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STRUCTURAL INVESTIGATIONS ON THE LIPOPOLYSACCHARIDE ISOLATED FROM *Vibrio cholera* OGAWA G-2102

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ABSTRACT

A pure lipopolysaccharide was isolated from *Vibrio cholera* Ogawa G-2102. After removal of the lipid, the polysaccharide (PS) could be resolved on a column of Sephadex G-75 into antigenic PS and the core PS. Results of the structural investigations on these components are discussed.

INTRODUCTION

A structural study of a polymeric material in the lipopolysaccharide (LPS) of *Vibrio cholera* has been reported[1], but its proportion in the LPS was not carefully determined. Redmond[2] estimated the proportion of this polymeric material in LPS to be only 14%. He and co-workers[3] found that, when LPS from *V. cholera* was heated with 1% acetic acid, the immunological activity of the LPS gradually diminished. Several other groups[4–10] also studied the LPS of *V. cholera* in a preliminary way. In our earlier communications[11,12], we reported the results of detailed, structural studies on the O-antigen of *Vibrio cholera*, Inaba 569B. Because O-antigens are present in the endotoxic lipopolysaccharides located in bacterial cell-walls, and as easy interconversions of serotypes of *Vibrio cholerae* (Inaba to Ogawa, and *vice versa*) are possible, it was of interest to study the structure of the O-antigen present in the LPS of a strain of *V. cholera* serotype Ogawa. We now report the results of our investigation of the O-antigen of *V. cholera* Ogawa G-2102.

RESULTS AND DISCUSSION

Cells of *Vibrio cholera* Ogawa G-2102 were obtained from an 18-h growth of the strain on nutrient agar in roll bottles, and were then digested with 1:1 (w/w) phenol–water for 45 min at 65°. The LPS was liberated and was present in the

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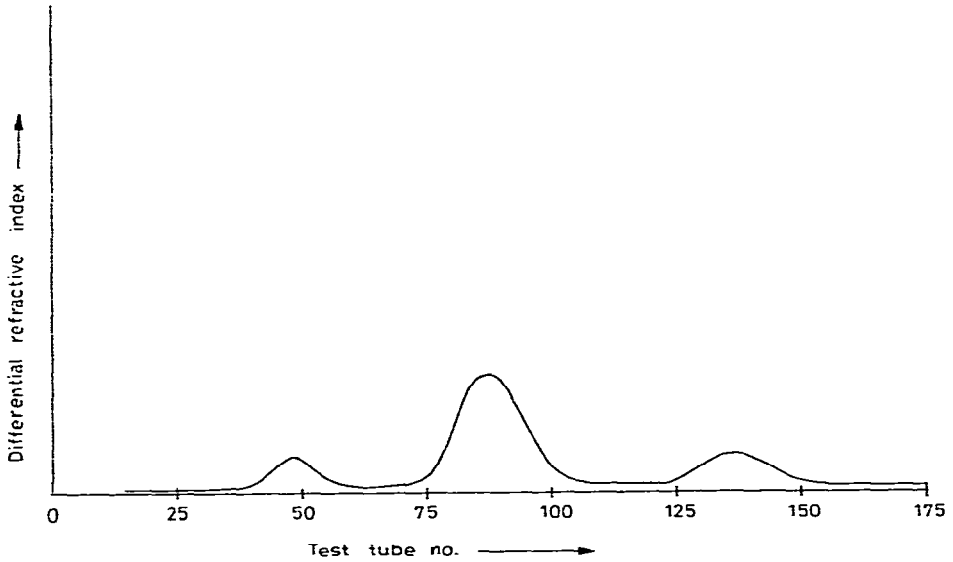

Fig. 1. Fractionation of the products obtained after delipidification of the LPS, using a column (1 m × 3 cm) of Sephadex G-75.

aqueous layer of this digest; it was fractionated with cetyltrimethylammonium bromide, and isolated by the procedure of Pant and Shrivastava[13]. The protein- and nucleic acid-free LPS had $[\alpha]_{589.5}^{23}$ +53° (Me_2SO). The LPS was further purified by dissolving it in 0.25M sodium chloride solution, removing the fine, insoluble matter by centrifugation, and precipitating the LPS from the supernatant liquor with ethanol (3 vol.). A solution of the LPS in distilled water was dialyzed against distilled water, and then lyophilized. The product had $[\alpha]_{589.5}^{23}$ +52° (Me_2SO). A small peak at 1735–1730 cm^{-1} in the i.r. spectrum (KBr) indicated the presence of *O*-acyl groups in the LPS. The LPS was found to be homogeneous; it gave a single precipitin line in the Ouchterlony gel-diffusion test[14,15] with antisera raised against the whole organism in rabbits, and showed a single spot, 2 cm from the starting line, towards the cathode.

