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STUDIES ON THE PARTIAL STRUCTURE OF THE O-ANTIGEN OF *Vibrio cholera*, INABA 569 B

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ABSTRACT

Five oligosaccharides were isolated in pure state from the lipopolysaccharide of *Vibrio cholera*, Inaba 569 B, and their structures were elucidated. More-detailed information regarding the partial structure of the lipopolysaccharide, containing glucose, mannose, glucuronic acid, 2-amino-2-deoxyglucose, D-*glycero*-L-*manno*-heptose, and D-*glycero*-L-*gluco*-heptose, was obtained through Smith degradation, chromium trioxide oxidation, and graded hydrolysis studies of the lipopolysaccharide and its derived products.

INTRODUCTION

In a previous communication[1], we reported the results of preliminary examination of the lipopolysaccharide (LPS), the carboxyl-reduced lipopolysaccharide (CR-LPS), and the polysaccharide (PS) derived from the LPS isolated from *Vibrio cholera*, Inaba 569 B. The types of linkages between the various sugar residues were also shown from methylation analysis of these materials. A structural study of a polymeric material in the LPS of *Vibrio cholera* has also been reported[2] recently, but its proportion in the LPS was not precisely determined. Redmond[3] estimated the proportion of this polymeric material in the LPS as only 14%, and also noted[4] that, when the LPS from *Vibrio cholera* was heated with 1% acetic acid, the immunological activity of the LPS gradually diminished. Several other groups[5] have also studied the LPS from *Vibrio cholera*, but no partial structures of the sugar residues reported[1] have been assigned. We now report the results of detailed, structural studies conducted on the O-antigen of *Vibrio cholera*, Inaba 569 B.

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RESULTS AND DISCUSSIONS

Both of the heptoses present in the LPS of *Vibrio cholera*, Inaba 569 B have been identified. The major heptose was identical in behavior to authentic D-*glycero*-L-*manno*-heptose (synthesized by the condensation of nitromethane[6] with D-galactose), both in g.l.c. (column *a*, R_{Glc} 2.21) and in paper chromatography (solvent *C*, R_{Glc} 0.95). The heptose was proved to be D-*glycero*-L-*manno*-heptose by isolating the compound in pure state by repeated resolution on paper, and determining its specific rotation, which was $[\alpha]_{589.5}^{23}$ −11.2° (water); lit.[7] $[\alpha]_{D}^{20}$ −13.7° (water).

The minor heptose behaved identically to authentic D-*glycero*-L-*gluco*-heptose (enantiomeric product of the previously mentioned synthesis) in g.l.c. (column *a*, R_{Glc} 2.54) and in paper chromatography (solvent *C*, R_{Glc} 0.78). The heptose was confirmed to be D-*glycero*-L-*gluco*-heptose by isolating the compound by resolution on paper, and determining its specific rotation, which was $[\alpha]_{589.5}^{23}$ −48.3° (water); lit.[7] $[\alpha]_{D}^{20}$ −50.7° (water).

The anomeric configurations of the various glycosyl groups were also ascertained. The LPS and CR-LPS were acetylated, and the acetates subjected to oxidation with chromium trioxide[8] in acetic acid at 50°, *myo*-inositol being used as the internal standard. During the oxidation (see Table I), the heptoses were oxidized rapidly, indicating that they have the β-anomeric configuration, the other sugars being α-linked. For the CR-LPS, it was noted that part of the glucose (that corresponded in amount to the glucuronic acid) was oxidized rapidly, along with the heptoses. As these glucose units originated from the glucuronic acid, this indicated that the glucuronic acid residues also have the β configuration in the lipopolysaccharide. The low specific rotation of the lipopolysaccharide indicates the presence of both the α- and the β-anomeric configuration of the sugar residues.

