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IMMUNOCHEMICAL STUDIES ON *Shigella dysenteriae* TYPE 9 BACTERIAL POLYSACCHARIDE

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ABSTRACT

On graded hydrolysis and Smith degradation, the O-somatic polysaccharide isolated from *Shigella dysenteriae* type 9 bacteria, strain NCTC 7919, yielded five oligosaccharides which were characterized. The positions of the *O*-acetyl and pyruvic acetal groups in the repeating unit were identified. Immunochemical studies indicated that D-galactose is the immunodominant sugar in the polysaccharide, and one of the oligomers, having the structure Gal-(1→3)-GlcNAc-(1→3)-Gal-(1→4)-Man, showed maximum inhibition of the homologous precipitation.

INTRODUCTION

Structural studies on the O-specific polysaccharide from *Shigella dysenteriae* type 9, strain NCTC 9348, have been reported by Dmitriev *et al.*[1]. Methylation and Smith degradation studies on the O-specific polysaccharide from a different strain (NCTC 7919) of the same type showed that both polysaccharides are composed of the same repeating-unit. In the present investigation, the polysaccharide was subjected to graded hydrolysis and periodate oxidation, and the oligosaccharides obtained were characterized and their immunochemical specificities determined.

RESULTS AND DISCUSSION

Sh. dysenteriae type 9 (strain, NCTC 7919) bacteria were grown in large quantities on brain-heart infusion–agar. On extraction with 45% phenol, the dried cells gave a mixture containing lipopolysaccharide (LPS) and nucleic acid, and the latter was precipitated as its cetavlon complex. The LPS was further purified by passing it through a column of Sephadex G-100. On electrophoresis, the material so obtained moved as a single substance, and gave a single band on Ouchterlony plates against homologous, rabbit antiserum, indicating its homogeneity. Results of preliminary analysis are listed in Table I. On hydrolysis, the LPS gave galactose, mannose, glucose, and 2-amino-2-deoxyglucose in the mole ratios of 2.9 : 1.0 : 0.8 : 0.9.

Lipids were precipitated from the purified LPS by treatment with 1% acetic

TABLE I

PHYSICAL CONSTANTS AND SUGAR COMPOSITION[a] OF LPS AND PS

	Purified LPS	*Purified PS*
Moisture (%)	4.5	3.2
$[\alpha]_D^{25}$ (*c* 0.5 water)	46	52
Galactose (%)	31.4	42.2
Mannose (%)	11.0	19.1
Glucose (%)	8.6	absent
2-Amino-2-deoxyglucose (%)	9.9	16.8

[a]Estimated, as the alditol acetates, by g.l.c.

acid, and the degraded material resulting was fractionated on a column of Sephadex G-75 into a high-molecular-weight fraction (O-somatic polysaccharide, PS) and an oligomeric fraction. The PS was found to be homogeneous in electrophoresis and in the Ouchterlony gel-diffusion[2] test. The results of a preliminary analysis of PS are summarized in Table I. In the i.r. spectrum, the PS showed a small band in the region of 1738 cm^{-1} and a band at 1650 cm^{-1}, indicating the presence of *O*-acetyl groups and primary amide linkages. The n.m.r. spectrum of a 2% solution of the PS in D_2O showed signals for *N*-acetyl, *O*-acetyl, and pyruvic acetal groups. The proportions of hexosyl and hexosamine residues were estimated to be 79.2 and 17.0%, respectively. On hydrolysis, the PS gave galactose, mannose, and 2-amino-2-deoxyglucose in the mole ratios of 2.2:1.0:0.9 (see Table I). Glucose was present as a constituent of the LPS, whereas it was not found in the PS. This sugar residue might have originated from the oligosaccharide or glycolipid fraction.

The PS was fully methylated by the Hakomori method[3], followed by the Purdie method[4]. The results of the analysis of the hydrolyzate of the methylated PS (as alditol acetates) by g.l.c. are shown in Table II. From the results, it was found that one of the two D-galactopyranosyl residues and the D-mannopyranosyl residue are (1→4)-linked, and the 2-amino-2-deoxyglucosyl residue is (1→3)-linked. Although the other D-galactopyranosyl residue was found to be 1,3,4,6-linked, no methylated sugars corresponding to nonreducing ends were detected in the hydrolyzate of the fully methylated PS. ^{1}H-N.m.r. studies of the PS indicated the presence of pyruvic acetal groups in the molecule. Therefore, the 2-*O*-methyl-D-galactose originated from the D-galactosyl unit bearing the pyruvic acetal group.