The polysaccharide and lipid moieties of LPS were cleaved[16] by heating it (100 mg) in 1% acetic acid for 3 h at 105°. The solution was washed with chloroform to remove the lipid, and the aqueous part, containing the degraded polysaccharide (DPS), was centrifuged, and lyophilized. The DPS (60 mg) was resolved by gel filtration on G-75, with elution with pyridine acetate buffer, pH ~5.75, the eluate being monitored with a differential refractometer; a small peak corresponding to 3 mg appeared at the void volume, and the rest of the material was obtained in two major fractions, *viz.*, the O-antigenic polysaccharide (OPS, 32 mg) and the core polysaccharide (CPS, 14.5 mg); see Fig. 1. Both the OPS, $[\alpha]_{589.5}^{23}$ +13° (water), and the CPS, $[\alpha]_{589.5}^{23}$ −8.6° (Me_2SO), gave a single spot when electrophoresed in

TABLE I

RESULTS OF ESTIMATION OF SUGARS IN DIFFERENT FRACTIONS OF THE LIPOPOLYSACCHARIDE OF *V. cholera* OGAWA G-2102

Sugars detected[a]	*Hydrolysis products*[b] (%)		
	Pure LPS	*OPS*	*CPS*
Glucose	2.9	4.25	7.35
Mannose	0.4	1.7	0.20
Galactose	0.25	1.4	0.36
Major heptose[c]	2.2	3.68	6.98
Minor heptose[d]	0.5	1.75	0.50
2-Amino-2-deoxyglucose	4.5	8.5	—
Glucuronic[e]	6.5	13.5	14
acid[f]	5.5	12	12.1

[a]Estimated by g.l.c. as alditol acetates. [b]Hydrolyzed with 0.5M H_2SO_4 for 24 h with *myo*-inositol as internal standard. [c]Behaves like L-*glycero*-D-*gluco*-heptose. [d]Behaves like L-*glycero*-D-*manno*-heptose. [e]Estimated by the carbazole–sulfuric acid method. [f]Estimated, by g.l.c., from the difference in percentage of glucose in the hydrolyzate of carboxyl-reduced LPS and of pure LPS.

different buffers (see the Experimental section), but only the OPS gave a single precipitin line in the Ouchterlony gel-diffusion test.

The LPS, OPS, and CPS were separately hydrolyzed (with *myo*-inositol as the internal standard), and, after the usual treatment, the sugars were analyzed by paper chromatography (p.c.), g.l.c., and g.l.c.–m.s. Table I shows the amounts of the different sugars present in these fractions. Glucuronic acid was detected by p.c., and by obtaining a comparatively larger quantity of glucose from carboxyl-reduced (CR-LPS), CR-OPS, and CR-CPS. It was estimated both by the carbazole–sulfuric acid test[17], and by estimating the difference in glucose content in the polysaccharide fractions and the corresponding, carboxyl-reduced fractions[18]. Hexosamine was detected, and estimated (using the same internal standard) with column *c*. A trace of fructose[19] was also detected on hydrolyzing the LPS with 0.2M acetic acid and analyzing the hydrolyzate by paper electrophoresis in borate buffer using other monosaccharides as standard. Analysis by paper electrophoresis was essential, as (*a*) p.c. using a number of solvents failed to resolve fructose from glucose and mannose, and (*b*) clear resolution by g.l.c. as the trimethylsilyl derivatives, or as the alditol acetates, was not possible in the presence of glucose and mannose. In paper electrophoresis, a spot corresponding to fructose (R_G 0.9) was obtained (mannose has R_G 0.41). The hexosamine was identified, by g.l.c. in column *c*, as 2-amino-2-deoxyglucose (using *myo*-inositol as the internal standard). The amino sugars in LPS and OPS were also estimated by using the Ehrlich reagent[20]. The CPS contained no 2-amino-2-deoxyglucose. The major heptose component was detected by p.c. in solvent *D*, g.l.c., and g.l.c.–m.s., and was found to be identical in behavior with L-*glycero*-D-*gluco*-heptose, both by p.c. in solvent *D* and by g.l.c. in column *a* (R_T 2.30)

