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STUDIES ON THE PARTIAL STRUCTURE OF THE O-ANTIGEN OF *Vibrio cholera* OGAWA G-2102

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ABSTRACT

Detailed information was obtained regarding the partial structure of the lipopolysaccharide (LPS), containing glucose, glucuronic acid, 2-amino-2-deoxy-glucose, L-*glycero*-D-*gluco*-heptose, and small proportions of L-*glycero*-D-*manno*-heptose, mannose, and galactose, isolated from *Vibrio cholera* Ogawa G-2102. Structures of three oligosaccharides were determined. Results of deamination experiments established the sequence of the linkages between the amino sugar and heptose residues in the O-antigenic polysaccharide.

INTRODUCTION

In a previous communication[1], we reported the results of preliminary examinations of the lipopolysaccharide (LPS), the O-antigenic polysaccharide (OPS), the core polysaccharide (CPS), and the carboxyl-reduced O-antigenic polysaccharide (CR-OPS) derived from the LPS isolated from *Vibrio cholera* Ogawa G-2102. The nature of the linkages of the different sugar residues was shown by methylation analysis and periodate-oxidation studies. The perosamine polymer[2], which resists hydrolysis under normal conditions of acid hydrolysis, is present in both the Ogawa G-2102 and Inaba 569B[3,4] strains of *Vibrio cholera*. The partial structures of other sugar residues of the strain Ogawa G-2102, some of which inhibit the antigen–antibody reaction between LPS/OPS and the antisera raised against the whole organism in rabbits, have not yet been ascertained. (In the case of Inaba 569B, some of the oligosaccharides[4] inhibit the antigen–antibody reaction.) We now report the results of detailed, structural studies conducted on the O-antigen of *V. cholera* Ogawa G-2102.

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RESULTS AND DISCUSSION

The major heptose present in the LPS, OPS, and CPS of *Vibrio cholera*, Ogawa G-2102, has been identified. The heptose, which was shown to be identical in behavior to authentic L-*glycero*-D-*gluco*-heptose (synthesized by the condensation of nitromethane[5] with L-galactose), both by g.l.c. (column *a*, R_{Hep} 2.30) and paper chromatography (solvent *B*, R_{Glc} 0.90), was proved to be L-*glycero*-D-*gluco*-heptose by isolating the compound in pure state, by resolution on paper, and determining its specific rotation; it had $[\alpha]^{23}_{589.5}$ −24.8° (in water); lit.[6] $[\alpha]_D^{23}$ −26.7° (in water).

Perosamine (4-amino-4,6-dideoxy-D-mannose) was identified in the LPS by hydrolyzing the LPS with 10M HCl for 30 min at 80°, and then analyzing the product by t.l.c. using 9:1 diethyl ether-acetone[7]; R_F 0.26. It was estimated by using the Ehrlich reagent[8], and was found to contain 9.5% of perosamine.

Guided by the results of pilot experiments, the OPS (200 mg) was hydrolyzed partially with 0.5M HCl for 1.5 h at 100°. The acidic, neutral, and basic oligosaccharides were resolved by chromatography on columns of ion-exchange resins. Paper chromatography of the acidic part, recovered from the anion-exchange resin column, in solvent *B* showed one spot, having R_{Glc} 0.55, along with some faint spots of higher oligomers. This oligosaccharide (**1**) was isolated by preparative paper-chromatography (yield 1.9 mg); it had $[\alpha]^{23}_{589.5}$ +10.6° (in water).

Paper chromatography of the neutral fraction in solvent *B* showed one major spot of an oligosaccharide having R_{Glc} 0.72, in addition to heavy spots of monosaccharides and some faint spots of higher oligomers. The oligomer was separated, and isolated by preparative paper-chromatography. The oligosaccharide (**2**; 3.8 mg) had $[\alpha]^{23}_{589.5}$ +16° (in water).

On paper chromatography in solvent *B*, the basic fraction, isolated from the cation-exchange resin column, showed one major spot of oligosaccharide, along with a spot for 2-amino-2-deoxyglucose. This oligosaccharide (**3**), having R_{Glc} 0.24, was also isolated by preparative paper-chromatography in the same solvent. The oligosaccharide (2.5 mg) had $[\alpha]^{23}_{589.5}$ +10° (in water).

