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Note

The structure of the chemical repeating-unit of the O-specific polysaccharide chain of *Shigella boydii* 6 lipopolysaccharide

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Bacterial lipopolysaccharides of the Shigella genus, unlike those of the Salmonella[1], have not been studied in detail The structures proposed for *Sh flexneri* lipopolysaccharides[2] were recently reinvestigated[3] We have chemotyped the *Sh dysenteriae*[4] and *Sh boydii*[5] serotypes on the basis of the monosaccharide composition of the respective lipopolysaccharides We now report data on the structure of the O-specific chain of *Sh boydii* 6 lipopolysaccharide

Isolation of the O-specific polysaccharide by mild hydrolysis of the lipopolysaccharide[6] and its characterization and monosaccharide composition have been described previously[7] The polysaccharide is composed of D-galactose, D-mannose, 2-acetamido-2-deoxy-D-galactose, and D-glucuronic acid residues in the ratios 1 2·1 1 When the polysaccharide was methylated (Hakomori[8]) and then methanolysed, and the product mixture subjected to ion-exchange chromatography, the methyl glycosides of partially methylated amino and neutral sugars were obtained The former was identified as methyl 2-deoxy-4,6-di-*O*-methyl-2-methylamino-D-galactoside, since the mass spectrum of its *N,O*-diacetyl derivative was closely similar to that of the corresponding *gluco* isomer, prepared from benzyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-glucopyranoside[9] Both mass spectra were in agreement with the literature data[10] The methyl glycosides of the partially methylated, neutral sugars were converted into the corresponding alditol acetates by hydrolysis, followed by reduction with borohydride and acetylation G l c and g l c–m s of the products revealed derivatives of 3,4,6-tri-*O*-methylmannose, 2,3,4-tri-*O*-methylmannose, and 2,6-di-*O*-methylgalactose in the ratios 1 1 0 7 Methylation analysis of the carboxyl-reduced polysaccharide[7] gave 2,3,4,6-tetra-*O*-methylglucitol diacetate together with the above components in approximately equivalent amounts

That glucuronic acid was linked to galactose followed from the isolation of a glucuronosylgalactose upon acid hydrolysis of the polysaccharide[7] Base-catalyzed

degradation of the glucuronic acid moiety in the permethylated polysaccharide, followed by mild hydrolysis with acid, deuteriomethylation[11], and g l c and g l c–m s of the derived methylated alditol acetates, revealed the presence of 2,6-di-*O*-methyl-4-*O*-trideuteriomethylgalactitol triacetate with concomitant loss of 2,6-di-*O*-methylgalactitol tetra-acetate Thus, the glucuronic acid residue is linked to position 4 of galactose

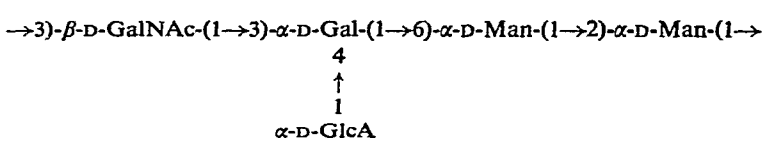
On treatment of the polysaccharide with periodate, all of the mannose was oxidized and the ratio of surviving galactose to 2-amino-2-deoxygalactose was 1 1 Periodate oxidation of the Smith-degraded polysaccharide changed this ratio to 1 0 3 This result, together with the methylation data, indicates the presence of a 2-amino-2-deoxygalactosyl-(1→3)-galactose fragment in the polysaccharide The same fragment has been found in *Sh dysenteriae* 3 polysaccharide[12]

Partial, acid hydrolysis of the polysaccharide gave a mixture of acidic components, from which an aldotriouronic acid consisting of mannose, galactose, and glucuronic acid was isolated, this acid reacted with the triphenyltetrazolium reagent, thus excluding a (1→2)-link between galactose and mannose.

Reduction of the aldotriouronic acid with $NaBT_4$ and oxidation of the product with periodate gave radioactive formaldehyde (isolated as the dimedone derivative), thus confirming the presence of a galactosyl-(1→6)-mannose fragment

The configurations of the glycosidic bonds in the polysaccharide were determined by application of the chromium trioxide-oxidation procedure[13,14] Analysis of the acetylated, carboxyl-reduced polysaccharide revealed the loss of 2-amino-2-deoxygalactose on oxidation An α configuration may therefore be assigned to all the glycosidic bonds except that of 2-amino-2-deoxygalactose The $[\alpha]_D$ value (+90°, water) of the polysaccharide accords with this conclusion

