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IMMUNOCHEMICAL STUDIES ON *Shigella dysenteriae* TYPE 10 BACTERIAL POLYSACCHARIDE

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ABSTRACT

On graded hydrolysis and Smith degradation, the O-somatic polysaccharide isolated from *Shigella dysenteriae* type 10 bacteria yielded four oligosaccharides which were characterized by methylation studies. Immunochemical studies using constituent monosaccharides and the oligosaccharides indicated that the sugar grouping →3)-ManNAc-(1→3)-Rha-(1→4)-GlcNAc-(1→ was the immunodominant part in the polysaccharide molecule, and that 2-acetamido-2-deoxy-D-mannose contributed maximally to the immunological specificity of the macromolecule.

INTRODUCTION

Based on the results of methylation, Smith degradation, and oxidation with chromium trioxide, the repeating unit of the O-somatic polysaccharide of *Shigella dysenteriae* type 10 was reported by Dmitriev *et al.*[1], but it has been observed in our laboratory that the structures of O-somatic polysaccharides of some serotypes of this organism differ from those published earlier; one of them is[2] type 7. In the present investigation, the polysaccharide isolated from *Sh. dysenteriae* type 10 bacteria (strain 2050.52) was subjected to methylation and periodate oxidation with a view to checking whether the structure of the polysaccharide isolated in the present studies was identical with that used by the earlier workers[1]. The polysaccharide was subjected to graded hydrolysis and Smith degradation, and the oligosaccharides obtained were used to enhance understanding of the immunochemical specificities of different sugar groupings in the macromolecule.

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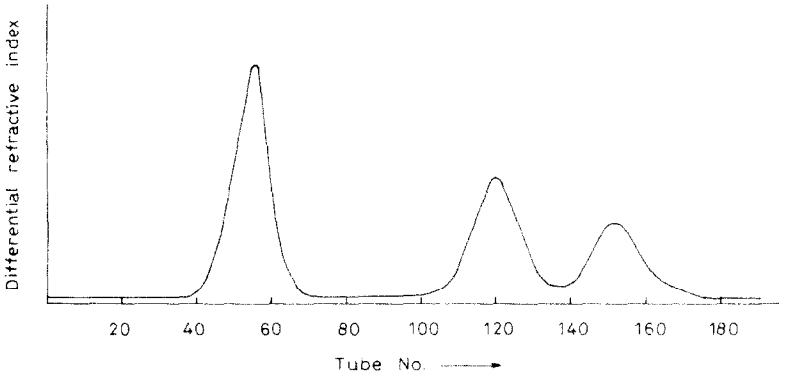

Fig. 1. Fractionation of the products obtained, after delipidification of the LPS, by using Sephadex G-75.

RESULTS AND DISCUSSION

Cells of *Shigella dysenteriae* type 10 (strain 2050.52) were obtained from a 72-h growth of the strain on brain-heart infusion-agar in Roux bottles. The proteinaceous part of the bacterial cells was removed by treatment with 45% aqueous phenol at 65–68°. The resulting product contained nucleic acid and lipopolysaccharide (LPS). The nucleic acid was precipitated as a complex with Cetavlon, and the LPS was isolated. It was further purified by passing it through a column of Sephadex G-100, to remove the remaining nucleic acid. The LPS was eluted as a single substance in 87% yield, and it had $[\alpha]_D^{25}$ +39°.

The LPS was cleaved by heating in 1% acetic acid for 1.5 h at 100°. After removing the lipid by centrifugation, the material was fractionated on a column of Sephadex G-75 (see Fig. 1). Three fractions, designated $\mathbf{F_1}$ (107 mg, $[\alpha]_D^{25}$ +85°), $\mathbf{F_2}$ (85 mg, $[\alpha]_D^{25}$ +83°), and $\mathbf{F_3}$ (55 mg, $[\alpha]_D^{25}$ +49°), in the order of their elution from the column, were obtained, $\mathbf{F_1}$ being eluted first. Each fraction was found to be electrophoretically homogeneous. In the Ouchterlony gel-diffusion[3] test against