TABLE II

METHYL ETHERS OF SUGARS FROM THE HYDROLYZATES OF METHYLATED LPS (A), METHYLATED CR-LPS (B), METHYLATED OPS (C), AND METHYLATED CR-OPS (D)

Sugars[a]	R_T[b]	*Approximate mole proportion*				*Mode of linkage*
		A	*B*	*C*	*D*	
2,3,4,6-Glc	1	4	4	6	6	Glc*p*-(1→
2,3,4-Tri-*O*-methylGlc	2.25	3	2.8	4.5	4.7	→6)-Glc*p*-(1→
2,3,6-Tri-*O*-methylGlc	2.50		11.8		18.7	→4)-Glc*p*-(1→
2,3,4,6,7-Penta-*O*-methylheptose	2.00	2.0	2.0	3.2	3.1	Hep*p*-(1→
2,4,4,7-Tetra-*O*-methylheptose	2.30	2.5	2.5	4.7	4.7	→6)-Hep*p*-(1→
2,3-Di-*O*-methylheptose	3.25	2.0	2.0	2.9	2.9	→4,6,7)-Hep*p*-(1→
2,6-Di-*O*-methylGlc	3.50	1.0	1.0	1.7	1.6	→3,4)-Glc*p*-(1→
3,6-Di-*O*-methylGal	4.48	0.6	0.6	1.0	1.0	→2,4)-Gal*p*-(1→
3,6-Di-*O*-methyl(2-*N*-Me)GlcNAc	1.65	9.6	9.5	13.2	13.2	→4)-Glc*p*NAc-(1→

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. [b]Retention times of the corresponding alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in a column of SP-2330 at 165°.

using different, authentic heptoses. A minor heptose, identified by the same procedure, was found to be identical with L-*glycero*-D-*manno*-heptose (R_T 2.15).

Samples of LPS, OPS, CR-LPS, and CR-OPS were separately permethylated, first by the Hakomori method[21], and then by two methylations by the Kuhn procedure[22a,b]. The products showed no hydroxyl absorption bands in their i.r. spectra. They were formylated with 85% formic acid, and the products hydrolyzed with 0.5M sulfuric acid. After the usual treatment, the partially methylated, amino sugar fraction was separated from the partially methylated, neutral (and acidic) sugars with a cation-exchange resin. After being dried, all of these fractions were converted into their alditol acetates[23,24], and these were analyzed by g.l.c., and some of the fractions, by g.l.c.–m.s. The results, given in Table II, showed that all of the nonreducing end units are either glucosyl or heptosyl groups, as all of the hydrolyzates contained 2,3,4,6-tetra-*O*-methylglucose and 2,3,4,6,7-penta-*O*-methylheptose. 2,3,6-Tri-*O*-methylglucose appeared only in the hydrolyzates of the carboxyl-reduced fractions, indicating that the glucuronic acid residues in the polymer are present in the interior part of the molecules and that they are linked through O-1 and O-4. The 2-amino-2-deoxyglucose residues are also present as (1→4)-linked sugars, as the basic fractions contained only 2-deoxy-3,6-di-*O*-methyl-2-(methylamino)glucose. The interior part also contained (1→6)-linked glucose and heptose residues, and other branched residues of these two sugars. The branch points are at O-1, O-3, and O-4 of glucose, and at O-1, O-4, O-6, and O-7 of the heptose residues.

The LPS was subjected to Smith degradation[25], after oxidation with sodium metaperiodate. The product was treated as usual, and analyzed as the alditol acetates by g.l.c. The chromatogram showed spots corresponding to glycerol, threitol, ery-

TABLE III

OXIDATION OF PERACETYLATED OPS, AND PERACETYLATED, CARBOXYL-REDUCED OPS WITH CHROMIUM TRIOXIDE

Material[a] *from*	*Time of oxidation (h)*	*Glucose*	*Heptose*		*2-Amino-2-deoxy-glucose*	*Galactose*	*Mannose*	myo-*Inositol*
			Major	*Minor*				
Acetylated CPS	0	6.8	5.0	2.1	12.0	1.7	1.5	20
	1	5.9	0.5	1.9	11.4	0	1.0	20
	2	4.5	0	1.8	10.7	0	0.8	20
Acetylated CR-OPS	0	17.5	6.1	2.9	12.6	1.9	1.6	20
	1	9.0	0	2.5	11.3	0	1.2	20
	2	6.5	0	1.9	10.8	0	0.7	20