The PS was treated with sodium metaperiodate at 5° in the dark, and the

TABLE I

OXIDATION OF PERACETYLATED LPS AND CARBOXYL-REDUCED LPS WITH CHROMIUM TRIOXIDE

Material[a]	*Time of oxidation (hours)*	*Mannose*	*Glucose*	D-glycero-L-manno-*Heptose*	D-glycero-L-gluco-*Heptose*	*2-Amino-2-deoxy-glucose*	myo-*Inositol*
A	0	6.6	15.8	9.0	1.4	3.7	10
	1	5.8	14.2	5.4	0.6	3.1	10
	2	4.8	11.5	2.7	0.2	2.7	10
B	0	5.7	21.3	8.4	1.3	3.4	10
	1	5.1	16.1	5.2	0.8	2.89	10
	2	4.6	11.8	2.4	0.3	2.55	10

[a]A: Alditol acetates obtained from peracetylated LPS oxidized with chromium trioxide. B: Alditol acetates obtained from peracetylated, carboxyl-reduced LPS oxidized with chromium trioxide.

amount of periodate consumed during the reaction was measured spectrophotometrically[9]. The PS consumed only 0.79 mol of periodate per mol of hexosyl unit in ~12 h, after which, the consumption of periodate remained reasonably constant. This result showed a much lower consumption of periodate than would be expected from the types of linkages indicated[1] by the methylation studies; this is evident from the fact that the PS contains only 30.7% of carbohydrate (as found by g.l.c. analysis). Of the rest of the material, the polymer established by Kenne *et al.*[2] (which constitutes ~14%) does not consume any periodate. The nature of the remaining material and its behavior towards periodate are not yet known, and therefore, it is not possible to calculate the theoretical consumption of periodate per mol of hexosyl residues. However, the results of Smith degradation[10] of the PS, followed by g.l.c. analysis, were quite satisfactory. As expected from the methylation data, all of the sugar residues are vulnerable to periodate, except the trace of heptose that yielded unidentified mono- or di-*O*-methylheptoses in the methylation analysis; and actually, after Smith degradation, it was found that practically no sugar residues survived. Again, after Smith degradation, the 1,2,6-linked heptose residue and the 1,4-linked 2-amino-2-deoxyglucose residue would be expected to liberate threitol and erythritol, respectively, and, from most of the remaining units, glycerol should result. Actually, after Smith degradation, glycerol, threitol, and erythritol were obtained in the molar ratios of ~9:1:1, in good agreement with the theoretically calculated values of 9:0.6:1.

The LPS (200 mg) was partially hydrolyzed with 0.5M hydrochloric acid for 2 h, and the acidic, basic, and neutral oligosaccharides were separated by ion-exchange resins. In paper chromatography in solvent *B*, the acidic part showed two distinct spots, having R_{Lact} 0.58 and 0.82. The faster-moving disaccharide (**1**, 2.2 mg) had $[\alpha]_{589.5}^{23}$ +13.2°, and the slower-moving (**2**, 2.6 mg) had $[\alpha]_{589.5}^{23}$ −9.8°.

In paper chromatography in the same solvent, the neutral fraction showed, in addition to the spots of monosaccharides, two distinct spots of disaccharides having R_{Lact} 0.4 and 0.56 (besides some other faint spots in the higher-oligosaccharide region). The disaccharides were separated, and isolated, by preparative paper-chromatography. The slower-moving disaccharide (**3**, 2.45 mg) and the faster-moving disaccharide (**4**, 2.0 mg) had $[\alpha]_{589.5}^{23}$ −5.8° and +24.3°, respectively.

In paper chromatography in solvent *B*, the basic portion showed, in addition to a trace spot of 2-amino-2-deoxyglucose, another spot having R_{Lact} 0.31; this oligosaccharide (**5**, 3.2 mg, $[\alpha]_{589.5}^{23}$ +68.2°) was also isolated by preparative paper-chromatography in the same solvent.