From the foregoing evidence, the repeating unit of the O-specific polysaccharide of *Sh boydii* 6 lipopolysaccharide can be assigned the following structure



EXPERIMENTAL

General methods — G l c. was performed with a Pye series 104 (Model 64) inst ument equipped with a dual, heated, hydrogen flame-ionisation detector coupled with a Vidar Autolab 6300 digital integrator Separations were carried out on a glass column (0 4 × 90 cm) packed with 3% of ECNSS-M on 100–120 mesh GasChrom Q (column *A*), or a steel column (0.6 × 150 cm) packed with 3% of SE-30 on 100–120 mesh Diatomite CQ (column *B*) G l c–m s was performed with a Varian MAT 111 "GNOM" instrument, using the same columns Paper electrophoresis was carried out on Filtrak FN-11 paper in a 25mM pyridinium acetate buffer (pH 4 5) at 28 V/cm Solutions were concentrated *in vacuo* at 40°

Methylation analysis of the polysaccharide — Using the conventional procedure[8], the methylated polysaccharide (~2 mg) was heated in a sealed tube with 1 ml of M hydrogen chloride in methanol for 20 h at 100° The solution was then concentrated to dryness and a solution of the residue in 50% aqueous methanol was passed through a column (0 7 × 5 cm) of Dowex-50(H^+) resin by elution with 30 ml of 50% aqueous methanol to give the methyl glycosides of the partially methylated, neutral monosaccharides The eluate was concentrated to dryness, the residue was heated with 1 ml of 85% formic acid for 1 h at 100°, the hydrolysate was concentrated, and the residue was heated with 1 ml of 0 3M hydrochloric acid for 16 h at 100°. The resulting sugars were converted into the corresponding alditol acetates by the conventional method G l c and g l c–m s revealed three peaks, with *T* values (relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol) of 1 94, 2 47, and 3 92 (column *A*, 155°), which were identified from their mass spectra[15] as the acetates of 3,4,6-tri-*O*-methylmannitol, 2,3,4-tri-*O*-methylmannitol, and 2,6-di-*O*-methylgalactitol The same components were detected on hydrolysis of the methylated polysaccharide with formic and hydrochloric acids, followed by borohydride reduction and acetylation

The Dowex-50 column was next eluted with 15 ml of 2M ammonia in 50% aqueous methanol, the eluate was concentrated, and the residue was treated with 0 5 ml of acetic anhydride and 1 ml of pyridine at room temperature overnight The mixture was concentrated to dryness and the residue was analysed by g l c and g l c–m s A single peak was detected with a *T* value (relative to methyl 2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-α-D-galactoside) of 1 18 (column *B*, 210°), the mass spectrum contained the following peaks *m/e* 43 (65%), 45 (43), 56 (32), 71 (36), 73 (91), 98 (59), 100 (28), 101 (48), 102 (20), 115 (100), 142 (53), 157 (9 3), 169 (24), 186 (2 7), 187 (2 7), 214 (8), 228 (2 7), and 246 (4)

Methylation[8] of benzyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-glucopyranoside[9], followed by methanolysis, isolation of the amino sugar fraction, and acetylation, afforded methyl 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-*N*-methylacetamido-D-glucopyranoside, the mass spectrum of which coincided with that of the 2-amino-2-deoxygalactose derivative isolated from the polysaccharide

The methylated polysaccharide (~2 mg) was dissolved in 3 8 ml of methanol and 0 2 ml of 2,2-dimethoxypropane, a catalytic amount of toluene-*p*-sulphonic acid was added, and the mixture was boiled under reflux for 30 min Sodium (~50 mg) was added and boiling was continued for 2 h Treatment[11] of the reaction product with dilute acetic acid, followed by trideuteriomethylation and sugar analysis (g l c–m s of alditol acetates), revealed the formation of 1,3,5-tri-*O*-acetyl-2,6-di-*O*-methyl-4-*O*-trideuteriomethylgalactitol, *T* (relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol) 1 44 (column *B*, 180°)

The carboxyl-reduced polysaccharide was obtained by treatment[16] of a solution in methyl sulphoxide with diazomethane The resulting methyl ester was reduced by dissolution in 0 2M borate buffer (pH 7 7) and treatment with potassium borohydride at room temperature for 1 h The product was isolated by gel filtration

Periodate oxidation of the polysaccharide — Polysaccharide (2.9 mg) was

treated with 3 ml of 0 1M sodium periodate for 48 h at room temperature in the dark Potassium borohydride (100 mg) was added and, after 1 h, the reaction mixture was passed through a column (2 3 × 35 cm) of Sephadex G-25 The fraction eluted with the void volume of the column was freeze-dried and the residue was subjected to sugar analysis[7] as follows