Graded hydrolysis of the PS. – To obtain the oligosaccharides, the PS was subjected to graded hydrolysis. The hydrolyzate was found to contain, besides monosaccharides, four oligosaccharides, designated A-1, A-2, A-3, and A-4 in the order of decreasing mobility in p.c. The mixture was separated on thick filter-papers, and the oligosaccharides that were isolated were purified by gel filtration.

On hydrolysis, oligosaccharide A-1 {R_{Gal} 0.53, $[\alpha]_D^{25}$ +4 (*c* 0.5, water)} gave galactose and mannose in the mole ratio of 1.0:1.1. On reduction with $NaBH_4$,

TABLE II

RESULTS OF METHYLATION ANALYSIS OF THE PS AND OLIGOSACCHARIDES (A-1 TO A-4)

Methyl sugars[a]	*Retention times*[b]		*Mole proportion*				
	3% ECNSS-M	*3% OV-225*	*PS*	*A-1*	*A-2*	*A-3*	*A-4*
2,3,4,6-Tetra-*O*-methylgalactose	1.25	1.19	—	—	—	—	1.0
2,3,6-Tri-*O*-methylgalactose	2.42	2.22	1.0	0.9	0.9	—	—
2,4,6-Tri-*O*-methylgalactose	2.28	2.03	—	—	—	0.8	0.9
2-*O*-Methylgalactose	8.10	—	0.8	—	—	—	—
2,3,4,6-Tetra-*O*-methylmannose	**1.00**	**1.00**	—	1.0	1.0	—	—
2,3,6-Tri-*O*-methylmannose	2.20	2.03	1.0	—	—	1.0	1.0
2-Amino-2-deoxy-3,4,6-tri-*O*-methylglucose	1.00	1.00	—	—	—	0.8	—
2-Amino-2-deoxy-4,6-di-*O*-methylglucose	2.32	2.32	0.9	—	0.8	—	0.8

[a]The methyl sugars were identified as 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° for amino sugars with respect to 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucitol.

followed by hydrolysis and p.c. examination using spray reagent *c*, the material gave a spot corresponding to mannose. The remaining portion of the hydrolyzate of the $NaBH_4$-reduced material was acetylated, and g.l.c. analysis of the product gave a peak corresponding to galactitol hexaacetate; other peaks did not correspond to any other sugars present in the PS. These results indicated the presence of a galactose residue at the reducing end of the oligomer. The fully methylated A-1 was hydrolyzed, and the methylated sugars resulting were identified, and estimated, by g.l.c. (see Table II, column A-1). Based on these results, the following structure was assigned to this oligomer.

Man-(1→4)-Gal

A-1

Hydrolysis of oligosaccharide A-2 {R_{Gal} 0.32, $[\alpha]_D^{25}$ +4° (*c* 0.6, water)} gave galactose, mannose, and 2-amino-2-deoxyglucose in the mole ratios of 1.1:1.0:0.9. P.c. and g.l.c. examination of the hydrolyzate of the $NaBH_4$-reduced oligomer, as before, showed that a 2-amino-2-deoxyglucose residue constituted the reducing end of the oligomer. On methylation, hydrolysis, and g.l.c. analysis, it yielded the methylated sugars shown in Table II, column A-2. From these results, the following structure was assigned to this oligomer.

Man-(1→4)-Gal-(1→3)-GlcNAc

A-2

On hydrolysis, oligosaccharide A-3 {R_{Gal} 0.13, $[\alpha]_D^{25}$ +12° (*c* 0.6, water)} gave mannose, galactose, and 2-amino-2-deoxyglucose in the mole ratios of 1.0:0.9:0.8. The reducing end was identified as mannose. The fully methylated material was hydrolyzed, and the methylated sugars resulting were identified, and estimated

TABLE III

COMPOSITION OF OLIGOSACCHARIDES A-1 TO A-4

Oligo-saccharide	*Yield (mg)*	*Sugars present and their mole proportion*			*Sugar present at the reducing end*
		Galactose	*Mannose*	*2-Amino-2-deoxyglucose*	
A-1	7	1.0	1.1	absent	galactose
A-2	7	1.1	1.0	0.9	2-amino-2-deoxyglucose
A-3	6	0.9	1.0	0.8	mannose
A-4	8	2.2	1.0	0.9	mannose

(see Table II, column A-3) in the usual way. From these results, the structure assigned to this oligomer was as follows.