TABLE I

SUGAR COMPOSITIONS OF $\mathbf{F_1}$, $\mathbf{F_2}$, AND $\mathbf{F_3}$

Sugars	$\mathbf{F_1}$		$\mathbf{F_2}$		$\mathbf{F_3}$	
	%	*mol*	*%*	*mol*	*%*	*mol*
Rhamnose	19.2	0.9	—	—	—	—
Glucose	—	—	45.3	1.0	24.5	1.9
Galactose	—	—	48.2	1.0	28.5	2.2
Mannose	21.6	1.0	—	—	13.0	1.0
2-Amino-2-deoxyglucose	21.2	1.0	—	—	—	—
2-Amino-2-deoxymannose	19.0	0.9	—	—	—	—

TABLE II

RESULTS OF METHYLATION ANALYSIS OF THE PS AND OLIGOSACCHARIDES **O-1** TO **O-3**

Methyl sugar[a]	*Retention time*[b] *on 3% ECNSS-M*	*Mole proportion*			
		PS	**O-1**	**O-2**	**O-3**
2,3,4-Tri-*O*-methylrhamnose	0.46	—	1.00	—	1.00
2,4-Di-*O*-methylrhamnose	0.99	1.00	—	0.96	—
3,4,6-Tri-*O*-methylmannose	1.95	0.90	1.00	1.00	1.00
2-Amino-2-deoxy-3,6-di-*O*-methylglucose	1.71	0.84	0.93	0.86	0.95
2-Amino-2-deoxy-3,4,6-tri-*O*-methylmannose	1.00	—	—	0.94	—
2-Amino-2-deoxy-4,6-di-*O*-methylmannose	2.85	0.87	—	—	0.92

[a]The methyl sugars identified are the corresponding alditol acetates. [b]Retention times are relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, at 170°, for neutral sugars; and at 190°, with respect to 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol, for amino sugars.

the homologous rabbit antiserum, $\mathbf{F_1}$ gave a single band, whereas the other two did not give any band.

All of the fractions were separately hydrolyzed, and the constituent sugars were identified and their amounts estimated (see Table I). As only $\mathbf{F_1}$ gave a band in the Ouchterlony gel-diffusion test, it was considered that this material contained the specific sugar groupings. Hence, further investigations were conducted on this fraction. Fraction $\mathbf{F_1}$ (PS) showed absorption bands at 1650 and 1565 cm^{-1} in the i.r. spectrum, indicating the presence of primary amide linkages.

The PS was fully methylated by the Hakomori method[4] followed by the Purdie method[5]. The product showed no hydroxyl band in its i.r. spectrum. The permethylated PS was hydrolyzed, the partially methylated sugars were converted into their alditol acetates[6,7], and these were analyzed by g.l.c. in column *1*. From the results, given in Table II, it was concluded that the structure of the repeating unit of the PS is the same as that reported by earlier workers[1], as follows.

$$\rightarrow 2)\beta\text{-D-Man}p(1\rightarrow 3)\alpha\text{-D-ManNAc}(1\rightarrow 3)\beta\text{-L-Rha}p(1\rightarrow 4)\alpha\text{-D-GlcNAc}(1\rightarrow$$

When the PS was subjected to periodate oxidation, it was found that 0.29 mol of the oxidant was consumed per mol of hexosyl residue in 8.5 h; the value calculated for the proposed structure is 0.25 mol. On hydrolysis, the periodate-oxidized and then reduced PS gave glycerol, rhamnose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxymannose in the mol ratios of 1.89:1.00:0.95:0.82. All of the sugar residues, except the mannose units, were resistant to periodate. Identification of glycerol in the hydrolyzate indicated that this compound resulted from oxidation of a (1→2)-linked mannose unit. Thus, the results of the periodate-oxidation studies are in good agreement with those expected from the structure assigned to the PS.