[a]Alditol acetates obtained from peracetylated OPS and peracetylated CR-OPS.

thritol, and small amounts of glucose and mannose, as expected from the methylation data. The molar ratios of the glycerol, threitol, and erythritol obtained after Smith degradation were 3:0.8:2.9. Heptose residues linked (1→6) and (1→4)-linked 2-amino-2-deoxyglucose residues would be expected to liberate threitol and erythritol, respectively, and, from most of the remaining units, glycerol should result; only the 2,6-di-*O*-methylglucose units survived the oxidation. L-Lyxose, expected to be liberated from the residue corresponding to 2,3-di-*O*-methyl-L-*glycero*-D-*gluco*- or -L-*glycero*-D-*manno*-heptose could not, however, be identified in the chromatogram.

The low, positive rotation of the LPS, OPS, and CPS indicated the presence of both the α- and β-anomeric configuration of different sugar residues, and this was confirmed by chromium(VI) trioxide oxidation[26] of the OPS at 50°. The results, given in Table III, showed that the glucose and 2-amino-2-deoxyglucose residues have the α configuration, whereas the heptose residues have the β configuration, as they were oxidized rapidly. The results of oxidation of the CR-OPS showed that the increased proportion of glucose (due to reduction of glucuronic acid) is vulnerable to chromium trioxide oxidation, indicating that the glucuronic acid residues also have the β configuration.

EXPERIMENTAL

General methods. — All evaporations were conducted at 40° (bath temperature) under diminished pressure. Aqueous solutions of small volumes were lyophilized. A differential refractometer (Water Associate model R-403) was used for monitoring eluates. Optical rotations were measured at 23 ±1° and 589.5 nm with a Perkin–Elmer model 241 MC spectrophotometer for solutions in water and Me_2SO. Ultraviolet and visible spectra were recorded with a Yanaco SP-1 spectrophotometer. Infrared spectra were recorded with a Beckman I.R.-20A instrument for KBr discs.

A Shandon, high-voltage electrophoresis apparatus, model L-24, was used for electrophoresis of materials in different buffers. Paper chromatography was performed by the descending technique, using Whatman No. 1 and No. 3 paper, with the following solvent-systems (v/v): (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) the upper layer of 4:1:5 1-butanol–acetic acid–water, (*C*) 5:5:1:3 ethyl acetate–pyridine–acetic acid–water, and (*D*) 6:4:3 1-butanol–ethanol–water. The spray reagents used were (*1*) alkaline silver nitrate, (*2*) 4% pentaerythritol[27] in 0.5M sodium hydroxide solution and silver nitrate solution in acetone, and (*3*) 2% ninhydrin in acetone.

For g.l.c., a Hewlett–Packard 5730A gas chromatograph with flame-ionization detector was used. Resolutions were performed in glass columns (1.83 m × 6 mm) containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100–200 mesh) at 190° (for alditol acetates of neutral sugars), (*b*) 1% of OV-225 on Gas Chrom Q (80–100 mesh) at 175° (for alditol acetates of partially methylated sugars), (*c*) 3% of Poly A-103 on Gas Chrom Q (100–120 mesh) at 180° (for alditol acetates of amino sugars), and (*d*) an SP-2330 column was used for g.l.c.–m.s. (in Prof. G. O. Aspinall's laboratory, York University, Canada). For quantitative evaluation of gas–liquid chromatograms, a Hewlett–Packard 3370B integrator was used.

Isolation of lipopolysaccharide. — Eighteen-h growths of the strain *V. cholera* Ogawa G-2102 on nutrient agar in roll bottles at 37° were harvested in 0.85% cold saline, and the suspension was filtered through muslin. The filtrate was passed through a Sharples centrifuge, and the sediment was suspended in cold saline and recentrifuged. The washed cells were treated with 1:1 (w/w) water–phenol for 45 min on a water bath at 65°, with occasional stirring. This material was kept in a refrigerator (5°). After centrifugation, the material in the aqueous layer was precipitated with 96% ethanol (1.5–2.0 vol.). The suspension was then centrifuged, and the precipitate was dissolved in water and fractionated with cetyltrimethylammonium bromide, pH ~8, and further purified as described by Pant and Shrivastava[13]. The neutral LPS fraction, $[\alpha]^{23}_{589.5}$ +53° (*c* 0.56, Me_2SO), was used in all of the experiments. The LPS preparations were confirmed to be free from protein and nucleic acid, respectively, and the degraded LPS, by the biuret test, the ultraviolet absorption at ~260 nm, and the Ouchterlony gel-diffusion test.