The polysaccharide (~1 mg) was hydrolysed with 1 ml of 2M HCl at 100° for 4 h The hydrolysate was concentrated to dryness, and the residue was dissolved in 0 3 ml of water and treated with 0 5 ml of 33% acetic acid and 0 5 ml of 5% aqueous sodium nitrite for 40 min at room temperature KU-2(H^+) resin was then added and the solution was freeze-dried A solution of the residue in water (1 ml) was treated with 5 mg of potassium borohydride for 1 h, then deionized with KU-2(H^+) resin, and concentrated to dryness, and boric acid was removed from the residue by distillation of methanol (3 × 2 ml) therefrom The resulting alditols were acetylated with acetic anhydride–pyridine at 100° for 15 min, and the product mixture was subjected to g l c (column *A*, 190°)

A solution of the oxidised–reduced polysaccharide in 2 ml of 0 5M hydrochloric acid was kept overnight at room temperature and then freeze-dried The residue was dissolved in 2 ml of water, and treated with 30 mg of sodium metaperiodate for 16 h at room temperature and then with 20 mg of potassium borohydride for 2 h. The reaction mixture was passed in succession through columns containing 5 ml each of Amberlite IRA-401(AcO^-) and KU-2(H^+) resins The eluate was concentrated to dryness and the residue was subjected to sugar analysis as described above

Partial, acid hydrolysis of the polysaccharide — The polysaccharide (12 9 mg) was heated with 1 3 ml of 0 3M hydrochloric acid for 2 h at 100° The solution was freeze-dried, and the residue was dissolved in water and passed through a column of Amberlite IRA-401(AcO^-) resin (5 ml) The column was eluted with water (20 ml), and the acidic components were desorbed by elution with 10 ml of 15% acetic acid This fraction was subjected to preparative paper electrophoresis for 90 min Two zones (R_{GlcA} 0 7, 0 5), detected after staining of guide strips with alkaline silver nitrate, were eluted with water Acid hydrolysis of the fast-moving component, with 2M hydrochloric acid for 16 h at 100°, afforded glucuronic acid and galactose, mannose was obtained as an additional product on hydrolysis of the second component

A solution of the aldotriouronic acid (R_{GlcA} 0 5, ~1 μmole) in 100 μl of 0 01M borate buffer (pH 9 25) was added to a tube containing ~0 5 μmole of tritium-labelled sodium borohydride After 2 h at room temperature, sodium borohydride (~2 mg) was added, and after a further 2 h, the solution was acidified with acetic acid and concentrated to dryness Boric acid was removed from the residue by evaporation of methanol (5 × 2 ml) therefrom The product was subjected to paper electrophoresis, and the acidic component (located by staining of guide strips with periodate–benzidine) was eluted with water (5 ml) to give the reduced, radioactive aldotriouronic acid A solution of this product in 1 ml of water was treated with 70 mg of periodic acid dihydrate for 30 min, galactitol (9 66 mg, 53 μmoles) was then added followed,

after 2 h, by a solution of dimedone in phosphate buffer[17] The precipitate was thrice recrystallized from methanol to give the dimedone–formaldehyde complex, m p 190–192°, activities of 5300, 4470, 4170, and 4460 counts min^{-1} $\mu mole^{-1}$ were obtained for the crude preparation and the three crystalline samples, respectively

Determination of the configurations of the glycosidic bonds — The carboxyl-reduced polysaccharide[16] (15 mg) was dissolved in freshly distilled formamide (3 ml) and treated with 2 ml of acetic anhydride and 2 ml of pyridine for 24 h at room temperature The light-brown solution was dialysed against distilled water, and the product was isolated by freeze-drying A portion of this acetylated polysaccharide was subjected to methanolysis (M hydrogen chloride in methanol, 100°, 30 min) followed by hydrolysis (2M hydrochloric acid, 100°, 4 h) The hydrolysate, after deamination, borohydride reduction, and acetylation, was analysed by g l c (column *A*, 190°)

The acetylated polysaccharide (~10 mg) was dried over P_2O_5 at 50°/0 1 torr for 2 h and then dissolved in 0 3 ml of glacial acetic acid, chromium trioxide (30 mg) was added, and the mixture was kept at 50° for 2 h The reaction mixture was diluted with water, and the product was extracted with chloroform The extract was washed with water and concentrated to dryness, and the residue was subjected to sugar analysis as described above

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