The oligosaccharides **1**–**3** (~0.5 mg each) were separately hydrolyzed with

TABLE I

THE SUGARS PRESENT IN THE HYDROLYZATES OF THE OLIGOSACCHARIDES **1** TO **3**

Sugars as alditol acetates[a]	*Proportion in oligosaccharides (mol %)*		
	1	**2**	**3**
Glucose		47.5	28
L-*glycero*-D-*gluco*-Heptose	100	52.5	47
2-Amino-2-deoxyglucose			25

[a]Analyzed by g.l.c. in column *a*, at 180°.

0.5M sulfuric acid for 18 h on a boiling-water bath, and the hydrolyzates, after the usual treatment, were converted into their alditol acetates, and these analyzed by g.l.c. using column *a*. The acidic oligosaccharide (**1**) showed only a peak corresponding to L-*glycero*-D-*gluco*-heptose. The neutral oligosaccharide (**2**) showed peaks of glucose and L-*glycero*-D-*gluco*-heptose in the ratio of 1:1. The basic oligosaccharide (**3**) showed peaks of glucose, 2-amino-2-deoxyglucose, and L-*glycero*-D-*gluco*-heptose in the ratios of 1:1:2. Interestingly, none of these major oligosaccharides contained L-*glycero*-D-*manno*-heptose, mannose, or galactose. The results are shown in Table I.

In order to ascertain which were the reducing ends in these oligosaccharides, the acidic, neutral, and basic oligosaccharides were reduced with sodium borohydride for 6 h; after neutralization of the base, the products were hydrolyzed with 0.5M H_2SO_4 for 18 h at 100°. The hydrolyzates, after being made neutral, were acetylated by the usual method. G.l.c. analysis in column *a* revealed the presence of only L-*glycero*-D-*gluco*-heptose in all of the oligomers, showing that this heptose is present as reducing sugar in these oligosaccharides.

The oligosaccharides **1–3** were separately methylated by the Kuhn method[9]. Each methylated oligosaccharide showed no peak for OH absorption in its i.r. spectrum. Portions of these methylated oligosaccharides were hydrolyzed with 0.5M H_2SO_4 and the hydrolyzates converted into their alditol acetates by following the usual procedure. G.l.c. analysis (column *a*) of these partially methylated alditol acetates showed the presence of 2,3,4,7-tetra-*O*-methyl-L-*glycero*-D-*gluco*-heptose in the methylation product of oligosaccharide **1** (confirmed by g.l.c.–m.s.). Oligosaccharide **2** yielded in this way 2,3,4,6-tetra-*O*-methylglucose (TMG) and 3,4,6,7-tetra-*O*-methyl-L-*glycero*-D-*gluco*-heptose (confirmed by g.l.c.–m.s.). Analysis of the alditol acetates of the methylated sugars obtained from the methylation product of the basic oligosaccharide **3** in column *a* at 190° showed four peaks, corresponding to 2,3,4,6-tetra-*O*-methylglucose, 2-deoxy-3,6-di-*O*-methyl-2-(methylamino)glucose, 3,4,6,7-tetra-*O*-methyl-L-*glycero*-D-*gluco*-heptose, and 2,3,4,7-tetra-*O*-methyl-L-*glycero*-D-*gluco*-heptose.

As the glucose unit had been shown[1] to have the α configuration, from these results the structure of the only neutral oligosaccharide **2** could be established as a disaccharide having the following structure.

α-Glc-(1→2)-L-*glycero*-D-*gluco*-heptose

2

To determine the linkage of the uronic acid in the acidic oligomer, the methylation product from oligosaccharide **1** was reduced[10] with lithium aluminum hydride, and the product hydrolyzed; the alditol acetates were prepared by the usual method. G.l.c. analysis of the alditol acetates from the permethylated and reduced oligosaccharide **1** showed the presence of about equal proportions of 2,3,4-tri-*O*-methylglucose and 2,3,4,7-tetra-*O*-methylheptose. The results are given in

TABLE II

RESULTS OF METHYLATION ANALYSIS OF THE OLIGOSACCHARIDES **1** TO **3**

Methylated sugars[a] *as alditol acetates*	*Retention time (column a)*	*Alditol acetates (mol %)*[b]			
		1	*2*	*3*	*4*
2,3,4,6-Glc	1			51	28
2,3,4,7-Hep	2.30	100	54		25
2,3,4-Glc	2.49		46		
3,4,6,7-Hep	2.42			49	25
3,6-GlcNMe	1.69[c]				

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methylglucose, *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 acetates from permethylated disaccharide **2**. 4, Alditol acetates obtained from permethylated tetrasaccharide **3**. [c]Retention time at 190°.