Purification of the lipopolysaccharide. — The crude LPS (350 mg) was purified by dissolving it in 0.25M sodium chloride solution, removing the fine, suspended matter by centrifuging, and precipitating the LPS with ethanol (3 vol.). The LPS was collected by centrifugation, dissolved in water, and the solution dialyzed to remove salts, and lyophilized; yield 90%, $[\alpha]^{23}_{589.5}$ +52° (*c* 0.56, Me_2SO).

Removal of lipid. — A mixture of the LPS (100 mg) with 1% acetic acid (7 mL) was heated[16] for 3 h at 105°. The polysaccharide was separated from the lipid by partitioning between water and chloroform (three times). The aqueous part, containing the degraded polysaccharide (DPS), was centrifuged at low speed, and the supernatant liquor was lyophilized; yield, 60 mg. The DPS was dissolved in water (5 mL), and was resolved by passing the solution through a column of Sephadex G-75

and eluting with pyridine acetate buffer, pH ~5.75. The void volume contained a small peak (3 mg). Two major peaks containing the O-antigenic and the core polysaccharide appeared later (see Fig. 1). These fractions were collected, dialyzed against distilled water, concentrated to a small volume, and lyophilized. The O-antigenic polysaccharide (OPS), $[\alpha]^{23}_{589.5}$ +13° (*c* 0.54, water), weighed 32 mg, and the core polysaccharide (CPS), $[\alpha]^{23}_{589.5}$ −8.6° (*c* 0.35, Me_2SO), weighed 14.5 mg.

High-voltage electrophoresis. — High-voltage electrophoresis experiments were performed in (*a*) borate buffer, pH ~9.1 (0.05M and 0.01M, 50 V/cm, 1 h), and (*b*) pyridine acetate buffer, pH ~5.75 (0.05M, 50 V/cm, 1 h) on LPS, OPS, and CPS. For each sample, a single spot was obtained.

LPS moved 2 cm towards the cathode in *a*; OPS moved 2.5 cm in *b*, and 19 cm in *a*, towards the cathode; and CPS moved 3.1 cm towards the anode in *b*, and 17.5 cm in 0.01M *a* towards the anode. In all of these experiments, silver nitrate in acetone plus 4% pentaerythritol in 0.05M sodium hydroxide was used as the spray reagent.

Preparation of antiserum. — Antiserum was produced by immunizing rabbits (two per group) intravenously with a suspension of bacteria (0.25 mL) containing ~1400 × 10^6 organisms per mL. Increasing doses were given twice a week for three weeks. The animals were bled one week after the last injection, and the serum was stored at −20°. Gel-diffusion studies were performed with the serum.

Ouchterlony gel-diffusion test[14,15]. — Original LPS, purified LPS, and OPS, in different concentrations in physiological saline, were added to the gel-diffusion plates. In all instances, only a single precipitin-line could be observed. The CPS did not show any precipitin line.

Sugar analysis. — The purified LPS, O-antigenic PS (OPS), and core PS (CPS) were separately hydrolyzed with 0.5M sulfuric acid for 24 h on a boiling-water bath. The hydrolyzates were made neutral with barium carbonate, and the suspensions were filtered through Celite. The neutral, basic, and acidic sugars were detected by paper chromatography using solvents *A*, *B*, and C, respectively. The spray reagents used were *1* for neutral and acidic sugars, and *3* for amino sugars. Neutral sugars, as their alditol acetates, were detected and estimated by g.l.c.–m.s. and g.l.c. in column *a*, using *myo*-inositol as the internal standard. Hexosamine was estimated in column *c* with the same internal standard. The uronic acids in the LPS and OPS were estimated by the carbazole–sulfuric acid method[17] (with D-glucuronic acid as the standard), and also from the difference in the percentage of glucose in the alditol acetates of LPS and OPS and the corresponding carboxyl-reduced[18] material as determined by g.l.c. (column *a*), with *myo*-inositol as the internal standard.

Preparation of carboxyl-reduced LPS (CR-LPS) and carboxyl-reduced OPS (CR-OPS)[18]. — LPS (15 mg) and OPS (10 mg) were respectively dissolved in 15 and 10 mL of water. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (0.38 and 0.270 g) was added to the stirred solutions of LPS and OPS respectively. During the esterification, the pH was kept at 4.75 by adding 0.01M hydrochloric acid dropwise. After 2 h, a 2M aqueous solution of sodium borohydride

(25 and 17 mL) was added, during 1 h, and the pH was maintained at ~7.00 by simultaneous addition of 4M hydrochloric acid. The solutions were dialyzed against distilled water for 48 h and lyophilized. The whole procedure was repeated once.

