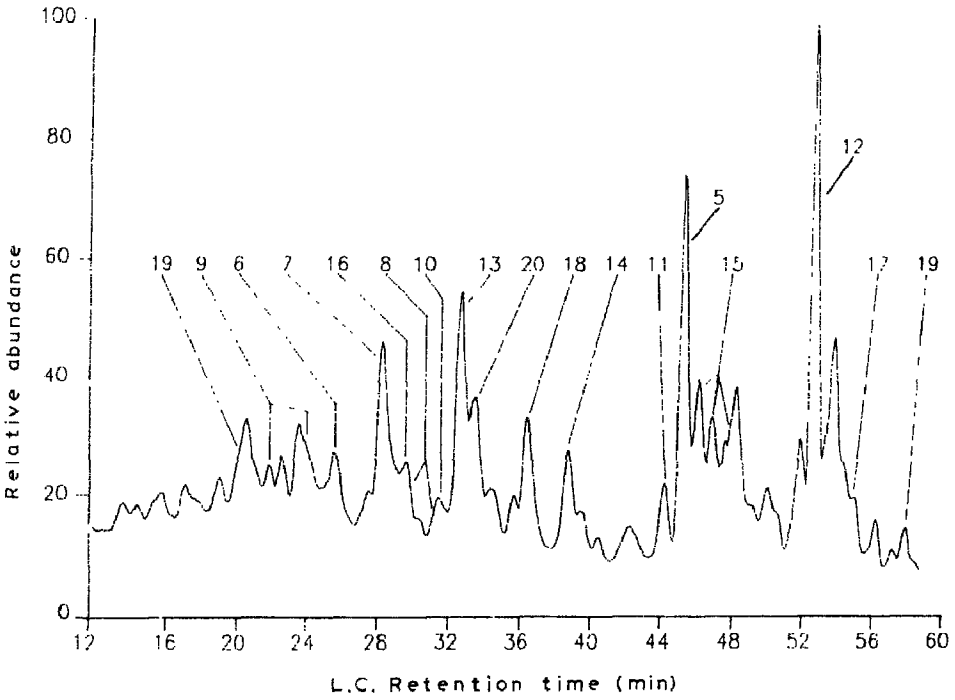


Fig. 5. Reversed-phase h.p.l.c.–m.s. elution profile of the per-*O*-methylated oligoglycosylalditols of Fraction III. The total ion chromatogram is plotted *vs.* the retention time. The numbers above the total ion chromatogram indicate the location of the individual oligoglycosylalditols listed in Table III.

attached to the deoxyhexitol residue was 3-linked. The ^{1}H-n.m.r. spectrum had a broad singlet at a chemical shift of 5.18 p.p.m., corresponding to an α-arabinofuranosyl residue and a doublet (coupling constant of 7.6 Hz) at a chemical shift of 4.33 p.p.m. corresponding to a β-galactopyranosyl residue (see Table VI). Glycosyl-linkage analysis of **5** confirmed that the terminal residue was an arabinofuranosyl residue, that the internal residue was a 3-linked galactopyranosyl residue, and that the reducing terminus was a 4-linked rhamnitol (see Table III).

Per-*O*-methylated diglycosylalditols **6** and **7** gave the same thermospray and c.i.-mass spectra. Both contained thermospray pseudomolecular $(M + NH_4)^+$ ions at *m/z* 662 and c.i. $(M + H)^+$ ions at *m/z* 645, which established that **6** and **7** were each composed of two galactosyl residues and one rhamnitol residue. The $aJ_2 + 18$ fragment-ion at *m/z* 223 and the cA_1 fragment-ion at *m/z* 219 in the mass spectra of **6** and **7** indicated that the deoxyhexosyl residues were at the reducing ends of the diglycosylalditols, and that the hexosyl residues were respectively terminal and internal.

