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STRUCTURAL STUDIES ON A CARBOHYDRATE CHAIN ISOLATED FROM THE LIPOPOLYSACCHARIDE OF *Shigella boydii* TYPE 8

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ABSTRACT

The lipopolysaccharide isolated from the cells of *Shigella boydii* type 8 bacteria gave precipitin bands against homologous antisera on Ouchterlony plates, whereas the carbohydrate-containing fractions obtained from it did not. One of the fractions was obtained in major proportion and contained 23.5% of sugars. A structure was assigned to the carbohydrate chain in this material by using the results of methylation, periodate oxidation, and deamination studies.

INTRODUCTION

The sugar compositions of the lipopolysaccharides (LPS) derived from the fifteen types of well-defined *Shigella boydii* bacteria were reported[1] to contain D-glucose, D-galactose, 2-amino-2-deoxy-D-glucose, heptose, and 3-deoxyoctulosonic acid. In addition to these basal sugars, D-mannose. L-quinovosamine, 2-amino-2-deoxy-D-galactose, and 3,6-dideoxyhexose were also detected as constituents of some of the materials.

Detailed, structural elucidation of the O-specific polysaccharide of various strains of *Shigella boydii*, except that[2] of type 6, have not been conducted. The present communication contains the results of structural investigations of carbohydrate material obtained from the LPS of *Shigella boydii* type 8.

RESULTS AND DISCUSSION

The dried cells of *Shigella boydii* type 8 were extracted with 45% phenol at 65–68°. On centrifugation in the cold, the extract gave three layers. The upper, aqueous layer, containing LPS and nucleic acid, was dialyzed and lyophilized. The

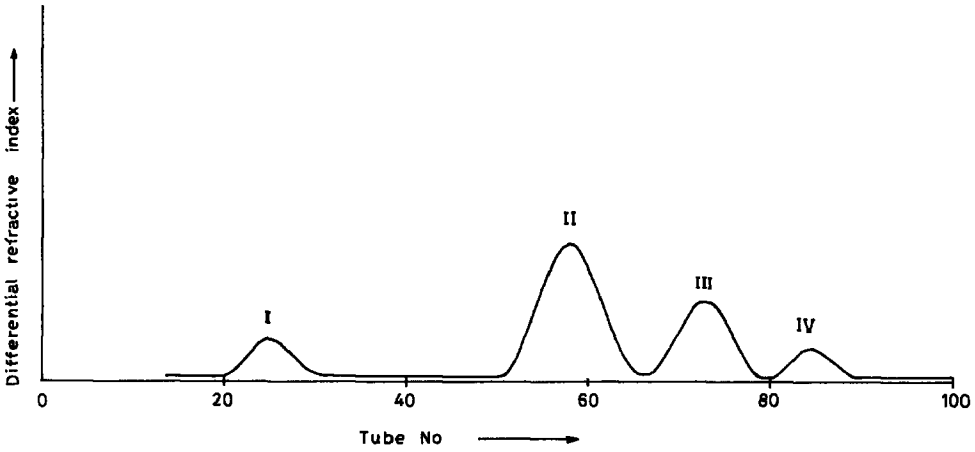

Fig. 1. Fractionation of the products, obtained after delipidification of the LPS, by use of a column of Sephadex G-100.

nucleic acid was separated as an insoluble complex with Cetavlon. The LPS was further purified by gel filtration through a column of Sephadex G-100. It contained D-glucose (3.9), D-galactose (2.3), L-*glycero*-D-*manno*-heptose (1.6), 2-amino-2-deoxy-D-glucose (1.4), and 2-amino-2-deoxy-D-galactose (0.6%). The LPS was heated with 1% acetic acid, and then centrifuged. The upper, aqueous layer was lyophilized, and then fractionated on a column of Sephadex G-100, to give four fractions (see Fig. 1).

