The Type-Specific Substance from *Pneumococcus* Type 29

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1. A pentasaccharide, corresponding to the dephosphorylated repeating unit of the specific substance, S.29, from *Pneumococcus* type 29, was obtained by hydrolysis with alkali followed by enzymic dephosphorylation. 2. The pentasaccharide was shown to be *O*-2-acetamido-2-deoxy- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-galactofuranosyl-(1 $\rightarrow$ 3)-*O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-galactofuranosyl-(1 $\rightarrow$ 1)-ribitol. 3. The phosphodiester linkages in S.29 join the hydroxyl group at position 5 of ribitol and the hydroxyl group at position 3 or 4 of a 2-acetamido-2-deoxy-D-galactose residue in the next repeating unit. 4. A partial structure for S.29 was deduced from these experiments.

Type 29 *Pneumococcus* was first characterized by Cooper, Rosenstein, Walter & Peizer (1932), and further serological investigations have been published by Kauffmann, Mørch & Schmith (1940), Walter, Guevin, Beattie, Cotler & Bucca (1941), Mørch (1942, 1943) and Eddy (1944). Type 29 is known as such both in Denmark and the United States (Kauffmann, Lund & Eddy, 1960). The type-specific substance, S.29, was isolated by Brown (1939), who carried out a preliminary chemical investigation.

S.29 was one of the type-specific substances examined by Shabarova, Buchanan & Baddiley (1962), who showed it to be a ribitol phosphate derivative, containing galactose and galactosamine residues. It thus belongs to the ribitol phosphate class of capsular compounds, several of which have been studied in detail (Rebers & Heidelberger, 1959, 1961, 1963; Roberts, Buchanan & Baddiley, 1963; Rao, Buchanan & Baddiley, 1966*a,b*; Dixon, Buchanan & Baddiley, 1966; Chittenden, Roberts, Buchanan & Baddiley, 1968; Dixon, Roberts, Mills, Buchanan & Baddiley, 1968; Roy & Glaudemans, 1968).

By hydrolysis with alkali and subsequent enzymic dephosphorylation S.29 has been degraded to a phosphorus-free repeating unit. This paper describes a structural investigation of the repeating unit of S.29 and its mode of linkage through phosphodiester bridges in the intact molecule.

EXPERIMENTAL

Materials

The specific substance from *Pneumococcus* type 29 was a generous gift from Dr Rachel Brown. It is described as the soluble specific substance of type 29, preparation no. 1b

(Brown, 1939). Type 29 rabbit antiserum (dated 1961) and type 35B rabbit antiserum (dated 1953) were kindly given to us by Dr. Erna Lund. Intestinal phosphomonoesterase was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., almond β -glucosidase (known to contain several glycosidases; Pigman, 1944) from L. Light and Co. Ltd., Colnbrook, Bucks., and Galactostat reagent (D-galactose oxidase) from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Chromatography

Paper chromatography was carried out on Whatman no. 1 or no. 4 paper, with the following solvent systems: *A*, propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); *B*, butan-1-ol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951); *C*, ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer & Nebel, 1955); *D*, butan-1-ol-ethanol-water-aq. NH_3 (sp.gr. 0.88) (40:10:49:1, by vol.; organic phase) (Hirst, Hough & Jones, 1949). Compounds were detected by: the periodate-Schiff reagents for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956); molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949); aniline phthalate reagent for reducing sugars (Partridge, 1949); ninhydrin reagent for hexosamines (Consden & Gordon, 1948); alkaline AgNO_3 for sugars and polyols (Trevelyan, Procter & Harrison, 1950); Elson-Morgan reagents for hexosamines (Partridge, 1948). When experiments were carried out on materials eluted from paper chromatograms appropriate determinations on equivalent paper extracts were made.

For adsorption chromatography silica gel (Hopkin and Williams Ltd., Chadwell Heath, Essex) was used. 'Neutral silica' refers to silica treated with aq. NH_3 as described by Buchanan & Schwarz (1962). For thin-layer chromatography the adsorbent was Kieselgel G (E. Merck A.-G., Darmstadt, Germany), and compounds were detected by spraying the plate with aq. 25% (v/v) H_2SO_4 and heating at 120° until charred spots appeared.