Graded hydrolysis of the PS. — To gain understanding of the immunochemi-

cal specificities of different sugar groupings in the PS, attempts were made to obtain oligosaccharides from it. The PS was subjected to mild hydrolysis with acid, and the hydrolyzate was found to contain, besides monosaccharides, three oligosaccharides, designated **O-1**, **O-2**, and **O-3** in the order of decreasing mobility in p.c. The mixture was separated on thick filter-papers, and the oligosaccharides isolated were purified by gel filtration.

On hydrolysis, oligosaccharide **O-1** {$R_{Maltose}$ 0.59; yield, 8 mg; $[\alpha]_D^{25}$ +5° (*c* 0.5, water)} gave rhamnose, mannose, and 2-amino-2-deoxyglucose in the mol ratios of 0.95:1.00:0.92. On reduction with $NaBH_4$, followed by hydrolysis and p.c. examination using spray reagent *c*, **O-1** gave spots corresponding to rhamnose and 2-amino-2-deoxyglucose. The rest of the hydrolyzate material was acetylated, and g.l.c. analysis of the products gave a peak corresponding to mannitol hexaacetate; other peaks did not correspond to any of the other sugars present in the PS. This indicated the presence of a mannose residue at the reducing end of the oligomer. The fully methylated **O-1** was hydrolyzed, and the resulting methylated sugars were identified, and estimated, by g.l.c. (see Table II, column **O-1**). Based on these results, the following structure was assigned to this oligomer.

Rha-(1→4)-GlcNAc-(1→2)-Man
O-1

Hydrolysis of oligosaccharide **O-2** {$R_{Maltose}$ 0.37; yield, 9 mg; $[\alpha]_D^{25}$ +10° (*c* 0.5, water)} gave rhamnose, mannose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxymannose in the mol ratios of 0.92:1.00:1.00:0.94. P.c. and g.l.c. examination of the hydrolyzate of the $NaBH_4$-reduced oligomer, as already described, showed that the mannose residue occupied the reducing end of the oligomer. On hydrolysis, the fully methylated material yielded partially methylated sugars which were identified, and estimated, by g.l.c. (see Table II, column **O-2**). From these results, and from the structure of **O-1**, the following structure was assigned to this oligomer.

ManNAc-(1→3)-Rha-(1→4)-GlcNAc-(1→2)-Man
O-2

On hydrolysis, **O-3** {$R_{Maltose}$ 0.15; yield, 20 mg; $[\alpha]_D^{25}$ +24° (*c* 0.7, water)} gave rhamnose, mannose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxymannose in the mol ratios of 1.10:1.00:0.98:0.96. P.c. and g.l.c. examination of the hydrolyzate of $NaBH_4$-reduced oligomer **O-3** showed that the 2-amino-2-deoxymannose unit occupied the reducing end of the oligomer. The results of methylation studies on this oligomer are given in Table II, column **O-3**. Based on these results, and from the structures of **O-1** and **O-2**, the following structure was assigned to this oligomer.

Rha-(1→4)-GlcNAc-(1→2)-Man-(1→3)-ManNAc
O-3

When the PS was subjected to Smith degradation[8], it yielded an oligomer which was designated **O-4**. It was purified by passing a solution of it through a column of Sephadex G-25, and the purified material, on hydrolysis, gave glycerol, rhamnose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxymannose in almost equimolar proportions. On methylation, followed by hydrolysis and g.l.c. analysis, **O-4** gave 2,4-di-*O*-methylrhamnose (1.00 mol), 2-amino-2-deoxy-3,6-di-*O*-methylglucose (0.92 mol), 2-amino-2-deoxy-3,4,6-tri-*O*-methylmannose (0.87 mol), and 1,3-di-*O*-methylglycerol (1.00 mol). The 2-amino-2-deoxymannose occupies the nonreducing end of the oligomer, and the rhamnosyl and 2-amino-2-deoxyglucosyl residues are respectively (1→3)- and (1→4)-linked. Based on these results, and from the structure of **O-2**, the following structure was assigned to this oligomer.