Table II. As the glucuronic acid unit had been shown[1] to have the β configuration, these results indicated that oligosaccharide **1** has the following structure.

β-GlcA-(1→6)-L-*glycero*-D-*gluco*-heptose

1

Deamination[11] studies were conducted in order to determine the position and linkage of the amino sugar residue in the polysaccharide. The OPS (20 mg) was first *N*-deacetylated[12] by heating it with 2M dimsyl sodium and sodium benzenethioxide for 17 h at 100° in a sealed tube. After dialysis against distilled water, the contents of the dialysis bag were lyophilized. The *N*-deacetylated material was deaminated with nitrous acid, and the excess of acid was removed by means of a vacuum pump. The deaminated OPS thus obtained was reduced with $NaBH_4$. The yield of deaminated OPS was 11.7 mg, and it had $[\alpha]_D^{23}$ +4.5° (in water).

The deaminated OPS (3 mg) was hydrolyzed with 0.5M H_2SO_4 for 18 h at 100°. The hydrolyzate was converted into alditol acetates by following the usual procedure. G.l.c. analysis, in column *a*, of the alditol acetates showed the peaks of rhamnose and 2,5-anhydromannitol, the latter being identified by preparing the authentic compound from 2-amino-2-deoxy-D-glucose hydrochloride (R_1 value, 0.403). The ratios of rhamnose:2,5-anhydromannitol:glucose were 1.3:1:1.5. The peak for rhamnose was derived from the deamination of perosamine (4-amino-4,6-dideoxy-D-mannose). The ratio of 2,5-anhydromannitol:glucose was lower than that of 2-amino-2-deoxyglucose:glucose in the original OPS, due to the fact that 2,5-anhydromannitol is one of the possible products from the deamination of 2-amino-2-deoxyglucose.

Another portion of deaminated OPS was methylated by the Hakomori[13] and then by the Kuhn[9] method. The permethylated material was hydrolyzed with 0.5M H_2SO_4 for 16 h at 100°. The hydrolyzate was converted into the alditol acetates by

the usual methods. G.l.c. analysis of the products showed the presence of 2,5-anhydro-1,3,6-tri-*O*-methylmannitol acetate (confirmed by co-injection with an authentic sample) and 4-*O*-methylrhamnose. The identification of 2,5-anhydro-1,3,6-tri-*O*-methylmannitol acetate further proved that the 2-amino-2-deoxyglucose is (1→4)-linked. The proportion of 3,4,6,7-tetra-*O*-methyl-L-*glycero*-D-*gluco*-heptose was very much lower (in comparison with penta-*O*-methylheptose) than in the original, methylated OPS. The methylation results indicated that the methylated 2-amino-2-deoxyglucose is linked through O-1 to O-2 of 3,4,6,7-tetra-*O*-methylheptose. These results indicate two possible structures for oligosaccharide **3**, as follows, where Hep is L-*glycero*-D-*gluco*-heptose.

α-Glc-(1→6)-β-Hep-(1→4)-α-GlcNAc-(1→2)-Hep

or

α-Glc-(1→4)-α-GlcNAc-(1→2)-β-Hep-(1→6)-Hep

3

EXPERIMENTAL

Materials and methods. — Paper partition-chromatography was performed on Whatman Nos. 1 and 3 MM papers. The solvent systems (v/v) used were (*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 spray reagents used were (*1*) alkaline silver nitrate, and (*2*) 0.2% ninhydrin in acetone, at 110°. All solvents were distilled before use, and all evaporations were conducted below 50°. Optical rotations were measured with a Perkin–Elmer model 241MC spectropolarimeter at 23 ±1° and 589.5 nm. Colorimetric estimations were conducted with a Yanaco Model Sp-1 spectrophotometer.

Gas–liquid chromatography (g.l.c.) was performed with a Hewlett–Packard 5730A gas chromatograph equipped with a flame-ionization detector. 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 180° (for alditol acetates of sugars), and at 155° (for partially methylated alditol acetates), (*b*) 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 165° (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).

