Carbohydrate Research, 64 (1978) 215–223

STRUCTURAL STUDIES OF A SPECIFIC POLYSACCHARIDE ISOLATED FROM NON-AGGLUTINABLE *Vibrio*

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(Received October 28th, 1977; accepted for publication, November 18th, 1977)

ABSTRACT

The purified, specific polysaccharide from *Vibrio cholera* type *NAG, NV* 384, *O-antigen, 2A, 2B human*, contains glucose (5.14%), galactose (4.21%), mannose (64.8%), xylose (3.16%), arabinose (1.98%), fucose (1.50%), mannuronic acid (14.3%), phosphate (0.32%), 2-amino-2-deoxy-D-glucose (2.9%), and 2-amino-2-deoxy-D-galactose (1.0%). Various reactions have shown that the material comprises a phosphoric diester-linked polysaccharide containing mainly (1→2)-linked manno-pyranose residues that are highly branched with other sugar residues.

INTRODUCTION

The classical discovery of Avery and Heidelberger[1] of the importance of polysaccharides in the pathogenicity and antigenicity of *Pneumococci* stimulated interest in *Vibrio* polysaccharides. In the 1930's, much work, particularly on the preparation of specific polysaccharides and immunochemical studies on these preparations, was published. From these studies, Linton *et al.*[2,3] concluded that precipitin reactions of the polysaccharides with antisera to whole organisms showed that, in general, serology expresses the underlying chemical pattern of these organisms. It was, therefore, to be expected that there would be some difference in the specific polysaccharides of the different serotypes of *Vibrio cholerae*. Also, as interconversions of serotypes of *V. cholerae* are possible[4], it would be of interest to ascertain whether this is due to any alteration in the specific polysaccharides present in these serotypes of *V. cholerae*. Attempts have also been made[5] to characterize some of the polysaccharides prepared from the *Ogawa, Inaba*, and *Rough* strains, but no detailed, structural studies have been made on the specific polysaccharides of any of the types of *V. cholerae*, and consequently, there was no information regarding any of the specific groupings in these polysaccharides. With this in mind, some structural studies have now been made on a specific polysaccharide isolated from a non-agglutinable *V. cholera NV* 384, *O-antigen, 2A, 2B human*, and the results are reported here.

RESULTS AND DISCUSSION

The crude polysaccharide (PLPS) material, $[\alpha]_{589.5}^{30}$ +28°, isolated from *V. cholera NAG, NV* 384, *O-antigen, 2A, 2B human* by the method described by Shrivastava and Seal[6], contained some protein and lipid, but showed no significant i.r. absorption in the 1735-cm^{-1} region, demonstrating the absence of *O*-acyl groups. The protein was removed by pronase treatment[7a] of the PLPS, to yield a material

TABLE I

RESULTS OF PRELIMINARY ANALYSIS OF DIFFERENT FRACTIONS OF *V. cholera* POLYSACCHARIDE

	PLPS	*Crude LPS*	*Pure LPS*	*PS*
Moisture (%)	9	10	10	10.5
$[\alpha]_{589.5}^{30}$ (degrees)	+28	+30	+30	+32
Nitrogen[a] (%)	3.42	trace	trace	trace
Phosphate[b] (%)	0.28	0.3	0.3	0.32
Total hexose[c] (%)		57.5	57	73
Total uronic acid[d] (%)		11.25	11.25	14.3

[a]Estimated by the Dumas method. [b]Estimated by the Ames method[7b]. [c]Estimated by the L-cysteine–sulfuric acid method[8]. [d]Estimated by the carbazole–sulfuric acid method[9]. All results were corrected for moisture.

TABLE II

PROPORTIONS OF SUGARS IN DIFFERENT FRACTIONS OF *V. cholera* POLYSACCHARIDE

Sugars detected	*Hydrolyzed with 0.5M H_2SO_4 for 18 h (with myo-inositol as the internal standard)*				*PS hydrolyzed with 3M HCl for 12 h*
	Crude LPS	*Pure LPS*	*PS*	*Carboxyl-reduced PS*	
Glucose (%)	5.2	3.9	5.14	6.1	
Galactose (%)	3.48	3.34	4.21	4.72	
Mannose (%)	48.56	49.7	64.8	75.6	
Xylose (%)	2.7	2.23	3.16	2.95	
Arabinose (%)	1.76	1.52	1.98	2.22	
Fucose (%)	1.2	1.15	1.50	1.3	
Glycerol	trace	trace	trace		
2-Amino-2-deoxy-glucose[a] (%)					2.9
2-Amino-2-deoxy-galactose[a] (%)					1.0
Mannuronic acid[b] (%)	11.25	11.25	14.3		

[a]Estimated by the Elson–Morgan method with the Boas modification[7c], and by Levvy and McAllan's method[7d]. [b]Estimated by the carbazole–sulfuric acid method[9]. Average values of three estimations (corrected for moisture) are given.