ManNAc-(1→3)-Rha-(1→4)-GlcNAc-(1→2)-Glycerol
O-4

For precipitin reactions, the antiserum was raised against killed, whole cells of *Sh. dysenteriae* type 10 in rabbits whose preserum was free from homologous and nonspecific antibodies. The results of the homologous, precipitin reaction (see Fig. 2) showed that 600 μg of the PS precipitated the maximum amount (140 μg) of antibody nitrogen from 1.0 mL of antiserum. The inhibition of immune precipitation was conducted by using the constituent monosaccharides, *viz.*, L-rhamnose, D-mannose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-mannose, and the results are given in Table III and Fig. 3. These results show that the D-mannose is the least immunospecific.

As expected, all of the oligomers (**O-1** to **O-4**) were found to be better inhibitors (see Table III, and Fig. 4) than the monosaccharides used. Oligomer **O-1** gave 48% inhibition of immune precipitation at the 1.51-μmol level, whereas **O-3**

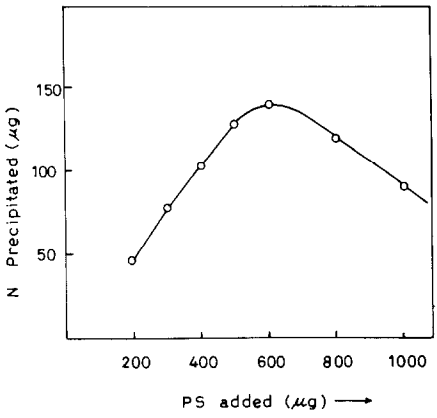

Fig. 2. Homologous precipitin-reaction (*Sh. dys.* type 10 PS).

TABLE III

INHIBITION, BY MONOSACCHARIDES AND OLIGOSACCHARIDES, OF THE PRECIPITATION OF RABBIT ANTISERUM BY *Sh. dysenteriae* TYPE 10 PS (RESULTS CALCULATED TO 1.0 mL OF UNDILUTED SERUM)

Inhibitor	*Micromoles added*	*Antibody N pptd* (μg)	*Inhibition* (%)
None	—	140	0
L-Rha	2.44	95	32
D-Man	2.78	119	15
D-GlcNAc	3.62	84	40
D-ManNAc	2.26	77	45
O-1	1.51	73	48
O-2	0.82	31	78
O-3	1.37	49	65
O-4	1.25	42	70

showed 65% inhibition at the 1.37-μmol level. Oligomers **O-2** (0.82 μmol), and **O-4** (1.25 μmol) respectively gave 78 and 70% inhibition. The increase in the % inhibitions of **O-2**, **O-3**, and **O-4** over **O-1** was obviously due to the presence of a 2-acetamido-2-deoxy-D-mannosyl group or residue in the first three. A 2-acetamido-2-deoxy-D-mannosyl group is present at the nonreducing end of **O-2** and **O-4**, and these oligomers showed greater inhibition of immune precipitation, even at lower concentrations. These two oligomers have almost the same structure, except that, in the former, the "reducing" end is occupied by a D-mannose residue, whereas, in the latter, it is glycerol. Oligomers **O-1** and **O-3** have similar structures, except that the latter contains an additional 2-acetamido-2-deoxymannose residue (at the reducing end). The difference in the % inhibition by oligosaccharides **O-1** and **O-3** is considerable.

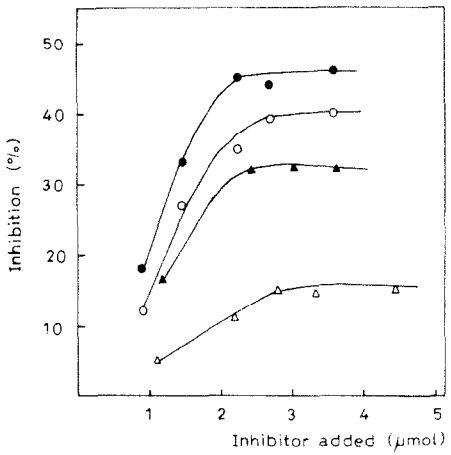

Fig. 3. Inhibition of homologous precipitation by monosaccharides. (Key: ●, 2-acetamido-2-deoxy-D-mannose; ○, 2-acetamido-2-deoxy-D-glucose; ▲, L-rhamnose; and △, D-mannose.)