GlcNAc-(1→3)-Gal-(1→4)-Man
A-3

Hydrolysis of oligosaccharide A-4 {R_{Gal} 0.07, $[\alpha]_D^{25}$ +52° (*c* 0.5, water)} gave galactose, mannose, and 2-amino-2-deoxyglucose in the mole ratios of 2.2:1.0:0.9. The reducing end was identified as mannose. Hydrolysis of the fully methylated material yielded the methylated sugars listed in Table II, column A-4. Based on these results, and the structures of A-2 and A-3, the structure assigned to this oligomer is

Gal-(1→3)-GlcNAc-(1→3)-Gal-(1→4)-Man.
A-4

From the structure of these oligosaccharides (see Table III), it may be seen that, of the two D-galactopyranosyl residues in the repeating unit of the PS, one is (1→3)-, and the other, (1→4)-linked. Methylation analysis showed that the pyruvic acetal group was not linked to the (1→4)-linked D-galactopyranosyl unit; hence, it is attached to the (1→3)-linked D-galactosyl residue through O-4 and O-6.

On periodate oxidation of the PS, 0.32 mol of oxidant was consumed per mol of hexosyl residue in 21 h. On hydrolysis, the periodate-oxidized, reduced PS gave erythritol, galactose, and 2-amino-2-deoxyglucose in the mole ratios of 1.0:2.0:0.9, indicating that both D-galactopyranosyl residues are resistant to oxidation; the erythritol obviously originated from a (1→4)-linked D-mannosyl unit.

Smith degradation of the PS yielded an oligomer which was designated A-5. It was purified by passing it through a Sephadex G-25 column, and on hydrolysis, the purified material gave erythritol, galactose, and 2-amino-2-deoxyglucose in the mole ratios of 1.0:2.0:0.9. On methylation, followed by hydrolysis and g.l.c. analysis, A-5 gave 2,3,4,6-tetra-*O*-methylgalactose (1.0 mol), 2,4,6-tri-*O*-methylgalactose (0.9 mol), 2-amino-2-deoxy-4,6-di-*O*-methylglucose (0.9 mol), and tri-*O*-methylerythritol (1.0 mol). Based on these data, and from the structure of oligomer A-4, the structure that could be assigned to A-5 was as follows.

Gal-(1→3)-GlcNAc-(1→3)-Gal-(1→Eryol

A-5

From the facts that one of the D-galactosyl units constituted the nonreducing end of A-5 [which contained a (1→3)-linked D-galactosyl residue], and that both of the D-galactosyl units resisted periodate oxidation, it was concluded that (*1*) the nonreducing end in A-5 originated from a (1→4)-linked D-galactosyl residue to which a D-mannosyl residue was linked glycosidically, and (*2*) an *O*-acetyl group was present at either O-2 or O-3 of this D-galactosyl residue.

Thus, based on all of these results, the structure that can be assigned to the repeating unit of the O-somatic PS of *Sh. dysenteriae* type 9 is as follows.

The consumption of periodate was in close agreement with the value (0.25 mol) expected from the structure of the repeating unit.

The antiserum used in the homologous precipitin reaction was raised against killed, whole cells of *Sh. dysenteriae*, type 9, in rabbits. The results of the homologous precipitin reaction (see Fig. 1) showed that 500 μg of the PS precipitated the maximum amount (270 μg) of antibody nitrogen from 1.0 mL of antiserum. In the cases of depyruvylated PS (see Fig. 2) and *O*-deacetylated PS (see Fig. 3), 600 μg of the material precipitated 260 and 265 μg of antibody nitrogen, respectively, from the same volume of the antiserum. From these results, it is clear that more of the *O*-depyruvylated or *O*-deacetylated PS is needed than of the intact PS in order to reach the equiv-

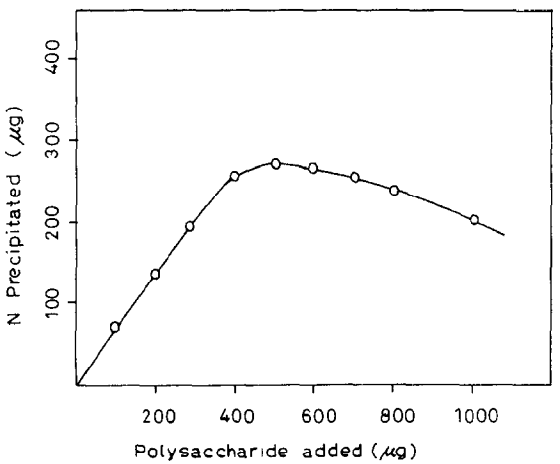

Fig. 1. Homologous precipitin reaction (*Sh. dys.* type 9 PS).