Gas-liquid chromatography was performed with a Pye

Series 104 Gas Chromatograph, with flame ionization detector. The column was composed of 15% polyethylene glycol succinate polyester on acid-washed Celite (85–100 mesh). The dimensions were 7 ft. $\times$ $\frac{1}{8}$ in., the operating temperature 158° and the flow rate 55–65 ml./min. with N₂ as carrier gas. The general operating procedure was similar to that of Aspinall (1963).

Analytical methods

Total phosphorus was determined by the method of Chen, Toribara & Warner (1956). Galactose, before or after acid hydrolysis, was determined by the phenol-H₂SO₄ method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954; Aspinall & Ferrier, 1957), the correction factor 0.14 being used to allow for the extinction of the IO₃⁻ ion, and formaldehyde by a modification of the chromotropic acid method (Roberts *et al.* 1963).

Characterization of S.29

Immunological study. The substance was examined by the Ouchterlony technique by using type 29 antiserum. It was highly reactive, giving only one line of precipitation. It also reacted, slightly less strongly, with type 35B antiserum, with which it is reported to give a strong cross-reaction (Mørch, 1942, 1943; Eddy, 1944). No immunological reaction was detected with a sample of S.29 that had been hydrolysed with aq. NaOH as described below.

Using the same technique Dr Jean M. Tyler kindly tested the preparation for the presence of C-substance (How, Brimacombe & Stacey, 1964). With two horse anti-pneumococcal sera (antisera types I and VII) with a high anti-(C-substance) content, and purified C-substance from a rough type II *Pneumococcus* as standard, it was shown that the S.29 preparation contained 5% of C-substance.

Optical rotation. Brown (1939) gives $[\alpha]_D -50.8^\circ$ (water), and we have found $[\alpha]_D^{25} -51.4^\circ$ (c 1 in water).

Infrared spectrum. The infrared spectrum of S.29 in a KBr disk showed weak, but characteristic, bands at 1638 and 1560 cm.⁻¹ (NH·CO·CH₃ group).

Quantitative analysis. S.29 (8.1 mg.) was dissolved in water (10 ml.) and total phosphorus determined on samples (Found: P, 3.05%). Brown (1939) found P, 3.22%. The same solution (1 ml.) was diluted to 10 ml. and samples were used for galactose determination (Found: galactose, 50.6%; phosphate/galactose molar ratio, 1:2.86).

Acid hydrolysis. S.29 (4.8 mg.) was hydrolysed with 2N-HCl (0.5 ml.) in a sealed tube at 100° for 3 hr. The acid was removed by evaporation *in vacuo* over KOH and the residue examined by paper chromatography in solvents A, B and C. The products were detected as follows: periodate-Schiff reagents for ribitol, anhydribose and ribitol phosphates; molybdate reagent for ribitol phosphates, galactosamine phosphates and inorganic phosphate; aniline phthalate for galactose; ninhydrin for galactosamine and galactosamine phosphates; alkaline AgNO₃ for ribitol, galactose and galactosamine.

An acid hydrolysate of S.29 (5 mg.), obtained as above, was chromatographed as a band in solvent A and the products were detected with ninhydrin and the molybdate reagent. Phosphate P₁ ($R_{\text{galactose}}$ 0.29) gave a blue colour

with molybdate reagent and a purple colour with ninhydrin. Phosphate P₂ ($R_{\text{galactose}}$ 0.43) gave a blue colour with molybdate reagent, but did not react with ninhydrin. Phosphates P₁ and P₂ were separately eluted from the paper with water and the concentrated solutions treated with intestinal phosphomonoesterase (1 mg.) and (NH₄)₂CO₃ (4 mg.). The solutions were covered with a drop of toluene and incubated at 37° for 20 hr. The solutions were then passed through small columns of Dowex 2 (CO₃²⁻ form) resin and material was eluted with water. The eluates were freeze-dried and the residues subjected to paper chromatography in solvent B. Phosphate P₁ yielded galactosamine, whereas phosphate P₂ yielded ribitol.

Configuration of galactose in S.29. S.29 (6.5 mg.) was hydrolysed with 2N-HCl (0.65 ml.) at 100° for 3 hr. The solution was passed through a column of Dowex 2 (CO₃²⁻ form) resin (4.0 ml.) and the aqueous eluate (40 ml.) evaporated to dryness *in vacuo*. The residue was chromatographed as a band in solvent B. The galactose was eluted from the appropriate area with water, the solution was diluted to 10 ml. with water and samples (0.1–0.3 ml.) were analysed for hexoses (by phenol-H₂SO₄) and D-galactose (by Galactostat) (Found: hexose/D-galactose molar ratio, 1:0.98).