The oligosaccharides (**1**–**5**) were separately hydrolyzed with 0.5M sulfuric acid for 18 h on a boiling-water bath, and the hydrolyzates were converted into the alditol acetates[11,12]. The acidic disaccharides (**1** and **2**) respectively showed mannose and D-*glycero*-L-*manno*-heptose; the neutral disaccharide (**3**), D-*glycero*-L-*manno*-heptose and mannose; and the neutral disaccharide (**4**), glucose and D-*glycero*-L-*manno*-heptose. The basic trisaccharide (**5**) showed glucose, 2-amino-2-deoxyglucose, and D-*glycero*-L-*manno*-heptose. The results are shown in Table II.

TABLE II

THE SUGARS PRESENT IN THE HYDROLYZATES OF THE OLIGOSACCHARIDES

Sugars as alditol acetates[a]	*Proportion in oligosaccharides (mole %)*				
	1	**2**	**3**	**4**	**5**
Mannose	100		54		
D-*glycero*-L-*manno*-Heptose		100	46	48	42
Glucose				52	29
2-Amino-2-deoxyglucose					29

[a]Analyzed by g.l.c. in column *a*.

TABLE III

RESULTS OF METHYLATION ANALYSIS OF THE OLIGOSACCHARIDES

Methylated sugar[a] *(as alditol acetate)*	*Retention time*		*Alditol acetates (mole %)*[b]						
	Column a	*Column* b	*1*	*2*	*3*	*4*	*5*	*6*	*7*
2,3,4,6-Glc	1.00	1.00						52	32
3,4,6-Man	1.95	1.82	1.00	49.2			51		
2,3,4,6,7-Hep	2.45	2.2					49		
2,3,4-Glc	2.49	2.22		50.8		51.7			
?[c]-Hep	4.01	3.3						48	
2,3,4,7-Hep	4.1	3.3			100	48.3			
3,4,6,7-Hep	4.49	3.75							38
3,6-GlcNMe	1.71[d]								30

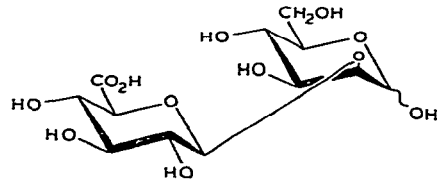
[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. [b]Key: 1. Alditol acetate from permethylated disaccharide **1**. 2. Alditol acetates from permethylated disaccharide **1** after reduction with lithium aluminum hydride. 3. Alditol acetate from permethylated disaccharide **2**. 4. Alditol acetates from permethylated disaccharide **2** after reduction with lithium aluminum hydride. 5. Alditol acetates obtained from permethylated neutral disaccharide **3**. 6. Alditol acetates obtained from permethylated neutral disaccharide **4**. 7. Alditol acetates obtained from permethylated trisaccharide **5**. [c]An unidentified heptose in the tetramethyl region. [d]Retention time at 190°.

In order to find out which was the reducing end in the neutral and basic oligosaccharides, the oligosaccharides **3**–**5** were reduced with sodium borohydride for 5 h, the solution was made neutral, and the products were hydrolyzed with 0.5M sulfuric acid for 18 h. The materials in the hydrolyzates were then acetylated by the usual procedure. G.l.c. analysis of the acetates showed the presence of mannose in disaccharide **3**, and of D-*glycero*-L-*manno*-heptose in oligosaccharides **4** and **5**.