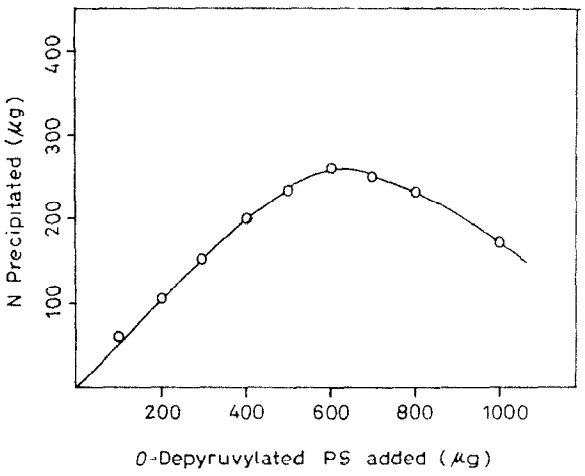

Fig. 2. Homologous precipitin reaction (*O*-depyruvylated *Sh. dys.* type 9 PS).

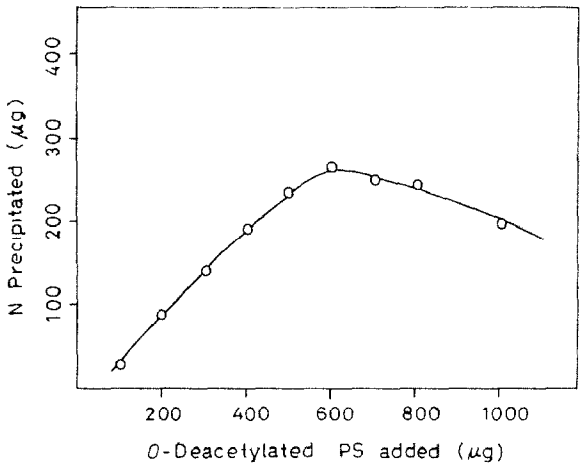

Fig. 3. Homologous precipitin reaction (*O*-deacetylated *Sh. dys.* type 9 PS).

alence zone; the amount of antibody nitrogen precipitated by the former two materials is slightly less than that by the latter, although the difference is small. It may therefore be concluded that the pyruvic acetal group and *O*-acetyl group make negligible contributions to the specificities in the immune precipitation.

Studies on the inhibition of immune precipitation were conducted by using the different sugars present in the PS, and the oligosaccharide obtained from the Smith-degraded product, and from the graded hydrolysis of the PS (see Table IV, and Figs. 4 and 5). D-Galactose inhibited 39% of the precipitation, whereas D-mannose and its methyl α-glycoside gave 9% inhibition. 2-Amino-2-deoxy-D-glucose and its *N*-acetyl derivative gave almost the same inhibition, 22 and 24%, respectively. The results show that, of the monosaccharides used, D-galactose is the best inhibitor.

TABLE IV

INHIBITION OF PRECIPITATION OF *Sh. dysenteriae* TYPE 9 POLYSACCHARIDE IN RABBIT ANTI (HOMOLOGOUS) SERA (1.0 mL) BY MONO- AND OLIGO-SACCHARIDES

Inhibitor	*Micromoles added*	*Antibody N ppt. (μg)*	*Inhibition (%)*
None	—	270	0
D-Gal	2.8	164	39
D-GlcNAc	2.3	204	24
D-GlcN	2.3	210	22
D-Man	3.3	246	9
Me α-D-Man	2.6	246	9
Oligo A-1	2.0	125	54
Oligo A-2	2.2	120	55
Oligo A-3	1.8	110	59
Oligo A-4	1.7	72	73
Oligo A-5	0.9	118	56

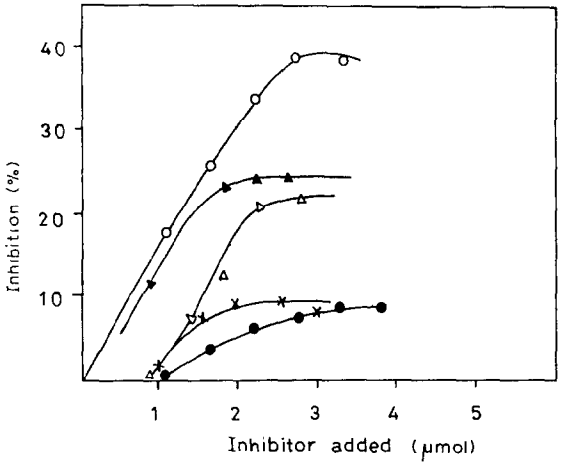

Fig. 4. Inhibition of homologous precipitation by monosaccharides and their derivatives. (Key: △, 2-amino-2-deoxy-D-glucose; ▲, 2-acetamido-2-deoxy-D-glucose; ○, D-galactose; ×, methyl α-D-mannoside; ●, D-mannose.