Fraction I contained nucleic acid, as it showed absorption at 260 nm. The sugar compositions of the other three fractions were determined, and the results are given in Table I. Of these three fractions, Fraction II, obtained in major proportion,

TABLE I

SUGAR COMPOSITION OF THE VARIOUS MATERIALS

Material	*Sugar content*[a]									
	Glucose		*Galactose*		*2-Amino-2-deoxyglucose*		L-glycero-D-manno-*Heptose*		*2-Amino-2-deoxygalactose*	
	%	*Molar proportion*	%	*Molar proportion*	%	*Molar proportion*	%	*Molar proportion*	%	*Molar proportion*
Lipopoly-saccharide	3.9	1.0	2.3	0.6	1.4	0.4	1.6	0.4	0.6	0.1
Fraction II	9.5	2.1	4.6	1.0	9.4	2.0				
Fraction III	5.3	1.0	4.8	0.9	1.5	0.3	2.4	0.5	4.1	0.8
Fraction IV	6.0									
IO_4-Oxidized material	4.5	1.0	4.6	1.0						

[a]Estimated by g.l.c., using *myo*-inositol as the internal standard.

TABLE II

METHYLATION ANALYSIS OF FRACTION II, ISOLATED FROM *Shigella boydii* TYPE 8 BACTERIA, AND ITS VARIOUS DEGRADATIVE PRODUCTS

Methylated sugars	*Retention time*[a]		*Mole proportion*			*Mode of linkage*
	column A	*column B*	*A*[b]	*B*[b]	*C*[b]	
2,3,4,6-Tetra-*O*-methylglucose	**1.00**	**1.00**	1.0		1.0	Glc*p*-(1→
2-Amino-2-deoxy-3,4,6-tri-*O*-methylglucose	1.31	1.53	1.1			Glc*p*NAc-(1→
2,4,6-Tri-*O*-methylglucose	1.95	1.83	1.3	1.0	1.3	→3)-Glc*p*-(1→
2-Amino-2-deoxy-3,4-di-*O*-methylglucose	2.44	2.32	1.1			→6)-Glc*p*NAc-(1→
3,6-Di-*O*-methylgalactose	4.35	3.81	1.2	1.1	1.2	→4)-Gal*p*-(1→ 2 ↑

[a]Retention times relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on column *A* (3% of ECNSS-M) at 170°, and column B (3% of OV-225) at 170°. [b]A, methylated Fraction II; B, IO_4-oxidized, methylated Fraction II; C, deaminated, methylated Fraction II.

was retained in the dialysis bag, whereas the other two materials passed through it. The LPS gave a single band in Ouchterlony gel-diffusion[3] against homologous antiserum, whereas fractions II, III, and IV did not give any band, indicating that none of them were of high molecular weight.

Further investigations were conducted on Fraction II. It had $[\alpha]_D^{26}$ +1°. The sugar content of this fraction was 23.5%; the remaining portion consisted of lipids that could not be removed under mild conditions. The material showed absorption bands at 1650 and 1570–1560 cm^{-1} in the i.r. spectrum, characteristic of an amide grouping.

On electrophoresis, Fraction II moved as a single substance, indicating its homogeneity. The fully methylated derivative of Fraction II was hydrolyzed, to yield a mixture of methylated sugars which were identified, and the proportions estimated, by g.l.c. The results are given in Table II. Isolation and characterization of 2,3,4,6-tetra-*O*-methyl-D-glucose (1 mol) and 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose (1 mol) indicated that the carbohydrate chain contains one nonreducing end each of D-glucopyranosyl and 2-amino-2-deoxy-D-glucosyl groups. It also contains 1 mol each of (1→3)-linked D-glucosyl and (1→6)-linked 2-amino-2-deoxy-D-glucosyl residues. The chain is branched, and the D-galactopyranosyl residue at the branch points is (1,2,4)-linked.