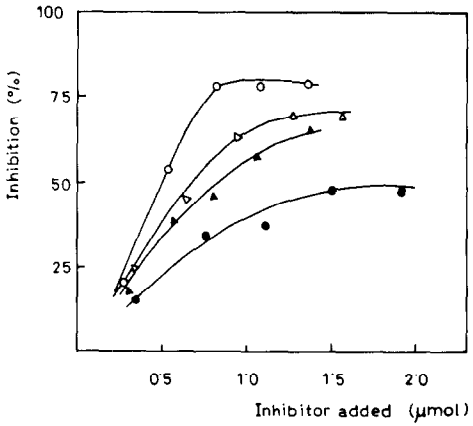

Fig. 4. Inhibition of homologous precipitation by oligosaccharides isolated from *Sh. dys.* type 10 PS. (Key: ●, oligosaccharide **O-1**; ○, oligosaccharide **O-2**; ▲, oligosaccharide **O-3**; and △, oligosaccharide **O-4**.)

Considering the facts that (*1*) 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-mannose showed better inhibition than other monosaccharides present in the PS, (*2*) the inhibition of precipitation by **O-3** is markedly greater than that by **O-1** due to the presence of a 2-acetamido-2-deoxy-D-mannose residue in **O-3**, and (*3*) the "reducing" ends of **O-2** and **O-4** are respectively occupied by a D-mannose residue and glycerol, whereas D-mannose itself has the lowest contribution (15%) to the immune specificity, it was concluded that the sugar grouping →3)-ManNAc-(1→3)-Rha-(1→4)-GlcNAc-(1→ is the immunodominant part in the PS molecule. 2-Acetamido-2-deoxy-D-mannose seems to contribute considerably to the immunological specificity of *Sh. dysenteriae* type 10 PS.

EXPERIMENTAL

The general methods used were those already reported[9].

Preparation of organism, and isolation of lipopolysaccharide (LPS). — *Shigella dysenteriae* type 10 bacteria (strain 2050.52) were grown for 72 h at 37° on brain-heart infusion-agar in 100 Roux bottles. The growths were harvested with saline solution. The bacterial-cell suspension was centrifuged, and the solid successively washed twice with saline and thrice with acetone. The acetone-dried cells (13 g) were disrupted with 45% aqueous phenol at 65–68°. The resulting mixture was centrifuged, giving three layers: a water layer, a phenol layer, and insoluble material at the phenol–water interface. The water layer (containing nucleic acid and the LPS) was siphoned off, dialyzed for three days against distilled water, and freeze-dried; yield, 1.69 g. The LPS was separated from the nucleic acid–LPS mixture by using Cetavlon in sodium chloride solution; the nucleic acid was precipitated at a salt concentration of 0.3M, whereas the LPS remained in solution. The LPS was precipitated by adding 10 volumes of ethanol; yield, 684 mg. The LPS was purified

by passing it through a column (100 × 2 cm) of Sephadex G-100, using pyridine acetate buffer (pH 4.5) as the eluant. The residual nucleic acid contaminant was eluted in the void volume, whereas the LPS constituted the major fraction; yield 600 mg.

Isolation of the polysaccharide. — The LPS (550 mg) was heated with 1:99 acetic acid–water (80 mL) for 1.5 h at 100°. The precipitated lipid A was removed by centrifugation, and the supernatant liquor was freeze-dried; yield, 275 mg. The material was fractionated on a column (90 ×2 cm) of Sephadex G-75 by using pyridine acetate buffer and collecting 5-mL fractions. Three fractions were obtained (see Fig. 1), and these were designated $\mathbf{F_1}$ (tube Nos. 40 to 70; 107 mg), $\mathbf{F_2}$ (tube Nos. 100 to 130; 85 mg), and $\mathbf{F_3}$ (tube Nos. 140 to 180; 55 mg). The homogeneity of these fractions was tested electrophoretically on t.l.c. plates, using 0.01M borate buffer, pH 9.2; a single spot moving towards the cathode was detected in each case. On Ouchterlony gel-diffusion using homologous rabbit antiserum, only $\mathbf{F_1}$ gave a single precipitin band.