It was also observed that all of the oligosaccharides are better inhibitors than the monosaccharides. Of them, 1.41 μmol of oligomer A-4 [having the structure Gal-(1→3)-GlcNAc-(1→3)-Gal-(1→4)-Man] inhibited the precipitation to the extent of 73%. With three other oligosaccharides (*viz.*, A-1, A-2, and A-3), the values ranged from 54 to 59%. The oligosaccharide A-5, which was obtained on Smith degradation of the PS, showed 56% inhibition at the 0.89-μmol level.

Oligomers A-4 and A-5 have almost the same structure, except that, in the former, the reducing end is a D-mannose residue, whereas, in the latter, the corre-

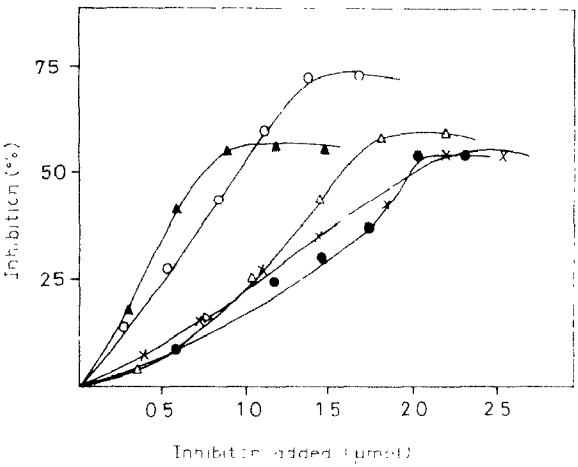

Fig. 5. Inhibition of homologous precipitation by oligosaccharides isolated from *Sh. dys.* type 9 PS. (Key: ●, oligosaccharide A-1; , oligosaccharide A-2; , oligosaccharide A-3; , oligosaccharide A-4; ▲, oligosaccharide A-5.)

sponding end is erythritol: the D-galactosyl portions in them are present at the nonreducing ends and as (1→3)-linked units. In compounds A-1 and A-2, a D-mannosyl group is the nonreducing end and the D-galactose or D-galactosyl residue is (1→4)-linked; the 2-acetamido-2-deoxyglucose residue in the latter is at the reducing end. Oligomer A-3 contains a 2-acetamido-2-deoxyglucosyl group at the nonreducing end, but the galactosyl residue is (1→3)-linked. Considering the facts that (*1*) A-5 (even at less than half the molar concentration) gave the same inhibition of precipitation as those given by A-1, A-2, and A-3, (*2*) the reducing end of A-4, and the corresponding end of A-5, are occupied by D-mannose and erythritol, respectively, which were in the fourth position from the nonreducing-end sugar units, and (*3*) D-mannose itself makes only a very small contribution (9%) to the immune specificity, it was concluded that the sugar grouping →4)-Gal-(1→3)-GlcNAc-(1→3)-Gal-(1→ that is present in the PS molecule is immunodominant. Because no oligosaccharide containing both (1→4)- and (1→3)-linked D-galactosyl residues could be isolated from any of the degradation products, it was not possible to adduce direct evidence for this contention, but the maximum inhibition (73%) given by A-4 provided ample support for this conclusion

EXPERIMENTAL

Materials and methods. — Whatman No. 1 filter paper was used for qualitative paper chromatography (p.c.), and large quantities of sugar mixtures were separated on Whatman No. 3 MM paper. The solvent systems (v.v) used for p.c. were (*A*) 4:1:5 1-butanol–acetic acid–water and (*B*) 8:2:1 ethyl acetate–pyridine–water. The spray reagents used were (*a*) alkaline silver nitrate, (*b*) 0.25% ninhydrin in acetone, and (*c*) saturated aniline oxalate in water.

Gel-filtration chromatography was conducted on columns of Sephadex G-100, G-25, and G-75 in pyridine acetate buffer (pH 4.5); elutions were monitored with a differential refractometer (Waters Associates; Model R-403). A Hewlett–Packard model 5710 A gas chromatograph, fitted with f.i.d. and glass columns (1.80 m × 6 mm) packed with (*1*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh), (*2*) 3% of OV-225 on Gas Chrom Q (100–120 mesh), and (*3*) 3% of Poly-A 103 on Gas Chrom Q (100–120 mesh), was used. A Hewlett–Packard 3380 A integrator was used for quantitization of the peaks.