The oligosaccharides (**1**–**5**) were separately methylated by the Kuhn procedure[13]. The methylated oligosaccharides were hydrolyzed, and the products converted into the alditol acetates. G.l.c. analysis (columns *a* and *b*) showed the respective presence of 3,4,6-tri-*O*-methylmannose and 2,3,4,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose

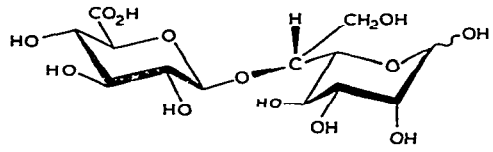
in the methylated disaccharides **1** and **2**. 2,3,4,6,7-Penta-*O*-methyl-D-*glycero*-L-*manno*-heptose and 3,4,6-tri-*O*-methylmannose were obtained from methylated disaccharide **3**. Disaccharide **4** yielded 2,3,4,6-tetra-*O*-methylglucose (TMG) and a methylated heptose in the tetramethyl region (R_{TMG} 4.01 and 3.3 in columns *a* and *b*, respectively). The heptose derivative could not be identified due to the lack of authentic compound. The alditol acetates obtained from the basic oligosaccharide **5** were analyzed in column *a* at 190°, and showed three peaks, corresponding to 2,3,4,6-tetra-*O*-methylglucose, 2-deoxy-3,6-di-*O*-methyl-2-(methylamino)glucose, and 3,4,6,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose.

In another experiment, the disaccharides **1** and **2** were separately permethylated, and reduced[14] with lithium aluminum hydride, the products hydrolyzed, and the



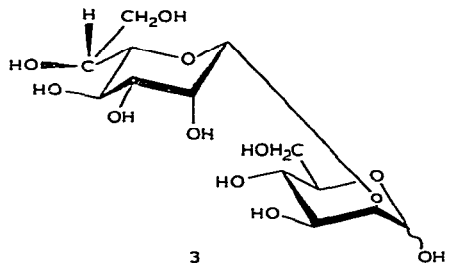
1

O-(β-D-Glucopyranosyluronic acid)-(1→2)-D-mannose



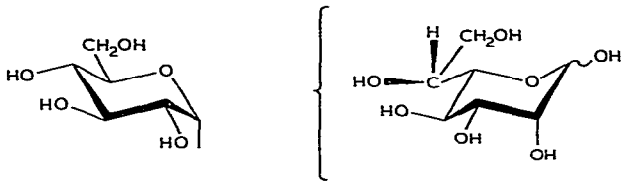
2

O-(β-D-Glucopyranosyluronic acid)-(1→6)-D-*glycero*-L-*manno*-heptose



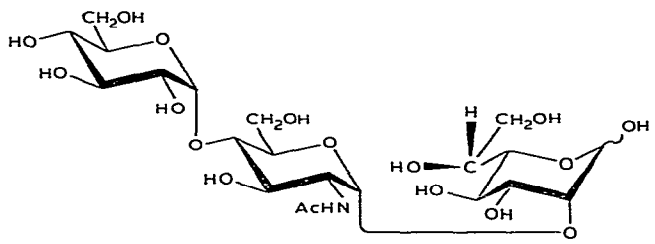
3

O-D-*glycero*-β-L-*manno*-heptopyranosyl-(1→2)-D-*mannose*.



4

O-α-D-Glucopyranosyl-(1→?)-D-*glycero*-L-*manno*-heptose



5

O-α-D-Glucopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-α-D-glucopyranosyl-(1→2)-D-*glycero*-L-*manno*-heptose

alditol acetates prepared in the usual way. On g.l.c. analysis, the alditol acetates from reduced, permethylated **1** showed peaks of almost equal intensity for 3,4,6-tri-*O*-methylmannose and 2,3,4-tri-*O*-methylglucose. The other acidic disaccharide (**2**) showed peaks identified as 2,3,4,7-tetra-*O*-methyl-D-*glycero*-L-*manno*-heptose and 2,3,4-tri-*O*-methylglucose. The results are shown in Table III.

From these results of methylation, and on consideration of the results of chromium trioxide oxidation, it became evident that the structures of the oligosaccharides are as shown in formulas **1**–**5**. None of these oligosaccharides contain any D-*glycero*-L-*gluco*-heptose, which was found in small proportion (0.8%) in the LPS.