Sugar composition. — All of the fractions (2.0 mg each) were separately hydrolyzed with 2M hydrochloric acid for 6 h at 100°. After the usual treatment, the sugars in the hydrolyzates were identified by p.c. (solvents *A* and *B*), and were estimated as alditol acetates by g.l.c., using *myo*-inositol as the internal standard. The results are shown in Table I. Fraction $\mathbf{F_1}$ was designated PS, and further work was conducted by using this material.

Methylation analysis of PS. — The PS (5 mg) was fully methylated by the Hakomori method[4] followed by the Purdie method[5]. The permethylated PS had no OH band in its i.r. spectrum. The product was hydrolyzed with 85% formic acid (2 mL) for 2 h at 100°, and then with 0.5M sulfuric acid for 18 h at 100°. The acid in the hydrolyzate was neutralized ($BaCO_3$), and, after the usual treatment, the resulting methylated sugars were converted into their alditol acetates, and these were identified, and estimated, by g.l.c. using column *I*. The results are given in Table II.

Partial hydrolysis of the PS. — From the results of a number of pilot experiments, the optimum conditions for maximal yield of oligomers were found to be: to heat the PS (50 mg) in 0.5M hydrochloric acid (35 mL) for 1 h at 100°. After evaporating the mineral acid, the neutral hydrolyzate was examined by p.c. using solvents *A* and *B*. Besides monosaccharides, spots corresponding to three slow-moving materials were obtained. The mixture was resolved on Whatman No. 3 MM filter paper, using solvent *A*. With the help of guide spots, the zones containing each oligosaccharide were excised, and eluted with 1:9 ethanol–water (150 mL each). The eluates were concentrated, and purified by passing them through a column (42 × 2 cm) of Sephadex G-25, using water as the eluant. The oligosaccharides had R_{Maltose} 0.59, 0.36, and 0.15 in solvent *A*, and were found to be chromatographically homogeneous. On electrophoresis (25 V/cm) in 0.01M borate buffer (pH 9.2), each fraction gave a single spot. The fractions were designated

fractions **O-1**, **O-2**, and **O-3**, in the order of decreasing $R_{Maltose}$ values. The yields and physical characteristics are summarized in Table III.

Characterization of the oligosaccharides. — The oligosaccharides (1.5 mg each) were hydrolyzed with 2M hydrochloric acid for 6 h at 100°. After removing the acid in the usual way, portions of the hydrolyzates were examined by p.c. (solvents *A* and *B*: spray reagents *a* and *b*). The sugars in the remaining portions were converted into their alditol acetates, and were identified, and estimated, by g.l.c.

The reducing-end residue in each oligosaccharide was detected by treatment with $NaBH_4$, followed by hydrolysis, and identification of the sugars in the hydrolyzate by p.c., using spray reagent *c*. A portion of the $NaBH_4$-reduced, and then hydrolyzed, product was acetylated, and the acetates were analyzed by g.l.c. using columns *1* and *3*.

The oligosaccharides (2 mg each) were methylated twice by Hakomori's method. The fully methylated products were extracted from the reaction mixtures with chloroform. They were purified by passing them through a column (25 × 2 cm) of Sephadex LH-20, using 2:1 chloroform–acetone as the eluant, and then hydrolyzed. The resulting methylated sugars were identified, and estimated, as their alditol acetates by g.l.c. The results are given in Table II.

Periodate oxidation and Smith degradation[8] *studies on the PS.* — The PS (2.0 mg) was treated with 8mM $NaIO_4$ in the dark at 4°. Consumption of the oxidant became constant in 8.5 h, at a value corresponding to 0.29 mol of the oxidant per mol of hexosyl residue.