Optical rotations were measured with a Perkin–Elmer Model 241 MC polarimeter. ^{1}H-N.m.r. spectra of materials in D_2O solution at 30° were recorded with a Varian XL-100 instrument, tetramethylsilane being used as an external standard. Spectrophotometric readings were taken with Yanaco SP-1 and Shimadzu 201 A spectrophotometers. Total hexose and hexosamine were respectively estimated by the phenol–sulfuric acid[5] and Blumenkrantz[6] methods. The homogeneity of the materials was tested by electrophoresis on t.l.c. plates, using 0.01M borate buffer (pH 9.2) at 25 V/cm for 1.25 h, and by the Ouchterlony gel-diffusion technique against homologous antisera. All methylations were conducted by the Hakomori method[3]. Solutions were evaporated *in vacuo* at bath temperatures below 40°.

Preparation of organism and isolation of lipopolysaccharide (LPS). — A strain of *Shigella dysenteriae* type 9, NCTC 7919, was grown for 72 h at 37° on brain-heart infusion–agar in 120 Roux bottles. The growth was harvested by gently shaking the broths with saline, and the cell suspension was kept with 0.5% (w/v) phenol. The suspension was then centrifuged at 15,000 r.p.m. for 45 min at 4°, in order to separate the cells. The cells were successively washed twice with saline and thrice with acetone. The yield of dry cells was 15 g. The dry cells (15 g) were disrupted with 45% aq. phenol at 65–68°. The resulting product was centrifuged when three layers were obtained: a water layer, a phenol layer, and an insoluble material at the phenol–water interface. The water layer (containing nucleic acid and the LPS) was siphoned off, dialyzed, and freeze-dried; yield, 1.2 g. The LPS was isolated from the nucleic acid–LPS mixture by using a cetavlon in sodium chloride medium; the nucleic acid was precipitated at a salt concentration of 0.3M, whereas the LPS remained in solution. Finally, the LPS was precipitated by adding 10 volumes of ethanol; yield, 527 mg. A solution of the LPS (100 mg) in 0.05M NH_4HCO_3 buffer, pH 7.8 (5 mL) was applied to a column (68 × 2.7 cm) of Sephadex G-100, and eluted with the same buffer at the rate of 9 mL/h. The eluate was collected in 5-mL fractions, and the carbohydrate content in each fraction was determined by the phenol–sulfuric acid method[5]. The LPS was eluted as a single substance (tubes No. 20 to 37); yield 80 mg.

In electrophoresis using a t.l.c. plate and 0.01M borate buffer, pH 9.2, at a voltage gradient of 25 V/cm for 1.5 h, the purified LPS moved, as a single substance, 1.3 cm towards the cathode. In Ouchterlony gel-diffusion[2] using homologous rabbit antiserum, it gave a single precipitin band. The results of a preliminary analysis of the LPS are summarized in Table I.

The LPS (3 mg) was hydrolyzed with 3M hydrochloric acid for 6 h at 100°

in a sealed tube. The acid was removed by repeated evacuation over KOH pellets P.c. examination (solvents *A* and *B*, and spray reagents *a* and *b*) of the hydrolyzate gave spots corresponding to galactose, mannose, glucose, and 2-amino-2-deoxy-glucose. The rest of the material in the hydrolyzate was reduced with $NaBH_4$, the alditols acetylated in the usual way, and the acetates analyzed by g.l.c. in columns *1* and *3* at 190°. Peaks corresponding to galactose, mannose, glucose, and 2-amino-2-deoxyglucose in the molar ratios of 2.9:1.0:0.8:0.9 were obtained. The amounts of constituent sugars of the LPS were estimated by g.l.c., using *myo*-inositol as the internal standard, and the results are given in Table I.

Isolation of the polysaccharide (PS). – The LPS (400 mg) was heated with 1:99 acetic acid–water (60 mL) for 1.5 h on a boiling-water bath, and the precipitated lipid A was removed by centrifugation. The supernatant liquor was freeze-dried, the product dissolved in pyridine acetate buffer, pH 4.5 (3 mL), and the solution applied to a column (90 × 2 cm) of Sephadex G-75. The column was eluted with the same buffer, 5-mL fractions being collected. The eluate afforded two fractions, one containing high-molecular-weight material (tube Nos. 24 to 38; 120 mg), and the other, an oligosaccharide fraction (tube Nos. 42 to 75; 100 mg). The results of preliminary analysis of fraction 1 are summarized in Table I.