It had already been mentioned that the LPS contains a polymer of a derivative of 4-amino-4,6-dideoxymannose[2]. Under Redmond's conditions of hydrolysis[3], our sample yielded this sugar. The high percentages of tetra-*O*-methylglucose and penta-*O*-methylheptose indicate that some mono- and oligo-saccharides are associated with this polymer. They are, presumably, attached to *O*-3 of the 4-amino-4,6-dideoxymannose residue, as this is the only free position. However, along with these structures, the feasibility of the presence of a polymer containing the repeating units (which cannot be predicted, as there are many possibilities) of some or all of the oligosaccharides mentioned cannot be excluded.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer model 241 MC spectropolarimeter at 23 ±1°C and 589.5 nm. Evaporations were conducted under diminished pressure at bath temperatures not exceeding 40°. Small volumes of aqueous solutions were lyophilized. Paper chromatography was performed on Whatman No. 1 paper, with the following solvent systems (v/v): (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) 9:2:2 ethyl acetate–acetic acid–water, and (*C*) 6:4:3 1-butanol–pyridine–water. The sugars were detected with (*1*) alkaline silver nitrate, and (*2*) 2% ninhydrin in acetone, at 110°. 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 (100–120 mesh) at 190° (for alditol acetates of sugars), and at 165° (for partially methylated, alditol acetates), (*b*) 1% of OV-225 on Gas Chrom Q (80–100 mesh) at 160° (for partially methylated sugars), and (*c*) 3% of Poly A-103 on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of amino sugars).

Chromium trioxide oxidation of lipopolysaccharide. — A mixture of LPS (16 mg) and *myo*-inositol (0.8 mg; internal standard) was dissolved in *N*,*N*-dimethylformamide (2 mL), and acetic anhydride (1.0 mL) and pyridine (1.0 mL) were added. After being stirred for 16 h at room temperature, the mixture was evaporated to dryness under vacuum, and the product reacetylated with pyridine (1.0 mL) and acetic anhydride (1 mL) with stirring for 16 h at room temperature. The pyridine and acetic anhydride were then removed in the usual way.

Powdered chromium trioxide[8] (75 mg) was added to a solution of the acetylated LPS (15 mg) in acetic acid (3 mL), and the resulting mixture was stirred in a water bath at 50°; aliquots were removed at 0, 1, and 2 h, and immediately diluted with water, the solution extracted with chloroform, and the extract washed with water, dried (anhydrous sodium sulfate), and evaporated to dryness. The samples were deacetylated with 0.2M sodium methoxide in methanol (0.2 mL) for 2 h, made neutral with dilute acetic acid, and evaporated to dryness. The materials were then hydrolyzed with 0.5M sulfuric acid during 18 h at 100°, the acid was neutralized, and the products were converted into the alditol acetates[11,12], which were analyzed by g.l.c. in columns *a* and *c*. The amounts of sugars in different aliquots were estimated from the respective, peak area.

Chromium trioxide oxidation of carboxyl-reduced lipopolysaccharide. — The LPS was reduced by the procedure of Taylor and Conrad[15]. A mixture of carboxyl-reduced LPS (15 mg) and *myo*-inositol (0.4 mg) was acetylated, and the product oxidized with chromium trioxide by the same procedure as described for the LPS.

Periodate oxidation and Smith degradation of polysaccharide. — The PS was treated with 0.04M sodium metaperiodate[9] in the dark at 5°, and the amount of periodate consumed was measured spectrophotometrically.

Polysaccharide (40 mg) was dissolved in water (80 mL) and a solution (80 mL) of 0.1M sodium metaperiodate was added[10]. The solution was kept in the dark for

24 h at 4°, an excess of ethylene glycol (5 mL) was added, and the mixture was kept for 3 h at room temperature, and dialyzed for two days against distilled water. Sodium borohydride (100 mg) was added to the solution from the dialysis bag, and after 5 h, the solution was made neutral with acetic acid, dialyzed to remove inorganic salts, and lyophilized. A portion of the product was hydrolyzed with 0.5M sulfuric acid for 16 h at 100°, the hydrolyzate made neutral with barium carbonate, and the product converted into the alditol acetates, and analyzed by g.l.c. in columns *a* and *c*.