(LPS) having $[\alpha]_{589.5}^{30}$ +30°. In an attempt to separate the acidic from the neutral and/or basic polysaccharides (if present), the LPS was passed through a column (15 × 1.5 cm) of DEAE-cellulose, and eluted first with water and then with a linear gradient of 0–1.0M sodium chloride solution. The polysaccharide was eluted as a single peak by the sodium chloride solution. The polysaccharide material was also passed through a column (100 × 2.25 cm) of Sephadex G-200, and was eluted as a single peak, showing the homogeneity of the product. On mild treatment with acetic acid at 100°, this pure LPS yielded the polysaccharide (PS), $[\alpha]_{589.5}^{30}$ +32°. The results of the preliminary analysis of these products are shown in Table I.

Different fractions of the polysaccharide material were hydrolyzed with 0.5M sulfuric acid. The neutral, basic, and acidic sugars obtained were identified by paper chromatography or by g.l.c. They were estimated by the g.l.c. technique[10], with *myo*-inositol as the internal standard, and also by colorimetric methods. The results are summarized in Table II. The uronic acid was identified as mannuronic acid by reducing the carboxyl group in the polysaccharide (PS) with diborane. After hydrolysis of the carboxyl-reduced PS, the content of mannose was found to increase by 10.8%, indicating that mannuronic acid had been present in the PS. As, in diborane reductions, a quantitative yield is not possible, this result is in good agreement with that obtained by the carbazole–sulfuric acid method.

The LPS did not react with acid phosphatase[11], but under the same conditions, the PS liberated 0.073% of phosphate out of the 0.32% present. When this material was treated with alkaline phosphatase[12], followed by acid phosphatase, the entire phosphate was removed from the polysaccharide. However, after the treatment with alkaline phosphatase, no dialyzable carbohydrate material could be detected, and the phosphate-free polysaccharide from the dialysis bag could not be resolved into fractions; this indicated that the phosphoric diester bond might be present somewhere near the middle of the whole molecule. The structural analyses described here would refer to the average structure on either side of the phosphorus atom, as the data available are insufficient to identify the two sides separately. The action of dilute alkali on the polysaccharide was similar to that of alkaline phosphatase on the polysaccharide.

The purified polysaccharide (PS) was methylated by the Hakomori[13,14] procedure, and part of the product was reduced with lithium aluminum hydride[15]. These two products were hydrolyzed, the partially methylated amino sugars were separated from the neutral part by passage through a column (10 × 1 cm) of Dowex-50W X-8 (H^+) ion-exchange resin, and then the partially methylated neutral sugars were analyzed as their alditol acetates[16], partly by g.l.c. and partly by g.l.c.–m.s. The partially methylated amino sugars[17] were eluted from the column with 2M hydrochloric acid followed by M and 0.5M hydrochloric acid, and were then identified, as their alditol acetates, by g.l.c. Quantitative evaluation of the chromatogram was not possible, due to the thermal lability and comparatively low volatility of the partially methylated amino sugars. Also, the response factors of partially methylated amino sugars differ considerably from those of the partially methylated neutral

TABLE III

METHYL ETHERS OF SUGARS FROM THE HYDROLYZATES OF METHYLATED PS (A), METHYLATED CARBOXYL-REDUCED PS (B), AND METHYLATED AMINO SUGARS (C)