The purified PS was subjected to electrophoresis under the conditions used for the LPS. A single spot, which moved 1.7 cm towards the cathode, was obtained. In Ouchterlony gel-diffusion against the homologous rabbit antiserum, the material gave a single precipitin band; the oligosaccharide fraction gave no precipitin band.

The i.r. spectrum of the PS in a KBr pellet showed bands at 1738 and 1650 cm^{-1}. For ^1H-n.m.r. spectroscopy, the sample was repeatedly dissolved in D_2O and freeze-dried, and a 2% solution in D_2O was finally prepared. Tetramethylsilane was used as the external standard. The spectrum showed signals for *N*-acetyl (δ 2.01), *O*-acetyl (δ 2.11), and pyruvic acetal (δ 1.54) groups. The total proportions of hexoses and hexosamines were estimated to be 79.2 and 17.0%, respectively.

Monosaccharide composition. — The PS (2 mg) was hydrolyzed with 3M hydrochloric acid for 6 h at 100° in a sealed tube. The acid was then removed by keeping the solution *in vacuo* over P_2O_5 and NaOH pellets. On p.c. examination using solvents *A* and *B*, and spray reagents *a* and *b*, the resulting material showed spots corresponding to galactose, mannose, and 2-amino-2-deoxyglucose. The constituent sugars, as their alditol acetates, were estimated by g.l.c., using *myo*-inositol as the internal standard. The results are given in Table I.

Methylation studies. The PS (5 mg) was methylated by the Hakomori method[3] followed by the Purdie method[4], to give a fully methylated product. The product was heated with 85% formic acid for 2 h at 100°. After removing the acid under diminished pressure, the material was hydrolyzed with 0.25M sulfuric acid for 20 h at 100°. The acid was neutralized with $BaCO_3$, and the suspension filtered. The resulting, methylated sugars were converted into their alditol acetates in the usual way, and were identified, and estimated, by g.l.c. using columns *1* and *2*. The results are given in Table II.

Partial hydrolysis of the PS. — Guided by the results of pilot experiments for the maximal yield of the oligosaccharides, the PS (55 mg) in 0.05M hydrochloric acid (30 mL) was heated for 3 h on a boiling-water bath, and the acid was removed under diminished pressure over NaOH pellets. The hydrolyzate, on p.c. examination using solvents *A* and *B* and spray reagents *a* and *b*, gave spots corresponding to galactose, mannose, and 2-amino-2-deoxyglucose, and four slow-moving materials. 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 cut, and eluted with 1:9 ethanol–water (150 mL for each). The eluates were each concentrated to a small volume, and freeze-dried. Each oligosaccharide was purified by passing it through a column (42 × 2 cm) of Sephadex G-25, eluted with water, and the eluate was freeze-dried. The oligosaccharides had R_{Gal} 0.53, 0.32, 0.13, and 0.07 in solvent *A*, and were found to be chromatographically homogeneous; they were designated fractions A-1, A-2, A-3, and A-4, in the order of decreasing mobility.

Characterization of the oligosaccharides. — The oligosaccharides (1 mg each) were hydrolyzed with 3M 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 these were identified, and estimated, by g.l.c. The results are given in Table III.

The reducing-end residues in the oligosaccharides were identified by treatment with $NaBH_4$, followed by hydrolysis, and the usual treatment; the sugars in the respective hydrolyzates were identified by p.c. using spray reagent *c*. The remaining portions of the hydrolyzates were analyzed in the usual way by g.l.c. using columns *1* and *3*. The sugar units present at the reducing ends in the oligosaccharides are given in Table III.

The oligosaccharides (1 mg each) were methylated by the Hakomori method[3], and the fully methylated derivatives were extracted with chloroform. They were hydrolyzed, and the resulting methylated sugars were identified, and estimated, as their alditol acetates, by g.l.c. The results are given in Table II, columns A-1 to A-4.

Periodate oxidation, and Smith-degradation[7] 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 21 h, at a value corresponding to 0.32 mol of the oxidant per mol of hexosyl residue.