Diglycosylalditols **6** and **7** were shown to be different molecules by their different elution volumes during liquid chromatography (see Table IV). G.l.c.–e.i.-m.s. analyses of **6** and **7** were similar but not identical. Differences were seen in the retention times of **6** and **7** and in the relative abundance of their abJ_2 fragment-ions

that lost methanol (see Table V). The e.i.-mass spectra of **6** and **7** confirmed the sequence of their glycosyl residues. The ^{1}H-n.m.r. spectrum of **6** had a pair of doublets at chemical shifts of 4.33 and 4.61 p.p.m., each with a coupling constant of 6.93 Hz; the doublets corresponded to two galactopyranosyl residues in the β-anomeric configuration. The ^{1}H-n.m.r. spectrum of **7** had a pair of doublets at chemical shifts of 4.33 and 4.62 p.p.m., with coupling constants of 6.57 and 7.0 Hz, respectively. These doublets corresponded to two galactopyranosyl residues in the β-anomeric configuration. The glycosyl-linkage compositions of **6** and **7** completed the structural analyses by establishing that **6** contained terminal and 6-linked galactopyranosyl residues and a 4-linked rhamnitol, whereas **7** contained terminal and 4-linked galactopyranosyl residues, and a 4-linked rhamnitol. The structures of **6** and **7** are presented in Table III.

Per-*O*-methylated oligoglycosylalditol **12** was shown by its thermospray-generated pseudomolecular $(M + NH_4)^+$ ion at *m/z* 778 to be a per-*O*-methylated triglycosylalditol composed of two arabinosyl residues, one galactosyl residue, and a rhamnitol residue. The glycosyl sequence of **12** was partially determined by the $aJ_2 + 18$ fragment ion at *m/z* 223, which established that a deoxyhexitol residue was at the reducing end of the molecule, and partially by the dA_1 fragment ion at *m/z* 175, which established that an arabinosyl was the terminal residue. The c.i.-mass spectrum established the rest of the glycosyl sequence by the c.i. rearrangement ions at *m/z* 557 and 397. These ions indicated that the remaining arabinosyl residue was attached to the galactosyl residue, which was attached to the rhamnitol.

G.l.c.–m.s. analysis indicated that **12** was eluted with a retention time consistent with that of a per-*O*-methylated triglycosylalditol. The e.i.-mass spectrum had an aJ_0 fragment-ion at *m/z* 251, indicative of a 3-linked galactosyl residue attached to the rhamnitol. The rest of the A and J series of fragment ions was consistent with the sequence derived from the c.i.-mass spectrum. The ^{1}H-n.m.r. spectrum of **12** had two broad singlets with chemical shifts of 5.16 and 5.32 p.p.m., respectively, which were assigned to two α-arabinofuranosyl residues; a doublet (coupling constant of 7.92 Hz) with a chemical shift of 4.31 p.p.m. was assigned to a β-galactopyranosyl residue. Glycosyl-linkage analysis of **12** confirmed its terminal arabinofuranosyl residue, and established that the other arabinofuranosyl residue was 2-linked, that the galactopyranosyl residue was 3-linked, and that the rhamnitol was 4-linked. The structure of **12** is shown in Table III.

Per-*O*-methylated triglycosylalditol **13** was shown to be composed of three hexosyl (galactosyl) and one "deoxyhexosyl" (rhamnitol) residues by the thermospray pseudomolecular $(M + NH_4)^+$ ion at *m/z* 866. The $aJ_2 + 18$ fragment ion at *m/z* 223 indicated that a deoxyhexitol residue was at the "reducing" end of the molecule, and the dA_1 fragment-ion at *m/z* 219 was diagnostic of a nonreducing terminal hexosyl group. Although the c.i.-mass spectrum did not have a pseudomolecular $(M + H)^+$ ion, it did have rearrangement ions at *m/z* 645 and 441 that confirmed the linear arrangement of the three galactosyl residues attached to the rhamnitol. The l.c. retention time of **13** was consistent with that of a per-*O*-

methylated triglycosylalditol. The e.i.-mass spectrum contained diagnostic A and J fragment-ions consistent with the linear sequence derived from the e.i.-mass spectrum. The ^{1}H-n.m.r. spectrum of **13** had two doublets in the anomeric-proton region. One doublet, which integrated for two protons, had a chemical shift of 4.32 p.p.m. and a coupling constant of 6.6 Hz. The other doublet, which integrated for one proton, had a chemical shift of 4.64 p.p.m. and a coupling constant of 6.93 Hz. These signals corresponded to three β-linked galactopyranosyl residues.