Isolation of oligosaccharides. — The O-antigenic PS (120 mg) was hydrolyzed with 0.5M hydrochloric acid (12 mL) for 1.5 h at 100°, the optimum conditions for a good yield of oligosaccharides having been obtained from pilot experiments [paper chromatography using solvent systems (*A*) and (*B*)]. The hydrochloric acid was removed over phosphorus pentaoxide and sodium hydroxide pellets in a vacuum desiccator. An aqueous solution of the hydrolyzate (~2 mL) was then successively passed through columns (10 × 1.0 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 oligosac-

charides, respectively. The columns were thoroughly eluted with water to collect the neutral oligosaccharides, and the eluate was concentrated to a small volume. The column of Dowex-50W X-8 (H^+) was eluted first with 1.5M hydrochloric acid (35 mL) and then with M hydrochloric acid (40 mL) in order to obtain the basic oligosaccharides.

The hydrochloric acid solution was evaporated to dryness and then freed of acid over anhydrous phosphorus pentaoxide and sodium hydroxide pellets in a vacuum desiccator. The anion-exchange resin column was eluted with 10% formic acid (75 mL). The eluate containing acidic oligosaccharide was evaporated under diminished pressure, and a trace of formic acid was removed by co-distillation with water. The neutral, acidic, and basic oligosaccharides were then isolated in pure state by preparative paper-chromatography in solvent *B*.

Acid hydrolysis of oligosaccharides. — The oligosaccharides (0.38 mg of **1**, 0.7 mg of **2**, and 0.5 mg of **3**) were hydrolyzed separately with 0.5M sulfuric acid for 18 h at 100°. The excess of acid was neutralized, and the various sugars in the hydrolyzates were converted into alditol acetates, which were then analyzed by g.l.c. using columns *a* and *c*.

Determination of reducing ends of oligosaccharides. — The oligomers (~0.4 mg of each) were reduced with sodium borohydride (15–20 mg). The solutions were made neutral with acetic acid, and boric acid was removed as volatile methyl borate. The reduced oligosaccharides were then hydrolyzed with 0.5M sulfuric acid for 18 h at 100°. The hydrolyzate was made neutral with barium carbonate, the suspension filtered through a bed of Celite, and the filtrate evaporated under diminished pressure. The alditols were then acetylated by the usual method, and the acetates analyzed by g.l.c. in columns *a* and *c*.

Methylation of oligosaccharides. — The oligosaccharides were methylated by the Kuhn method[9]. The oligosaccharides (1.1 mg of **1**, 2 mg of **2**, and 1.6 mg of **3**) were dissolved in *N,N*-dimethylformamide (0.5 mL), silver oxide (0.3 g) and Drierite (0.2 g) were added, and the suspension was stirred magnetically for 45 min. Methyl iodide (0.5 mL) was added, stirring was continued for 30 h in the dark, chloroform (15 mL) was added, and the mixture was vigorously stirred. The solids were filtered off through a Celite bed, and the filtrate was washed with water (3 × 20 mL), dried (anhydrous sodium sulfate), and evaporated to dryness. The process was repeated once, to ensure complete methylation. The products were hydrolyzed with 0.5M sulfuric acid for 16 h at 100° and the methylated sugars were converted into alditol acetates by the usual method. These were then examined by g.l.c. in column *a*.

Reduction of methylated oligosaccharide. — Methylated oligosaccharide **1** (0.6 mg) was reduced with lithium aluminum hydride[10] in 1:2 diethyl ether–dichloromethane, and the reduced, methylated oligosaccharide was hydrolyzed with 0.5M sulfuric acid for 15 h at 100°; the products were converted into alditol acetates, and these were analyzed by g.l.c. in column *a*. The results are given in Table II.

TABLE III

RESULTS OF METHYLATION ANALYSIS OF DEAMINATED OPS

Methyl sugars[a] *as alditol acetate*	*Retention time*[b] *in column* a	*Approximate mol proportion*	
		Deaminated OPS	*OPS*
2,3,4,6-Glc	1	2.3	3.6
2,3,4-Glc	2.49	3.9	2.8
2,3,4,7-Hep	2.30	3.2	2.85
3,4,6,7-Hep	2.42	0.6	1.4
1,3,6-Man-2,5-an	0.67	7.5	—
4-Rha	1.71	5.0	—
2,3,4,6,7-Hep	2.03	2.2	1.9
2,6-Glc	3.49	1.0	1.0

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methylglucose, *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 column *a* at 155°.