In a separate experiment, the PS (10 mg) in water (10 mL) was oxidized with 0.2M $NaIO_4$ solution (10 mL) for 21 h at 4°. The excess of periodate was decomposed with ethylene glycol, and the solution was dialyzed for three days against distilled water. The dialyzate was concentrated to 5 mL, and the product reduced with $NaBH_4$ (60 mg) for 4 h. The excess of borohydride was decomposed with acetic acid, and the solution was dialyzed, and lyophilized; yield 7.0 mg. A part (0.5 mg) of the periodate-oxidized, reduced PS was hydrolyzed by heating with 2M hydrochloric acid for 6 h at 100°. The hydrolyzate was analyzed by p.c. and g.l.c. in the usual way; erythritol, galactose, and 2-amino-2-deoxyglucose were found to be present in the mole ratios

of 1.0:2.0:0.9. The remaining portion was kept with 0.5M hydrochloric acid (10 mL) for 24 h at room temperature, and, after the usual treatment, was passed through a column (53 × 1.7 cm) of Sephadex G-25, the eluate being collected in 5-mL fractions. Tubes 11–14, containing the main fraction, were pooled, and lyophilized, to afford the Smith-degraded oligosaccharide; it was designated fraction A-5; yield, 5 mg. P.c. and g.l.c. analysis of the hydrolyzate of A-5 showed the presence of erythritol, galactose, and 2-amino-2-deoxyglucose in the mole ratios of 1.0:2.0:0.9. A portion (1.5 mg) of the material was fully methylated, and, on hydrolysis, the methylated product gave 2,3,4,6-tetra-*O*-methyl-D-galactose (1.0 mol), 2,4,6-tri-*O*-methyl-D-galactose (0.9 mol), 2-amino-2-deoxy-4,6-di-*O*-methyl-D-glucose (0.9 mol), and 1,3,4-tri-*O*-methyl-D-erythritol (1.0 mol).

Preparation of O-depyruvylated[8] *and O-deacetylated PS.* – The PS (4.0 mg) was heated with 1mM oxalic acid in 0.1M NaCl solution (2 mL) for 2 h at 100°. The hydrolyzate was dialyzed against distilled water for 3 days, and freeze-dried, yield 2.8 mg.

For *O*-deacetylation, the PS (5.0 mg) was dispersed in methanol (3 mL), and then the calculated volume of methanol containing sodium methoxide was added to it, so that the final concentration of MeONa was 0.1M. The solution was kept for 4 h at room temperature, dialyzed against distilled water for 3 days, and freeze-dried, yield, 4.0 mg.

Preparation of rabbit antiserum. – Healthy rabbits, whose pre-sera did no show nonspecific and homologous agglutinin, were used for immunization. The inoculation schedule consisted of 3 subcutaneous (inner side of the thigh) injections followed by 4 intravenous (marginal ear-vein) injections, starting from 0.2 mL, and rising by 0.1 mL, of bacterial-cell suspension (10^8 CFU/mL) in subsequent injections. One week after completing the immunization schedule, test bleeding was done from the ear vein (5 mL) for determining the antibody titer. Final bleeding (20–30 mL) from the heart was done under aseptic conditions, they were bled 2–3 times in the course of a week. The serum was separated from the blood in the usual way, and finally stored at 0° with sodium merthiolate (0.0001%) as a preservative. From the slide agglutination test, the titer values of antisera isolated from different rabbits were found to be 1:80 and 1:160. They were mixed, and used in further investigations.

Quantitative precipitin reaction. – Precipitin reactions[9,10] were set up, using the PS, *O*-depyruvylated PS, and *O*-deacetylated PS, and the rabbit antiserum. The reaction mixture contained 0.10 mL of the antiserum with various amounts of antigen (20–120 μg) and saline in a total volume of 0.50 mL. The mixtures, and a blank containing only serum, were set up in duplicate. The solutions were kept for 72 h at 1 to 3°. These conditions were maintained throughout the work. The precipitates were collected by centrifugation in the cold, washed twice with chilled saline (1 mL each time), and then dissolved in 2.0 mL of 0.25M acetic acid. The optical absorbance of each solution was measured at 280 nm, and the amounts of nitrogen in the precipitates were calculated from a standard curve drawn by using bovine

serum albumin (nitrogen, 15.03%). The amounts of nitrogen precipitated were plotted against the amounts of antigen added per 1.0 mL of antiserum (see Figs. 1, 2, and 3).

Inhibition studies. — Inhibition of the precipitin reaction was achieved by using, as the inhibitors, D-galactose, D-mannose, methyl α-D-mannoside, 2-amino-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose, and the oligosaccharides obtained from the Smith-degradation product and from the graded hydrolysis of the PS. They were added in increasing amounts to 0.10-mL portions of 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 to 3°. To each tube was then added a solution of the PS in saline (containing 50 μg of the material), to bring the system to equivalence. The final volume of each solution was 0.50 mL. Two controls, one containing the same amounts of antigen and antiserum 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 1 to 3°, and the amounts of precipitated antibody nitrogen were assayed as described earlier. The results are shown in Table IV, and Figs. 4 and 5.

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