Glycosyl-linkage analysis established that **13** was composed of a terminal galactopyranosyl group, a 6-linked galactopyranosyl residue, a 4-linked galactopyranosyl residue, and a 4-linked rhamnitol residue. The order of the 4-linked and the 6-linked galactosyl residues in **13** was established by the method of Svensson *et al.*[17]. This technique takes advantage of the fact that the ratio of the abundances of J_2:J_2 − MeOH:J_2 − 2 (MeOH) for a 4-linked galactopyranosyl residue is different from that of a 6-linked galactopyranosyl residue. Per-*O*-methylated triglycosylalditol **13** was found to fragment with a relative abundance of abJ_2:abJ_2 − MeOH:abJ_2 − 2 (MeOH) similar to that of per-*O*-methylated diglycosylalditol **7** (a 4-linked galactopyranosyl residue), namely, 1:1.3:1 for **13** compared to 1:1.2:1 for **7**. The ratios of the abundances of the $abcJ_2$:$abcJ_2$ − MeOH:$abcJ_2$ − 2 (MeOH) in the spectrum of per-*O*-methylated triglycosylalditol **13** were similar to those of the abJ_2:abJ_2 − MeOH:abJ_2 − 2 (MeOH) of per-*O*-methylated diglycosylalditol **6**, namely, 1:0:0.01 in **13** (see Table VIII) compared to 1:0.05:0.01 in **6**. Thus, the residue attached to the rhamnitol of **13** is the 4-linked galactopyranosyl, and the 6-linked galactopyranosyl group is attached to the 4-linked galactosyl residue. The primary structure of **13** is shown in Table III.

Per-*O*-methylated oligoglycosylalditol **14** was shown to contain two

TABLE VIII

COMPARISON OF J_2, J_2 − MeOH, AND J_2 − 2 MeOH E.I.-M.S. FRAGMENT IONS FOR THE DETERMINATION OF THE SEQUENCE OF SELECTED PER-*O*-METHYLATED OLIGOGLYCOSYLALDITOLS

Per-O-methylated oligoglycosylalditol	J_2, J_2 − *MeOH* J_2 − *2 MeOH*	J_2:J_2 − *MeOH* J_2 − *2 MeOH ratio*	*Linkage determined*	*Linkage deduced*
7	409, 377, 345	1:1.2:1	4-linked β-D-Gal*p*[a]	
6	409, 377, 345	1:0.05:0.01	6-linked β-D-Gal*p*[a]	
13	409, 377, 345	1:1.3:1		4-linked β-D-Gal*p*[b]
	613, 581, 549	1:0:0.01		6-linked β-D-Gal*p*[b]
14	409, 377, 345	1:1.1:1		4-linked β-D-Gal*p*[b]

[a]The linkage points in per-*O*-methylated diglycosylalditols **6** and **7** were unambiguously determined by glycosyl-linkage analysis. [b]These residues were, from ^{1}H-n.m.r. analysis, known to be in the β-anomeric configuration and were later confirmed to be galactopyranosyl residues by g.l.c. retention times obtained during glycosyl-linkage analysis.