Sugars[a]	*T*[b]	*Approximate mole %*[c]			*Mode of linkage*
		A	*B*	*C*[d]	
2,3,5-Ara	0.41	2	2		Ara*f*-(1→
2,3,4-Fuc	0.58	1	1		Fuc*p*-(1→
2,3,4,6-Man	0.99	29	26		Man*p*-(1→
2,3-Xyl	1.19	4	3		→4)-Xyl*p*-(1→
3,4,6-Man	1.82	33	29		→2)-Man*p*-(1→
2,3,6-Gal	2.22	3	3		→4)-Gal*p*-(1→
2,3,6-Glc	2.32	7	7		→4)-Glc*p*-(1→
2,6-Gal	3.14	3	2		→3,4)-Gal*p*-(1→
3,4-Man	4.36	17	19		→2,6)-Man*p*-(1→
2,3-Man	3.69		8		→4)-Man*p*-(1→
3-(2-*N*-Me)-Glc	3.75[e]			3	→4,6)-GlcNAc*p*-(1→
3-(2-*N*-Me)-Gal	4.07[e]			1	→4,6)-GalNAc*p*-(1→

[a]2,3,5-Ara = 2,3,5-Tri-*O*-methylarabinose, 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, on an OV-225 column at 170°. [c]Average values from three determinations. [d]Ratio of the two amino sugars. [e]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, on an ECNSS-M column at 190°.

sugars. The 2,3-di-*O*-methylmannose (which was obtained only from the carboxyl-reduced, methylated PS) is formed from the mannuronic acid residues. The results are summarized in Table III.

Characterization of 2,3,4,6-tetra-*O*-methylmannose, 2,3,4-tri-*O*-methylfucose, and 2,3,5-tri-*O*-methylarabinose in the hydrolyzate of the methylated polysaccharide indicated that mannopyranosyl, fucopyranosyl, and arabinofuranosyl groups constitute the nonreducing end-groups of the polysaccharide molecule. Identification of 3,4,6-tri-*O*-methylmannose, 2,3,6-tri-*O*-methyl-glucose and -galactose, and 2,3-di-*O*-methylxylose from the hydrolyzate indicated that (1→2)-linked mannopyranosyl, and (1→4)-linked glucopyranosyl, galactopyranosyl, and xylopyranosyl groups are present in the interior part of the molecule, the (1→2)-linked mannopyranosyl groups of which constitute more than two-thirds of the total, interior part. The presence of a relatively large proportion of di-*O*-methylhexoses showed that the molecule is highly branched, and that, at the branch points, the mannopyranosyl groups are linked through O-1, O-2, and O-6, and the galactopyranosyl groups, through O-1, O-3, and O-4. Normally, the ratio of single-branched residue to a nonreducing end-group is 1 : 1. There are comparatively larger proportions of tetra-*O*-methylmannose and tri-*O*-methyl-arabinose and -fucose than of the di-*O*-methylhexoses and mono-*O*- and *N*-methylhexosamines taken together. The presence of a small proportion of mono-*O*-methylhexose (unidentified) accounts for part of this deficiency. However, the general

structural features of the molecule of *V. cholera* polysaccharide are apparent from the types of bonds shown in Table III.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer model 241 MC spectropolarimeter at 30 ±1° and 589.5 nm for solutions in water. Infrared spectra were recorded with a Beckman IR-20-A instrument, with cesium bromide optics. Ultraviolet and visible spectra were recorded with Carl Zeiss VSU2-P and Yanaco SP-1 spectrophotometers. For g.l.c., a Hewlett–Packard 5730 gas chromatograph, with flame ionization detector, was used. Resolutions were performed on glass columns (1.83 m × 6 mm) containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of sugars) and at 170° (for partially methylated alditol acetates), and (*b*) 5% of OV-225 on SIL. RUB. (80–100 mesh) at 170° (for partially methylated sugars), and (*c*) on a steel column (1.83 m × 3 mm) of 3% of SE-52 on SIL. RUB. (100–120 mesh) at 180° (for silyl derivatives of *N*-acetylated amino sugars). For quantitative evaluation of the g.l. chromatograms, a Hewlett–Packard 3370 B integrator was used. Paper chromatography was performed on 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–ethanol–water, (*C*) 5:5:1:3 ethyl acetate–pyridine–acetic acid–water, (*D*) 10:1:2 1-butanol–ethanol–water, and (*E*) 4:1:1 1-butanol–ethanol–water. The compounds were detected with (*a*) alkaline silver nitrate, (*b*) 2% ninhydrin in acetone, at 110°, and (*c*) 3% *p*-anisidine hydrochloride in ethanol, at 120°. Evaporations were conducted under diminished pressure at bath temperatures not exceeding 40°. Small volumes of aqueous solutions were lyophilized.