Deamination[11] *studies.* — OPS (20 mg) was *N*-deacetylated[12] by heating it with sodium benzenethioxide (1.1 mg) in water (1 mL) and 2M dimsyl sodium in dimethyl sulfoxide (5 mL) in a sealed tube for 17 h at 100°. The mixture was diluted with water, the precipitate of diphenyl sulfide formed during the reaction was filtered off, and the filtrate was dialyzed against water. The dialyzate was concentrated, and lyophilized.

The *N*-deacetylated OPS (15 mg) was dissolved in water (0.5 mL), and treated successively with a 5% aqueous solution of sodium nitrite (0.9 mL) and 33% aqueous acetic acid (0.9 mL) in an ice bath. After being kept for 1.5 h at room temperature, the mixture was treated with Dowex-50W X-8 (H^+) resin, and freeze-dried. The residue was reduced with $NaBH_4$ (10 mg) in water (1 mL), and the base was neutralized with Dowex-50W X-8 (H^+) resin. Boric acid was removed as methyl borate by repeated codistillation with methanol. The yield of deaminated OPS was 11.7 mg, and it had $[\alpha]_{589.5}^{23}$ +4.5° (water). A part of the deaminated material (3 mg) was hydrolyzed with 0.5M H_2SO_4 for 18 h at 100°, and the alditol acetates, prepared in the usual way, were analyzed by g.l.c. in column *a*.

Another part (5 mg) of the deaminated material was methylated by the Hakomori[13] and then by the Kuhn method[9], to yield a product showing no OH absorption band in its i.r. spectrum. The methylated product was hydrolyzed with 0.5M H_2SO_4 for 16 h at 100°. From the hydrolyzate, the alditol acetates were prepared by following the usual procedure, and these were analyzed by g.l.c. in column *a*. The results are given in Table III.

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REFERENCES

1 M. MAJUMDER, A. K. MUKHERJEE, B. G. THAKURTA, A. DUTTA, AND D. SASMAL, *Carbohydr. Res.*, 108 (1982) 269–278.
2 (a) L. KENNE, B. LINDBERG, P. UNGER, T. HOLME, AND J. HOLMGREN, *Carbohydr. Res.*, 68 (1979) c14–c16; (B) L. KENNE, B. LINDBERG, AND P. UNGER, *Carbohydr. Res.*, 100 (1982) 341–349.
3 A. K. SEN, A. K. MUKHERJEE, B. G. THAKURTA, A. DUTTA, AND D. SASMAL, *Carbohydr. Res.*, 72 (1979) 191–199.
4 A. K. SEN, A. K. MUKHERJEE, B. G. THAKURTA, A. DUTTA, AND D. SASMAL, *Carbohydr. Res.*, 86 (1980) 113–121.
5 J. C. SOWDEN, *Adv. Carbohydr. Chem.*, 6 (1951) 291–318.
6 J. C. SOWDEN AND D. R. STROBACH, *J. Am. Chem. Soc.*, 82 (1960) 954–955.
7 J. W. REDMOND, *FEBS Lett.*, 50 (1975) 147–149; *Biochim. Biophys. Acta*, 542 (1978) 378–384.
8 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Clin. Biochem.*, 9 (1976) 269–274.
9 (a) R. KUHN, H. TRISCHMANN, AND I. LOW, *Angew. Chem.*, 67 (1955) 32–37; (b) H. G. WALKER, JR., M. GEE, AND R. M. MCCREADY, *J. Org. Chem.*, 27 (1962) 2102–2103.
10 B. LINDBERG, J. LONNGREN, AND W. NIMMICH, *Carbohydr. Res.*, 23 (1972) 45–55.
11 G. LEDDERHOSE, *Z. Physiol. Chem.*, 4 (1880) 139–159.
12 C. ERBIG, K. GRANATH, L. KENNE, AND B. LINDBERG, *Carbohydr. Res.*, 47 (1976) c5–c7.
13 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.