deoxyhexosyl residues and two hexosyl residues by the pseudomolecular $(M + NH_4)^+$ ion at *m/z* 836 in its thermospray mass spectrum. The thermospray $aJ_2 + 18$ fragment-ion at *m/z* 223 established that one of the "deoxyhexosyl" residues (rhamnitol) was the nonreducing terminus of the molecule, and the dA_1 fragment ion at *m/z* 189 established that the other deoxyhexosyl residue was at the other terminal end of the molecule. The pseudomolecular $(M + H)^+$ ion at *m/z* 819 (very weak) in the c.i.-mass spectrum confirmed the composition of per-*O*-methylated triglycosylalditol **14**; the c.i. rearrangement ion at *m/z* 615, the $aJ_2 + 18$ fragment-ion at *m/z* 223, and the dA_1 fragment-ion at *m/z* 189 confirmed the sequence derived from the thermospray mass spectrum. In liquid chromatography, **14** was eluted with a retention time consistent with that of a triglycosylalditol. The e.i.-mass spectrum A and J fragment-ions confirmed the glycosyl sequence of **14** (see Table V). The ^{1}H-n.m.r. spectrum of **14** had three anomeric-proton signals: a doublet with a chemical shift of 5.47 p.p.m. and a coupling constant of 2.45 Hz was assigned to an α-linked fucopyranosyl residue, and doublets with a chemical shift of 4.27 p.p.m. (coupling constant of 6.60 Hz) and 4.84 p.p.m. (coupling constant of 7.92 Hz) were assigned to β-linked galactopyranosyl residues.

Glycosyl-linkage analysis established that **14** was composed of a terminal fucopyranosyl group, a 2-linked galactopyranosyl residue, a 4-linked galactopyranosyl residue, and a 4-linked rhamnitol residue. The same technique that was used to establish the order of the 4- and 6-linked galactosyl residues of **13** was used to determine the sequence of the 2- and 4-linked galactosyl residues of **14**. The ratios of the abundances of the abJ_2:abJ_2 − MeOH:abJ_2 − 2 (MeOH) fragment-ions of **14** closely matched the ratios of the abundances of the abJ_2:abJ_2 − MeOH:abJ_2 − 2 (MeOH) fragment-ions of **7** (see Table V). This match indicated that the 4-linked galactosyl unit was attached to the 4-linked rhamnitol residue and that the 2-linked galactosyl residue must therefore have been attached to the 4-linked galactosyl residue.

Insufficient quantities of material prevented determination of the complete structures of per-*O*-methylated oligoglycosylalditols **8–10, 15, 16,** and **19**. Thermospray, c.i.- and e.i.-mass spectra, and g.l.c.–m.s. retention times permitted the sequence of the glycosyl residues of these oligoglycosylalditols to be determined (see Table III). Per-*O*-methylated diglycosylalditols **8** and **9**, triglycosylalditol **15**, and tetraglycosylalditol **19** were of particular interest. Per-*O*-methylated diglycosylalditols having the same glycosyl-residue sequence as **8** were eluted from the l.c. column at two different retention times, indicating the presence of two different di-*O*-arabinosylarabinitols having differently linked arabinosyl residues or with different anomeric configurations of their arabinosyl linkages, or both. Similarly, **9** represents two different di-*O*-galactosylgalactitols, **15** represents three different tri-*O*-arabinosylarabinitols, and **19** represents two different tetra-*O*-arabinosylarabinitols.

*The structures of per-*O*-methylated oligoglycosylalditols in Fraction IV.* — Thermospray l.c.–m.s. analysis of Bio-Gel P-2 Fraction IV (see Fig. 6) showed that