Isolation of the crude polysaccharide from Vibrio cholera, *strain* NAG, NV *384 O-antigen, 2*A, *2*B *human.* — Seventy-two-hour growths of the strain on nutrient broth with 1% peptone broth (pH 8.0) were treated with enough phenol to make 0.5% phenol, and allowed to stand for 1 h. The suspension was passed through a Sharples Supercentrifuge to remove the sediment. The supernatant liquor was concentrated to one-tenth its volume, and the polysaccharide material was isolated from this solution by the method of Shrivastava and Seal[6]. The polysaccharide showed $[\alpha]^{30}_{589.5}$ +28° (*c* 0.1, water), nitrogen 3.42%, and phosphate 0.28%. In the i.r. spectrum (KBr), practically no absorption was observed at ~1735 cm^{-1} (*O*-acyl region). There was significant absorption at ~280 nm, indicating the presence of protein.

Removal of protein from the protein–lipopolysaccharide complex[7a]. — PLPS was incubated at 37° with pronase (70,000 PUK/g; E. Merck, Darmstadt) in Tris–acetic acid buffer (pH 7.8) containing 0.01M calcium chloride. After 72 h, the enzyme was deactivated by heating the mixture for 20 min at 50°. The suspension was then centrifuged, to remove the precipitated enzyme, and the supernatant liquor was dialyzed in an 18/32 Visking cellophane tube at 4°, first against 0.1M sodium chloride for 6 h,

and then against distilled water. The product, isolated by lyophilization, was obtained in 73% yield, and showed $[\alpha]_{589.5}^{30}$ +30° (*c* 0.1, water); nitrogen, a trace; and phosphate, 0.3%. The u.v. spectrum (at 280 nm) showed the absence of a significant amount of protein.

Purification of the lipopolysaccharide (LPS). — (*a*) LPS (15 mg) in water was passed through a column (20 × 1.5 cm) of DEAE-cellulose. The column was eluted first with water and then with a linear gradient comprised of water (200 mL) and 1.0M sodium chloride (200 mL), the effluents being monitored by the phenol–sulfuric acid[20] method. Only one fraction was obtained, in 97% yield.

(*b*) A solution of LPS (25 mg) in 0.05M ammonium hydrogencarbonate solution (3 mL) was applied to a column (100 × 2.2 cm) of Sephadex G-200. The column was eluted with 0.05M ammonium hydrogencarbonate, and the effluents were collected in 3-mL portions. The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method, which showed only one peak. The LPS recovered by lyophilization was obtained in 95% yield. The two products (methods *a* and *b*) were similar.

Removal of lipid. — A 1% solution of LPS in 0.1M acetic acid was heated for 2 h in a boiling-water bath, cooled, and repeatedly washed with ethyl ether. The ether washings were then extracted with water, and the extracts were combined with the aqueous solution, dialyzed against distilled water overnight, purified by passing through a column (60 × 2.2 cm) of Sephadex G-50, and then lyophilized, to give an amorphous product in 77% yield. The PS showed $[\alpha]_{589.5}^{30}$ +32° (*c* 0.1, water), and phosphate, 0.32%.

Sugar analysis. — The LPS and PS were separately hydrolyzed with 0.5M sulfuric acid for 18 h (optimal conditions), the acid was neutralized with barium carbonate, and the suspension was centrifuged. The neutral, acidic, and basic sugars were separated from each other by using Dowex-50W X-8 (H^+) and Dowex-1 X-4 (HCO_3^-) resins. The neutral sugars were detected by paper chromatography (solvents *A*, *B*, and *D*, and spray reagents *a* and *c*) and by g.l.c. (column *a*). Acidic sugars were detected by paper chromatography (solvents *A* and *C*, and spray reagent *a*). For the detection of amino sugars, solvent *A* and spray reagents *a* and *b* were used. Amino sugars, as the per(trimethylsilyl) derivatives, were also identified by g.l.c. using column *c*, and by ninhydrin degradation[7e] followed by chromatography in solvent *E*. Total hexose was estimated by the primary L-cysteine–sulfuric acid[8] and anthrone–sulfuric acid[18] methods. Neutral sugars, as their alditol acetates[10], were estimated by g.l.c. (column *a*), with *myo*-inositol as the internal standard. Uronic acid was estimated by the carbazole–sulfuric acid method[9] (with D-glucuronic acid as the standard). 6-Deoxyhexose was estimated by the Dische–Shettles method[19]. After hydrolysis with 3M hydrochloric acid for 12 h, total hexosamine was determined by the Elson–Morgan method and the Boas modification[7c]. Relative amounts of 2-amino-2-deoxyglucose and 2-amino-2-deoxygalactose were estimated by Levvy and McAllan's method[7d]. Total phosphate was estimated by an ashing procedure[7b]. Inorganic or end-group phosphate was estimated by Chen's method[7f].