Isolation of oligosaccharides. — The lipopolysaccharide (200 mg) was hydrolyzed with 0.5M hydrochloric acid (20 mL) for 2 h at 100°, the optimum conditions for obtaining a good yield of oligosaccharides being found from pilot experiments. The hydrolyzate was washed with diethyl ether (to remove the lipid), made neutral with silver carbonate, and the suspension centrifuged. The precipitate was washed repeatedly, and the washings and the supernatant liquor were combined, and concentrated to ~2 mL. The hydrolyzate was then passed successively through columns (10 × 1.5 cm) of Dowex-50W X-8 (H^+) and Dowex-1 X-4 (HCO_3^-) ion-exchange resins, in order to trap the basic and acidic oligosaccharides, respectively. The columns were thoroughly washed, and the neutral oligosaccharides were collected, and concentrated to a small volume. The column of Dowex-50W X-8 (H^+) was eluted first with 1.5M hydrochloric acid (25 mL) and then with M hydrochloric acid (50 mL) to give the basic oligosaccharides.

The solution was then made neutral with silver carbonate, and the suspension centrifuged. The precipitate was thoroughly washed with water and centrifuged. The supernatant liquor and washings were combined, and concentrated to a small volume. The acidic oligosaccharides were eluted from the column of Dowex-1 X-4 (AcO^-) with 30% acetic acid (75 mL). The eluate was evaporated to dryness under diminished pressure, and a trace of acetic acid was removed by co-distillation with methanol. The neutral, acidic, and basic oligosaccharides were then isolated by preparative paper-chromatography in solvent *B*.

Acid hydrolysis of oligosaccharides. — The oligosaccharides (~0.5 mg each) were hydrolyzed with 0.5M sulfuric acid for 18 h at 100°. The excess of acid was neutralized, and the hydrolyzed sugars were converted into the alditol acetates, which were then analyzed by g.l.c. in columns *a* or *c*.

Determination of reducing ends of oligosaccharides. — The oligosaccharides (~0.5 mg each) were reduced with sodium borohydride (10–15 mg). The solutions were made neutral with acetic acid, and boric acid was removed as methyl borate. The reduced oligosaccharides were then hydrolyzed with 0.5M sulfuric acid for 18 h at 100°. The hydrolyzates were made neutral with barium carbonate, the suspension was filtered, and the filtrate was evaporated under diminished pressure. The samples were then acetylated by the usual procedure, and the acetates analyzed by g.l.c. in columns *a* and *c*.

Methylation of oligosaccharides. — The oligosaccharides were methylated by the Kuhn procedure[13]. To a solution of each of the oligosaccharides (~1 mg) in *N*,*N*-dimethylformamide (1 mL) were added silver oxide (0.4 g) and Drierite (0.25 g),

the mixture was stirred for 30 min, and methyl iodide (0.3 mL) was added. Stirring was continued for 20 h in the dark, chloroform (10 mL) was added, and the mixture was vigorously stirred. The solids were filtered off through a bed of Celite, and the filtrate was washed with water (4 × 25 mL), dried (anhydrous sodium sulfate), and evaporated to dryness. The products were then hydrolyzed under the same conditions, and the alditol acetates were prepared, and analyzed by g.l.c. in columns *a* and *b*.

Reduction of methylated, acidic oligosaccharides. — Methylated acidic oligosaccharides (~1 mg each) were reduced with lithium aluminum hydride[14] in 1:2 diethyl ether–dichloromethane, and the reduced, methylated oligosaccharides were hydrolyzed with 0.5M sulfuric acid for 12 h at 100°, converted into the alditol acetates, and analyzed by g.l.c. in columns *a* and *b*.

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