A solution of the PS (10 mg) in water (10 mL) was treated with 0.2M $NaIO_4$ solution (10 mL) for 8.5 h at 4°. The excess of periodate was decomposed with ethylene glycol, and the solution was dialyzed against distilled water. The dialyzate was concentrated to 4 mL, and reduced with $NaBH_4$ (60 mg) for 4 h. The excess of borohydride was decomposed with glacial acetic acid, and the solution was dialyzed; the dialyzate was concentrated to a small volume, and freeze-dried; yield, 6.5 mg. A part (1 mg) of the periodate-oxidized and reduced PS was hydrolyzed by heating with 2M hydrochloric acid for 6 h at 100°. The sugars in it were identified in the usual way (by p.c. and g.l.c.) to be glycerol, rhamnose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxymannose in the mol ratios of 1.89:1.00:0.95:0.82. The remaining portion was kept with 0.5M hydrochloric acid (10 mL) for 24 h at room temperature, and, after removing the acid, the material was passed through a column (53 × 1.7 cm) of Sephadex G-25, using water as the eluant, the eluate being collected in 5-mL fractions. The main fraction (tubes 13 to 17) was isolated, and was designated fraction **O-4**; yield, 4.5 mg. A small part of **O-4**, on hydrolysis followed by p.c. and g.l.c. analysis, gave glycerol, rhamnose, 2-amino-2-deoxyglucose, and 2-amino-2-deoxymannose in the mol ratios of 1.00:1.00:0.95:0.82. Another portion of **O-4** was fully methylated by the Hakomori method[4], and on hydrolysis, the product gave 2,4-di-*O*-methyl-L-rhamnose (1.00 mol), 2-amino-2-deoxy-3,6-di-*O*-methyl-D-glucose (0.92 mol), 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-mannose (0.87 mol), and 1,3-di-*O*-methylglycerol (1.00 mol).

Preparation of rabbit antiserum. — The antisera were raised in rabbits by injecting bacterial-cell suspension (10^8 CFU/mL) in saline, following the immunization schedule described earlier[9]. The serum was separated from the blood in the usual way, and stored at 0° with sodium merthiolate (0.0001%) as a preservative. The highest dilution of the serum giving visible agglutination of the cell suspension was found to be 1:80.

Quantitative precipitin-reaction[10,11] *and inhibition studies*[12]. — To 0.10 mL of antiserum in 4-mL, conical centrifuge-tubes were added increasing amounts (20–100 μg) of PS. The final volume of the mixtures in all of the tubes was made up to 0.50 mL with saline. The mixtures (and blanks containing only serum), in duplicate, were thoroughly mixed, and kept for 72 h at 1–2°. The tubes were centrifuged, and the precipitates were washed twice with chilled saline (1 mL each time) and then dissolved in 0.25M acetic acid (2.0 mL). The optical absorbance of each solution was measured at 280 nm, and the amounts of the antibody nitrogen precipitated were calculated from a curve calibrated by using bovine serum albumin (nitrogen, 15.03%). The results are given in Table III and plotted in Fig. 2.

Inhibition of homologous precipitation was determined by using, as the inhibitors, L-rhamnose, D-mannose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, and oligosaccharides isolated from the PS by Smith degradation and graded hydrolysis. They were added in increasing amounts to 0.10-mL portions of the antiserum, in duplicate, diluted with appropriate quantities of saline. The contents of the tubes were mixed, and allowed to stand for 1 h at 1–2°. To all the tubes, PS (60 μg) in saline was added. The final volume of the mixture was 0.50 mL. Two controls, one containing the same amounts of antigen and antibody as in the other tubes, and the other containing the antiserum alone, were included in each set. The tubes were kept for 72 h at 4°, and the amounts of precipitated antibody nitrogen were assayed as described earlier. The results are given in Tables III and plotted in Figs. 3 and 4.

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