Detection of fructose in the LPS[19]. — The LPS (15 mg) was dissolved in 0.2M acetic acid (1.5 mL), and hydrolyzed for 8 h at 100°. After centrifugation at low speed, the supernatant liquor was lyophilized. Electrophoresis was conducted with a 1% solution of this LPS hydrolyzate, using 0.01M borate buffer, pH ~9.1, at 2 kV (25 V/cm). Standard, 1% solutions of glucose, fructose, and mannose were spotted on the same paper. The paper was dried, and sprayed with 5% acetic acid and then with reagent *2*. Fructose was detected on the paper.

Methylation analysis. — LPS, CR-LPS, OPS, and CR-OPS (~8 mg each) were each dissolved in Me_2SO (2.5 mL), 2M methylsulfinyl sodium[21] (2.5 mL for each) was added under nitrogen, and the solutions were stirred overnight at room temperature. Methyl iodide (2 mL) was then added dropwise to each, with external cooling, and the mixtures were stirred for 3 h, dialyzed, and lyophilized. Once-methylated samples were twice remethylated by the Kuhn method[22a,b]. The i.r. spectra of the materials contained no bands for free hydroxyl groups at 3600–3300 cm^{-1}.

The methylated products were formylated with 85% formic acid for 2 h at 100°. The acid was removed by co-distillation with water, and the products were further hydrolyzed with 0.5M H_2SO_4 for 20 h at 100°. The solutions were made neutral, and passed through a column (10 × 1.5 cm) of Dowex 50-W X-8 (H^+) ion-exchange resin. The columns were thoroughly eluted with water, to remove all of the partially methylated, neutral and acidic sugars. After conversion into alditol acetates, these were examined by g.l.c. and g.l.c.–m.s.

The columns of Dowex 50-W X-8 (H^+) resin were then successively eluted with 2M (25 mL) and M (25 mL) hydrochloric acid, to obtain the partially methylated, basic sugars. After removal of the HCl in a vacuum desiccator (over P_2O_5 and NaOH pellets), they were converted into the alditol acetates, and these were analyzed by g.l.c. and g.l.c.–m.s.

Smith degradation. — A solution of the LPS (23.6 mg) in water (25 mL) was treated with 0.2M sodium metaperiodate[25] (25 mL) in the dark for 48 h at 5°. The excess of periodate was decomposed with ethylene glycol, and the solution was dialyzed against distilled water, and freeze-dried. The product was reduced with sodium borohydride in water, overnight at room temperature. The excess of borohydride was neutralized with glacial acetic acid, the solution was dialyzed, and the dialyzate concentrated to a small volume, and lyophilized. The product was hydrolyzed with 0.5M H_2SO_4 for 24 h at 100°, the sugars in the hydrolyzate were converted into alditol acetates by the usual procedure, and the acetates were analyzed by g.l.c. using columns *a* and *c*.

Chromium(VI) trioxide oxidation[26] *of OPS and CR-OPS.* — A mixture of OPS (12 mg) and *myo*-inositol (1.288 mg) was dissolved in formamide (1.5 mL). Acetic anhydride–pyridine (1:1) was added, and the solution was stirred for 18 h

at room temperature. The excess reagents were removed by evaporating the mixture to dryness, the partially acetylated product was reacetylated with 1 : 1 acetic anhydride–pyridine, and the excess reagents were removed by co-distillation with toluene.

The product was dissolved in glacial acetic acid (3 mL), and powdered chromium trioxide (300 mg) was added, with stirring, at 50°. Aliquots were removed at 0, 1, and 2 h, and immediately diluted with water. The solutions were thrice extracted with chloroform, and the extracts were combined, dried (anhydrous Na_2SO_4), and evaporated to dryness. The product was deacetylated with 0.2M sodium methoxide, the material was hydrolyzed with 0.5M H_2SO_4 for 22 h, and the sugars were converted into alditol acetates. These were analyzed by g.l.c., using columns *a* and *c*.

CR-OPS (8.5 mg, plus 0.9 mg of *myo*-inositol) was also subjected to chromium trioxide oxidation by the same procedure. The results are given in Table III.

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