Preparation of carboxyl-reduced PS. — PS (15 mg) was suspended in formamide (1 mL), and pyridine (1 mL) and acetic anhydride (0.5 mL) were added. The mixture was stirred for 24 h at room temperature, and the resulting solution was evaporated to dryness. The residue was dissolved in dry tetrahydrofuran (1 mL), an excess of diborane in the same solvent was added, the mixture was stirred for 16 h at room temperature, and the excess of diborane was decomposed by cautious addition of methanol. The mixture was evaporated to dryness, methyl borate was removed by co-distillation with methanol, and the product was treated with 2% sodium methoxide in methanol (0.2 mL) for 3 h. The reaction was stopped by adding a few drops of water, and the solution was dialyzed overnight. The carboxyl-reduced PS (9.6 mg) was recovered by lyophilization.

Carboxyl-reduced PS (2 mg), mixed with *myo*-inositol (0.1 mg), was hydrolyzed with 0.5M sulfuric acid for 18 h at 100°. The hydrolyzate was made neutral with barium carbonate, and then analyzed (as the alditol aceates) by g.l.c.

Action of acid phosphatase[11] *on LPS and PS.* — LPS or PS (20 mg) was dissolved in 50 mM acetate buffer (pH 5.6; 3 mL) containing 5 mM magnesium chloride. Purified, wheat-germ, acid phosphatase (from BDH Chemicals Ltd, Poole, England; 2 mg) in the same buffer (2 mL) was added, and the solution was incubated at 37°. After elapse of 3 days, and 6 days, a fresh quantity (1 mg in 1 ml of buffer) of the same enzyme was added. After 11 days, the reaction was stopped by precipitation of the enzyme (by adding an equal volume of acetone). The precipitate was centrifuged off, washed three times with 50% aqueous acetone, and discarded. The supernatant liquor and the washings were combined and dialyzed. The solutions from both inside and outside the dialysis bag were separately collected, concentrated, and lyophilized. For both the LPS and the PS, the solution outside the dialysis bag contained no carbohydrate, as tested by the phenol–sulfuric acid method[20]. All of the carbohydrate and the phosphate was present in the inner solutions. The amount of phosphate liberated was estimated by Chen's method[7f] on the 3rd (0.001% for PS, nil for LPS) and 6th day (0.02% for PS, nil for LPS), and after completion of the reaction (0.073% for PS, nil for LPS).

Action of alkaline phosphatase[12]. — PS (15 mg) treated with acid phosphatase was dissolved in 5 ml of glycine buffer (1.5% of glycine and 0.1% of magnesium chloride in 2M sodium hydroxide, and the pH adjusted to 8.9 with M hydrochloric acid), and to the solution was added a solution of 2 mg of alkaline phosphatase (Worthington Biochemical Corp., Freehold, New Jersey) in 1 ml of the same buffer, and the mixture was incubated at 37°. After 3 and 6 days, fresh enzyme (1 mg in 1 mL of buffer, each time) was added. After 11 days, the enzyme was deactivated, and removed, in the same way as in the case of the acid phosphatase treatment. The solution was then dialyzed, and the materials from both inside and outside the dialysis bag were collected, concentrated, and lyophilized. All of the carbohydrate was present in the solution inside the dialysis bag.

Action of acid phosphatase on the PS treated with alkaline phosphatase. — The preceding material (14 mg) was treated with acid phosphatase as already described.

After deactivation and removal of the enzyme, the material was dialyzed, and the solutions inside and outside the dialysis bag were collected, concentrated, and lyophilized. The inner solution contained phosphate-free carbohydrate, and the external solution contained no carbohydrate.

Degradation with alkali. — PS (15 mg) treated with acid phosphatase was dissolved in 0.2M sodium hydroxide (2 mL), and the solution was diluted with an equal volume of water, and kept for 96 h at 37°. The solution was then cooled, made neutral (dil. hydrochloric acid), and the volume made to 10 mL.