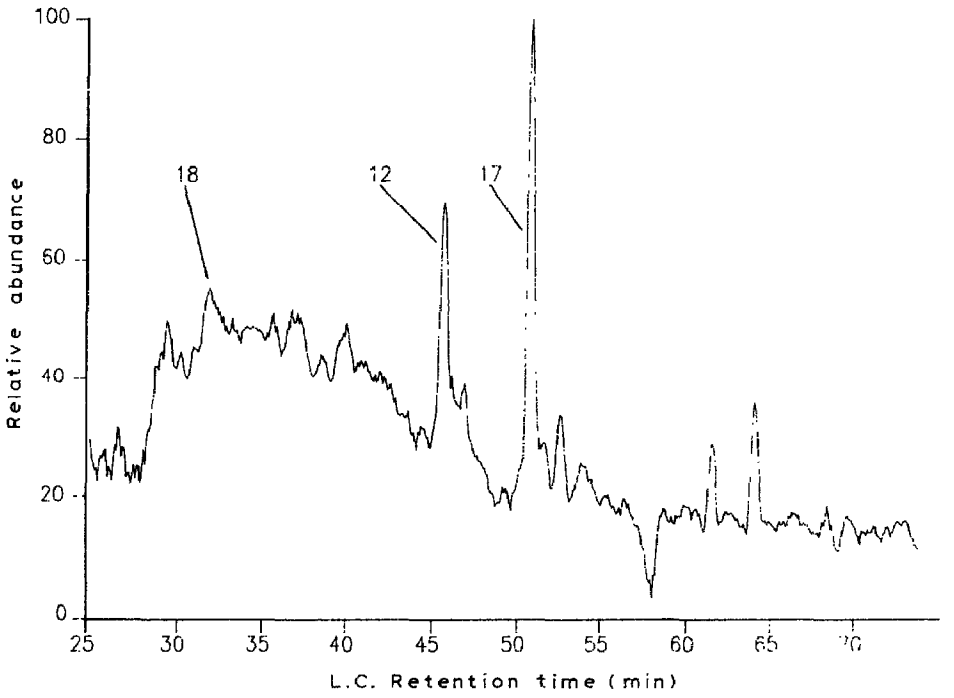


Fig. 6. Reversed-phase h.p.l.c.–m.s. elution profile of the per-*O*-methylated oligoglycosylalditols of Fraction IV. The total ion chromatogram is plotted *vs.* the retention time. The numbers above the total ion chromatogram indicate the location of the individual oligoglycosylalditols listed in Table III.

one per-*O*-methylated tri-*O*-glycosylalditol (**12**) and two per-*O*-methylated tetra-*O*-glycosylalditols (**17** and **18**) were present. The structure of per-*O*-methylated tri-*O*-glycosylalditol **12**, which was found in higher abundance in Bio-Gel P-2 Fraction III, has already been described.

The thermospray mass spectrum of **17** had a pseudomolecular $(M + NH_4)^+$ ion at *m/z* 938 that established the presence of three pentosyl (arabinosyl) residues, one hexosyl (galactosyl) residue, and one "deoxyhexosyl" (rhamnitol) residue. The aJ_2 + 18 fragment-ion at *m/z* 223 established that the rhamnitol residue was at the "reducing" end of the molecule. The eA_1 fragment-ion at *m/z* 175 and edA_1 fragment-ion at *m/z* 335 indicated that both the nonreducing terminal and the penultimate residues were arabinosyl residues. D.l.i.-m.s. failed to reveal a pseudomolecular $(M + H)^+$ ion or any c.i. rearrangement ions. C.i. fragment-ions (cA_1 at *m/z* 175 and cdA_1 at *m/z* 335) were present and were used, together with the l.c. retention time in the thermospray l.c.–m.s., to locate **17** in the l.c. effluent of the d.l.i. experiment. The l.c. gradients for the d.l.i. and thermospray experiments were identical. G.l.c.–m.s. analysis of the material diverted in the d.l.i. experiment showed that **17** had a g.l.c. retention time consistent with that of a per-*O*-methylated tetra-*O*-glycosylalditol. The c.i. mass spectrum of **17** had an aJ_2 fragment-ion at *m/z* 205, which was diagnostic of a deoxyhexitol (rhamnitol) at the "reducing"

end of the molecule. The aJ_0 fragment ion at *m/z* 251 was indicative of a 3-linked residue adjacent to the rhamnitol. The abJ_2 fragment ion at *m/z* 409 established that a hexosyl (galactosyl) residue was attached to the rhamnitol residue. The A fragment-ions (eA_1, edA_1, and $edcA_1$ at *m/z* 175, 335, and 495, respectively) defined the nonreducing end of the molecule as a linear sequence of arabinosyl residues. ^{1}H-N.m.r. spectroscopy failed to determine unambiguously the anomeric configuration of the glycosyl residues of **17**. Glycosyl-linkage analysis of **17** showed that it contained a terminal arabinofuranosyl residue, a 5-linked arabinofuranosyl (or 4-linked arabinopyranosyl) residue, a 2-linked arabinofuranosyl residue, a 3-linked galactopyranosyl residue, and a 4-linked rhamnitol residue (see Table III). Further attempts to define the structure of this per-*O*-methylated tetraglycosylalditol were unsuccessful due to lack of sufficient material.