Fraction II was subjected to periodate oxidation. On hydrolysis, a portion of the periodate-oxidized, reduced material gave glucose and galactose in the molar ratio of 1 : 1; 2-amino-2-deoxyglucose was absent. On methylation, hydrolysis followed by the usual treatment, and g.l.c. examination, this material gave peaks corresponding

to 2,4,6-tri-*O*-methylglucose and 3,6-di-*O*-methylgalactose. Mild hydrolysis of the periodate-oxidized, reduced material with acid, followed by gel filtration through a column of Sephadex G-25, gave a fraction containing a disaccharide composed of glucose and galactose in the molar ratio of 1 : 1. These results indicate the presence, in the carbohydrate chain, of the following fragment.

$$\rightarrow 3)\text{-Glc}p\text{-}(1\rightarrow 4)\text{-Gal}p\text{-}(1\rightarrow$$
$$\begin{array}{c} 2 \\ \uparrow \end{array}$$

Deamination[4] studies were conducted in order to identify the positions of the amino sugar residues in the carbohydrate chain. On g.l.c. examination of a portion, the deaminated product was found to contain 4.5% of 2,5-anhydromannitol, whereas the hydrolyzate had 9.1%. These results suggest that, of the two moles of 2-amino-2-deoxy-D-glucose, one is present as a (nonreducing) end-group, and the other is in the chain.

Methylation analysis of the deaminated product (see Table II, column C) showed the presence of 2,3,4,6-tetra- and 2,4,6-tri-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-galactose in almost equimolar proportions. The presence of 2,3,6-tri-*O*-methyl-D-galactose in this hydrolyzate (instead of the 3,6-di-*O*-methyl-D-galactose in the hydrolyzate of methylated Fraction II) confirms that one 2-amino-2-deoxy-D-glucosyl group is linked to O-2 of a D-galactosyl residue in the chain; the other one is obviously a residue at the "reducing" end of the chain and is glycosidically linked to the aglycon part of the molecule.

From all of these results, the structure assigned to the carbohydrate chain of Fraction II is as follows.

$$\text{Glc}p\text{-}(1\rightarrow 3)\text{-Glc}p\text{-}(1\rightarrow 4)\text{-Gal}p\text{-}(1\rightarrow 6)\text{-Glc}p\text{NAc-}(1\rightarrow\text{aglycon part}$$
$$\begin{array}{c} 2 \\ \uparrow \\ 1 \\ \text{Glc}p\text{NAc} \end{array}$$

EXPERIMENTAL

General. — All evaporations were conducted under diminished pressure at bath temperatures of not more than 40°. Optical rotations were recorded with a Perkin–Elmer model 241 MC Polarimeter at 26°. Partition chromatography was performed by the descending technique on Whatman No. 1 chromatography paper, using the following solvent systems (v/v): (*A*) 8:2:1 ethyl acetate–pyridine–water, and (*B*) butanol–ethanol–water (4:1:5, upper phase). The spray reagents used were (*1*) alkaline silver nitrate and (*2*) 0.2% ninhydrin in acetone. A Hewlett–Packard model 5730A gas chromatograph fitted with a flame-ionization detector was used; nitrogen was the carrier gas. The columns (1.8 m × 6 mm) used were (*a*) 3% of ECNSS-M on Gas Chrom Q (100–200 mesh), (*b*) 3% of OV-225 on Gas Chrom Q

(100–200 mesh), and (*c*) Poly-A on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of amino sugars). High-voltage electrophoresis was conducted in a Shandon model L-24 apparatus. I.r. spectra were recorded with a Beckman IR-120A instrument having cesium bromide optics, and u.v. and visible spectra with Cary 17D and Carl Zeiss VSU2-P spectrometers, respectively. Elutions were monitored with a Waters Associates Model R-403 differential refractometer.

A strain of *Shigella boydii* type 8 (NCTC-254) supplied by NCTC, London, was grown on a large scale in sterile media containing Difco bactoagar and brain-heart infusion (Difco) at pH 7.4 in Roux bottles. The bottles were incubated for 48 h at 37°, and the bacteria were collected by washing with saline. The cell suspension from 200 bottles was centrifuged at 18,000 r.p.m., and the sediment was successively washed with saline (twice) and acetone; weight of the dry cells, 18 g.