Treatment of a portion (5 mL) of this solution with acid phosphatase was conducted as already described. The material inside the dialysis bag contained polysaccharide that was free from phosphate.

The remaining solution was dialyzed for 24 h against distilled water. The solutions inside and outside the dialysis bag were concentrated, and examined for carbohydrate. Only the inner material contained carbohydrate and phosphate.

Methylation analysis[13] *of polysaccharide PS.* — PS (15 mg) in methyl sulfoxide (2 mL) was treated with 2M methylsulfinyl sodium[14] (2 mL) under nitrogen. The resulting solution was agitated in an ultrasonic bath for 1 h, and kept for 2 h at room temperature. Methyl iodide (4 mL) was then added dropwise, with external cooling. The turbid solution was agitated in an ultrasonic bath for 1 h, when a clear solution was obtained. The mixture was dialyzed against running tap-water, and then against distilled water. The methylated PS obtained after lyophilization was remethylated by the Purdie method[21], to yield fully methylated material (10.12 mg).

The methylated PS (10 mg) was hydrolyzed with 90% formic acid (3 mL) for 2 h at 100°. The acid was removed by co-distillation with water, and the residue was further hydrolyzed with 0.5M sulfuric acid (2 mL) for 18 h at 100°. The solution was made neutral with barium carbonate, and then passed through a column (15 × 1.5 cm) of Dowex-50W X-8 (H^+) resin. The column was eluted with water (500 mL), to remove all of the (neutral and acidic) partially methylated sugars.

The eluate was then concentrated to ~1 mL, the contents reduced with sodium borohydride, and the products converted into their alditol acetates[16], and examined by g.l.c. on columns *a* and *b*, and by g.l.c.–m.s.

The column was now successively eluted with 2M (25 mL), M (25 mL), and 0.5M (50 mL) hydrochloric acid, to isolate the basic sugars. The hydrochloric acid was partly removed by evaporation and then by neutralization with silver carbonate. The suspension was filtered, the filtrate was concentrated, and the contents were reduced with sodium borohydride for 3 h; the base was neutralized with glacial acetic acid, and the solution evaporated to dryness. The boric acid was removed by repeated addition and evaporation of methanol containing a few drops of acetic acid. The white residue of salts was dried over phosphorus pentaoxide in a vacuum desiccator. Acetic anhydride (0.5 mL) was added, and the mixture was heated for 2 h at 100°. The acetic anhydride was evaporated off under vacuum, final traces were removed by co-distillation with toluene, and the residue was extracted with chloroform. The

extract (containing the partially methylated amino sugars) was washed with water, dried (anhydrous sodium sulfate), and analyzed by g.l.c. (column *a*).

Carboxyl reduction[15] *of methylated PS.* — Methylated PS (5 mg) was dissolved in 1:2 ethyl ether–dichloromethane (5 mL), lithium aluminum hydride (30 mg) was added, and the mixture was refluxed for 4 h in a nitrogen atmosphere. The excess of lithium aluminum hydride was decomposed by adding ethyl acetate and then water, and the solution was made neutral with M phosphoric acid. The precipitate was filtered off, and the filtrate was extracted with chloroform. The extract was washed with water, dried (anhydrous sodium sulfate), and evaporated to dryness, to give the carboxyl-reduced, methylated polysaccharide (3.6 mg).

This material (2 mg) was treated with 90% formic acid for 2 h at 100°, the formic acid was removed, and the material was hydrolyzed with 0.5M sulfuric acid for 18 h at 100°. The acid was neutralized with barium carbonate, and the product converted into the alditol acetates. The components were identified by g.l.c.

ACKNOWLEDGMENTS

The authors thank Prof. B. Lindberg of Stockholm University, Sweden, for his help in identifying some of the tri- and di-*O*-methyl sugars by g.l.c.–m.s., and Dr. N. Roy of this laboratory for supplying authentic samples of some of the *O*-methyl sugars. Thanks are also due Dr. R. K. Sen, Serologist, Government of India, 3 Kyd Street, Calcutta, for making available to us a number of batches of the growths of the strain of *Vibrio*, and Mrs. T. Biswas of this laboratory is thanked for her help in the isolation of the crude polysaccharide.

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