Per-*O*-methylated tetraglycosylalditol **18** was present in small amounts in Fraction IV. The thermospray mass spectrum of **18** was weak. However, a pseudomolecular $(M + NH_4)^+$ ion at *m/z* 1070 was located by use of reconstructed selected-ion chromatograms. In this method, the computer searched for the ion of interest among all the scans recorded during the thermospray l.c.–m.s. experiment. A computer program written by William York and modified by Scott Doubet, both of this laboratory, automatically calculates the pseudomolecular ions of all possible per-*O*-alkylated oligoglycosylalditols, *i.e.*, all the pseudomolecular ions possible for per-*O*-methylated oligoglycosylalditols containing a specified number of glycosyl residues, the possible composition of which was determined by the glycosyl residues known to be present in RG-I. All such pseudomolecular ions in the mass spectra of the l.c.–m.s. experiments were routinely searched for by forming reconstructed ion chromatograms. Such a search located **18**.

The g.l.c. retention of **18** was consistent with that of a per-*O*-methylated tetraglycosylalditol. The glycosyl-residue sequence of **18** was determined by g.l.c.–m.s. analysis: the e.i.-m.s. J fragment ions (at *m/z* 205, 409, and 613, respectively) and A fragment-ions (at *m/z* 219 and 423, respectively; see Table V) established that **18** was composed of four linear galactosyl residues attached to a rhamnitol residue (see Table III). Further attempts to elucidate the structure of **18** were unsuccessful due to lack of sufficient material.

F.a.b-m.s. of the oligoglycosylalditol products of lithium-treated RG-I. — The partially separated oligoglycosylalditols in Bio-Gel P-2 Fractions I–IV were examined by f.a.b.-m.s. The per-*O*-methylated derivatives of the oligoglycosylalditols were more successfully analyzed by f.a.b.-m.s. than were the corresponding per-*O*-acetylated and underivatized oligoglycosylalditols. In particular, the f.a.b.-mass spectrum of per-*O*-methylated oligoglycosylalditols doped with ammonium acetate gave more intense pseudomolecular $(M + NH_4)^+$ and $(M + H)^+$ ions.

F.a.b.-m.s. analysis of the per-*O*-methylated oligoglycosylalditols in Fractions III and IV confirmed the presence of the oligoglycosylalditols **5–7, 9–14, 17,** and **18**, whose structures had been determined by the methods already described.

Due to the greater sensitivity of f.a.b.-m.s. (relative to the other analytical

TABLE IX

FAST ATOM BOMBARDMENT-MASS SPECTROMETRY OF PER-*O*-METHYLATED OLIGOGLYCOSYL ALDITOLS DERIVED FROM LITHIUM-TREATED RG-I

m/z [$(M + NH_4)^+$]	*Composition consistent with pseudomolecular ion*
574	$(pent)_2$ deoxyhex[a]
618	pent hex deoxyhex[a]
662	$(hex)_2$ deoxyhex[a]
692	$(hex)_3$[a]
778	$(pent)_2$ hex deoxyhex[a]
836	$(hex)_2$ $(deoxyhex)_2$[a]
866	$(hex)_3$ deoxyhex[a]
938	$(pent)_3$ hex deoxyhex[a]
1040	$(hex)_3$ $(deoxyhex)_2$[b]
1070	$(hex)_4$ deoxyhex[a]
1100	$(hex)_5$[b]
1244	$(hex)_4$ $(deoxyhex)_2$[b]
1274	$(hex)_5$ deoxyhex[b]
1304	$(hex)_6$[b]
1448	$(hex)_5$ $(deoxyhex)_2$[b], or $(pent)_6$ $(hex)_2$[b]
1464	pent $(hex)_6$[b]
1478	$(hex)_6$ deoxyhex[b]
1652	$(hex)_6$ $(deoxyhex)_2$[b], or $(pent)_6$ $(hex)_3$[b]
2264	deoxyhex $(hex)_9$, $(deoxyhex)_2$[b], or $(pent)_6$ $(hex)_6$[b]
2308	$(pent)_5$ $(hex)_7$[b]
2672	$(hex)_{11}$ $(deoxyhex)_2$[b], or $(pent)_6$ $(hex)_8$[b]
2846	$(hex)_{11}$ $(deoxyhex)_3$[b], or $(pent)_6$ $(hex)_8$ deoxyhex[b]
3080	$(hex)_{13}$ $(deoxyhex)_2$[b]