Isolation of the lipopolysaccharide. — The dry cells (11.5 g) were suspended in water (200 mL) at 65–68°, and an equal volume of 90% phenol at 65–68° was added to it, while stirring. After 15 min, the mixture was cooled to 4°, and centrifuged at 4000 r.p.m. for 40 min. The upper, aqueous layer was collected, exhaustively dialyzed against distilled water, and lyophilized; yield, 800 mg. The material was dissolved in water (120 mL), and an aqueous 2% Cetavlon solution (12 mL) was added while stirring. The precipitated nucleic acid–Cetavlon complex was removed by centrifugation, and the supernatant liquor was dialyzed, concentrated, and lyophilized, to yield a solid. The lipopolysaccharide (LPS) was dissolved in 0.5M sodium chloride solution (45 mL), and reprecipitated by adding ethanol. The precipitated LPS was collected at the centrifuge, dialyzed, and freeze-dried; yield, 574 mg.

Removal of lipid. — The LPS (400 mg) was heated with 1% acetic acid (40 mL) for 1.5 h at 100°, and the precipitated lipid was removed by centrifugation. The supernatant liquor was lyophilized, to yield a solid (173 mg). A solution of the material in pyridine acetate buffer, pH 4.5 (1.5 mL) was added to a column (70 × 3.5 cm) of Sephadex G-100 which was eluted with the same buffer. The material was separated into four fractions (see Fig. 1). Fraction I (test-tubes 20–30) showed u.v. absorption at 260 nm, and was identified as nucleic acid. Fraction II (52–66), obtained in major amount, was lyophilized, to yield a solid product; yield, 94 mg. Fraction III (67–78), yield, 25 mg, and Fraction IV (80–88), yield, 10 mg. On dialysis, Fraction II remained in the dialysis bag, whereas Fractions III and IV passed through it.

Test for homogeneity. — The LPS and Fraction II were subjected to high-voltage electrophoresis for 45 min, using Whatman No. 3 MM paper and pyridine acetate buffer, pH 4.5, at a potential gradient of 40 V/cm. On spraying with ninhydrin, a single spot moving towards the cathode was observed in both cases. When electrophoresis was conducted for 2 h on a plate of silica gel, with the same buffer, and 1% sulfuric acid as the spray reagent, a single spot was obtained. In Ouchterlony gel-diffusion, using rabbit antisera raised against whole cells, one band was obtained with the LPS, whereas the other fractions did not give any band.

Monosaccharide composition. — The LPS, and Fractions II, III, and IV were

hydrolyzed with 3M hydrochloric acid for 10 h at 100°. The excess of acid was removed under vacuum over P_2O_5 and KOH, and the last traces of acid were removed by codistillation with methanol. The hydrolyzates were examined by p.c. and g.l.c. (as the alditol acetates) for the sugar components and their amounts. The results are given in Table I. Further work was conducted by using Fraction II.

Methylation studies. — Fraction II (10 mg) was methylated by the Hakomori method[5], followed by the Purdie method[6], to give a product showing no OH absorption band in the i.r. spectrum. The material was purified by passing a solution through a column (20 × 1.5 cm) of Sephadex LH-20, using 2:1 chloroform–acetone as the eluant. The fully methylated product (7 mg) was boiled with 85% formic acid for 2 h, and the formic acid was removed in the usual way. The material was then heated with 0.5M hydrochloric acid for 5 h at 100°. After the usual treatment, the methylated sugars were converted into their alditol acetates, and these were analyzed by g.l.c. The results are given in Table II, column A.

Periodate oxidation. — To Fraction II (15 mg) in water (7.5 mL) was added 0.1M sodium metaperiodate solution (7.5 mL), and the mixture was kept for 48 h in the dark at 4°. The excess of periodate was decomposed by adding ethylene glycol (3 mL), and the solution was dialyzed against distilled water, and lyophilized; yield 14 mg. A portion (1 mg) of it was reduced with $NaBH_4$, and, after the usual treatment, the product was hydrolyzed with 3M hydrochloric acid for 10 h at 100°. The sugars in the hydrolyzate were converted into their alditol acetates, and these were identified by g.l.c. The results are given in Table I.