[a]The ion observed is consistent with a per-*O*-methylated oligoglycosylalditol. However, f.a.b.-m.s. offers little information on the identity of the reducing-end residue. In this case, the per-*O*-methylated oligoglycosylalditol had already been structurally characterized and the sequence is as shown. [b]The sequence of the per-*O*-methylated oligoglycosylalditol is not known. However, the glycosyl-composition analysis shows that, if a deoxyhexosyl residue is present, it is likely to be located at the reducing end.

techniques used) for detecting larger per-*O*-methylated oligoglycosylalditols, pseudomolecular ions for a variety of other per-*O*-methylated oligoglycosylalditols that had not previously been detected were observed (see Table IX). The types of structures observed with the oligoglycosylalditols of lower d.p. continued and were elaborated on at higher d.p. For example, per-*O*-methylated oligoglycosylalditols similar to **1, 6, 7, 13,** and **18**, having structures defined by the sequence $(hexosyl)_y$deoxyhexitol, were detected, where y was 5–7. Oligoglycosylalditols similar to **14**, defined by the structure deoxyhexosyl$_2$$(hexosyl)_x$deoxyhexitol, were also detected, where x was 3–6 and 10–13 (see Table IX). These larger oligoglycosylalditols may be larger homologs of the side chains already characterized.

F.a.b.-m.s. analysis also allowed the discovery of other oligoglycosylalditols that did not appear to be larger homologs of the side chains that had already been structurally characterized. In fact, as the d.p. increased, the number of structurally distinct side-chains appeared to increase (see Table IX). Insufficient quantities of the larger side-chain compounds prevented further structural characterization.

GENERAL DISCUSSION

The galactosyluronic acid residues of the pectic polysaccharide RG-I were selectively cleaved by dissolving RG-I in ethylenediamine and subjecting it to treatment with lithium metal for 1 h. The products of this cleavage were shown, by glycosyl-composition analysis, to contain only the neutral glycosyl residues known to be part of intact RG-I. The L-rhamnopyranosyl residues of the RG-I backbone were recovered as pre-reduced alditols, or reducing aldoses, which confirmed that they were the points of attachment of the majority[4] of the D-galactosyluronic acid residues in intact RG-I. This finding supports previous studies[4] in which alternating L-rhamnosyl and D-galactosyluronic acid residues were found to constitute the backbone of RG-I.

Of the non-rhamnosyl neutral glycosyl residues, ~10% (see Table I) were found, by glycosyl-composition analysis, to have been reduced at C-1 with hydrogen. It is likely that the reduction of these residues occurred during lithium treatment or immediately afterwards (with sodium borohydride). Even though the number of the neutral residues cleaved was relatively low, their presence indicated that neutral residues, in addition to rhamnosyl residues, were glycosidically linked to galactosyluronic acid residues, or else that lithium degradation (or the procedure for the isolation of products) was not restricted to the cleavage of galactosyluronic acid residues. Indeed, some galactosyluronic acid residues may be present in the RG-I side-chains, as had been proposed[3]. Side-chain galactosyluronic acid could account for the release of some of the arabinitol and galactitol, although some of these residues may have been branches on galactosyluronic acid residues of the backbone. If galactosyluronic acid residues are present in side chains, some of the side chains characterized herein are likely to represent only those parts of the side chains attached to galactosyluronic acid residues, or located on the reducing side of galactosyluronic acid residues.

Lithium treatment of RG-I produces a large distribution of molecule sizes. All of the more-abundant side-chains were attached to O-4 of a rhamnosyl residue, which provides additional evidence[3,4,18] that the side chains of RG-I are attached to O-4 of about half of the rhamnosyl residues of the →2)-α-L-Rha-(1→4)-α-D-GalA-(1→ repeating unit of the backbone.

Despite the wide variety and structural complexity of the side chains present in RG-I, the side chains are not random structures. There is, of course, an enormous number of possible side-chain structures that were not found. Furthermore, the oligoglycosylalditols recovered after lithium treatment of RG-I have been obtained

TABLE X

POSSIBLE RG-I SIDE-CHAIN FAMILIES[a]

2	Ara-(1→5)-Araol	**4**	Ara-(1→4)-Rhaol	**3**	Gal→Galol
8	Ara→Ara→Araol	**10**	Ara→Ara→Rahol	**9**	Gal→gal→Galol
15	Ara→Ara→Ara→Araol	**11**	Fuc→Ara→Rhaol	**16**	Gal→Gal→Gal→Galol
19	Ara→Ara→Ara→Ara→Araol				

1	β-D-Gal*p*-(1→4)-L-Rhaol	**1**	β-D-Gal*p*-(1→4)-L-Rhaol
6	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-L-Rhaol	**7**	β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol
		14	α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol

1	β-D-Gal*p*-(1→4)-L-Rhaol	**1**	β-D-Gal*p*-(1→4)-L-Rhaol
5	α-L-Ara*f*-(1→3)-β-D-Gal*p*-(1→4)-L-Rhaol	**7**	β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol
12	α-L-Ara*f*-(1→2)-α-L-Ara*f*-(1→3)-β-D-Gal*p*-(1→4)-L-Rhaol	**13**	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol
17	L-Ara*f*-(1→5)-L-Ara*f*-(1→2)-L-Ara*f*-(1→3)-D-Gal*p*-(1→4)-L-Rhaol	**18**	Gal→Gal→Gal→Gal→Rhaol

[a]The suggested families of side chains consist of those side chains that were characterized during the analysis procedures and whose structures are given in Table III.

in a reproducible manner from different preparations of RG-I and by different lithium treatments (data not presented). The glycosyl-linkage compositions of RG-I from the cell walls of different plants are also similar[19–22], and are consistent with the side-chain structures described herein.

The reproducibility of the lithium treatment described allowed us to create a procedure for obtaining a more definitive "fingerprint" of RG-I than is provided by glycosyl-linkage analysis, the procedure that has most commonly been used to identify the presence of RG-I in a sample. The lithium treatment was used recently[22a] to demonstrate that RG-I is present in the walls of suspension-cultured maize cells. In samples wherein a polysaccharide having a glycosyl-linkage composition consistent with that of RG-I was present, an arabinogalactan for example[23], great effort would be required for researchers to separate and identify the contaminating polysaccharides. Lithium treatment of such samples followed by methylation and g.l.c.–m.s. or l.c.–m.s. analysis of the resulting per-*O*-methylated oligoglycosylalditols can demonstrate the presence of per-*O*-methylated oligoglycosylalditols characteristically derived from RG-I.

The reproducible complexity of the side chains of RG-I raises questions about the biosynthesis of RG-I. The structures of the neutral side-chains of RG-I appear to exist as complex "families" (see Table X). For example, starting with oligoglycosylalditol **1**, attachment of a single glycosyl residue to the terminal galactosyl residue of **1** would yield oligoglycosylalditol **5, 6,** or **7**. The addition of arabinosyl residues to the proper positions of **5** will yield oligoglycosylalditols **12** and **17**. By the same process, oligoglycosylalditol **7** can be converted into **13** by the addition of a galactosyl residue, and the further addition of a fucosyl residue will yield **14**. Almost all of the relatively abundant side-chains found in RG-I are related in this way.

It is possible that individual molecules of RG-I contain only the members of a single family of side chains. It is also possible that the different side chains are not randomly arranged on a single RG-I molecule.

Recent evidence[24,25] indicates that oligosaccharide fragments of the polysaccharides of cell walls play important roles in the regulation of physiological processes in plants. Although the reasons for the diversity of the RG-I side chains are not yet known, it is possible that the different side-chains of RG-I, when enzymically released from RG-I, function as regulatory molecules in plant tissues.

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