Another portion (6 mg) was kept with 0.5M hydrochloric acid overnight. After removing the acid in the usual way, the hydrolyzate was examined by p.c. A spot having R_{Maltose} 1.01 was detected, along with low-molecular-weight components. The mixture was separated into its components in a column (20 × 2.5 cm) of Sephadex G-25, using pyridine acetate buffer, pH 4.5, and the eluate was monitored by the phenol–sulfuric acid test. The material, collected in fractions (15–25), was concentrated, and lyophilized; yield 0.2 mg. It was found to be homogeneous in paper electrophoresis.

A portion of the oligomer obtained was hydrolyzed with 2M hydrochloric acid for 10 h at 100°, and the alditol acetates of the sugars were examined by g.l.c. Glucose and galactose were found to be present in the molar ratio of 1:1.

The periodate-oxidized, reduced material (5 mg) was methylated by the Hakomori method, the product hydrolyzed, and the methylated sugars obtained were identified by g.l.c. The results are given in Table II, column B.

Deamination studies. — Fraction II (39 mg) in formamide (1.2 mL) was acetylated with acetic anhydride (1.2 mL) in pyridine (1.8 mL). The acetylated material (36 mg) was *N*-deacetylated[7] by heating it for 17 h at 100° with sodium thiophenoxide (1.8 g) in water (1.8 mL) and 2M dimsyl sodium anion in methyl sulfoxide (10 mL) in a sealed tube. The reaction mixture was diluted with water, filtered, and the filtrate concentrated to 2 mL. The solution was passed through a column (30 × 3.5 cm) of Sephadex G-100, using water as the eluant. Each fraction

(5 mL) was tested by the phenol–sulfuric acid method. Fractions 15–25 were collected, concentrated, and lyophilized; yield 6 mg.

The *N*-deacetylated product (5 mg) was dissolved in water (1 mL), and then treated with 5% aqueous sodium nitrite (2.6 mL) and 33% acetic acid (2.6 mL) at 0°. After being kept for 2 h at room temperature, the mixture was treated with Dowex 50W X-8 (H^+) resin, and freeze-dried. The material so obtained was reduced with $NaBH_4$ (10 mg) in water (1 mL), and the product was treated with acetic acid to pH 4.5. The solution was evaporated, and the boric acid was removed by repeated addition and evaporation of methanol. The material was dissolved in water, and the solution lyophilized; yield 3.2 mg.

A part of the deaminated material was acetylated in the usual way, and the product examined by g.l.c. A peak corresponding to 2,5-anhydromannitol was detected. Using inositol as the internal standard, the proportion of the anhydro sugar was estimated by g.l.c. to be 4.5%. Another portion was hydrolyzed, and the sugars were identified, and estimated, by g.l.c.: 2,5-anhydromannitol, 9.1; glucose, 9.4; and galactose, 4.5%.

Another portion (2 mg) of the deaminated material was methylated twice by the Hakomori method, to yield a product showing no OH absorption band in the i.r. spectrum. The methylated product was hydrolyzed, and the alditol acetates of the methylated sugars were examined in the usual way by g.l.c. The results are given in Table II, column C.

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REFERENCES

1 B. A. Dmitriev, L. V. Backinowsky, N. K. Kochetkov, and N. A. Khomenko, *Eur. J. Biochem.*, 34 (1973) 513–518.
2 B. A. Dmitriev, L. V. Backinowsky, V. L. Lvov, Yu. A. Knirel, N. K. Kochetkov, and N. A. Khomenko, *Carbohydr. Res.*, 41 (1975) 329–333.
3 (a) S. B. Misra and D. L. Shrivastava, *J. Sci. Ind. Res., Sect. C*, 18 (1959) 209–212; (b) E. A. Kabat and M. M. Mayer, *Experimental Immunochemistry*, Thomas, Springfield, Illinois, 1964, pp. 85–88.
4 G. Ledderhose, *Z. Physiol. Chem.*, 4 (1880) 139–159.
5 S.-I. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
6 T. Purdie and J. C. Irvine, *J. Chem. Soc.*, 85 (1904) 1049–1070.
7 C. Erbig, K. Granath, L. Kenne, and B. Lindberg, *Carbohydr. Res.*, 47 (1976) c5–c7.