O-Acetyl groups. S.29 (0.5 mg.) gave a negative reaction for O-acetyl groups by the method of Frèrejacque (1955).

Isolation and composition of the neutral and basic pentasaccharides

Hydrolysis of the specific substance with alkali. S.29 (130 mg.) was dissolved in N-NaOH (8 ml.) and the solution heated in a sealed tube for 1 hr. at 100°. The hydrolysate was diluted with water, passed through a column of Dowex 50 (NH₄⁺ form) resin (15 ml.) and eluted with water. The eluate (125 ml.) was evaporated in a rotary evaporator to a small volume and then freeze-dried. The residue was dissolved in water, silica removed by centrifugation and the solution evaporated *in vacuo* to 3 ml. A small portion was examined by chromatography in solvent A. Three groups of products, with R_{ribitol} values 0.11, 0.26 and 0.49, were detected. The last two gave a purple colour rapidly with the periodate-Schiff reagents, whereas the first gave a purple colour slowly. Only the first two contained phosphate (molybdate reagent). No inorganic phosphate was detected.

Enzymic dephosphorylation of the products of alkali hydrolysis. The remainder of the hydrolysate above (about 3 ml.) was treated with (NH₄)₂CO₃ (15 mg.) and alkaline phosphomonoesterase (10 mg.). After the addition of a few drops of toluene the solution was incubated at 37° for 16 hr. The enzymic hydrolysate was passed through a column of Dowex 2 (CO₃²⁻ form) resin (15 ml.) and material was eluted with water. The eluate (200 ml.) was evaporated to small volume *in vacuo* and then freeze-dried. The residue (98.4 mg.) was dissolved in water (10 ml.), passed through a column of Dowex 50 (H⁺ form) resin (10 ml.) and the column quickly washed with water followed by N-NH₃. The neutral eluate (125 ml.) and the basic eluate (125 ml.) were each evaporated under reduced pressure. The neutral residue (29.7 mg.) and the basic residue (67.1 mg.) were each examined chromatographically in solvent A and found to be homogeneous.

Hydrolysis of S.29 with N-NaOH at 100° for 3 hr. also

gave three groups of products identical with those obtained from hydrolysis for 1 hr. Enzymic dephosphorylation of the products gave two fractions, neutral and basic, approximately in the ratio 1:5.

Neutral pentasaccharide. The above neutral residue was dissolved in the minimum amount of water, and ethanol was added until a precipitate was obtained. The precipitation was hastened by scratching the sides of the flask and refrigeration for 4–8 hr. The solid was removed by centrifugation and washed with ethanol, ethanol–ether and finally ether. The micro-crystalline product was dried in high vacuum over P_2O_5 at room temperature and was not hygroscopic. It had R_{ribitol} 0.52 in solvent *A*, and gave a purple colour rapidly with the periodate–Schiff reagents. It had $[\alpha]_D^{25} - 46.9^\circ$ (*c* 0.59 in water) [Found: N, 1.69. $C_{31}H_{55}NO_{25}$ requires N, 1.66; $C_{31}H_{55}NO_{25} \cdot 3H_2O$ requires N, 1.56%].

Basic pentasaccharide. This was obtained from the basic residue above, by precipitation from aqueous ethanol, as a pale-yellow micro-crystalline powder, $[\alpha]_D^{25} - 58.7^\circ$ (*c* 0.76 in water), R_{ribitol} 0.44 in solvent *A*. It gave a purple colour rapidly with the periodate–Schiff reagents.

Hydrolysis of the neutral and basic pentasaccharides with acid. The pentasaccharides (3 mg. each) were separately treated with 2*N*-HCl (0.3 ml.) at 100° for 3 hr. The acid was removed in each case by means of a column of Dowex 2 (CO_3^{2-}) resin. The freeze-dried eluates were examined by paper chromatography in solvents *B* and *C*, the periodate–Schiff reagents, ninhydrin and alkaline $AgNO_3$ being used for detection. The main products of hydrolysis of the neutral pentasaccharide were galactose, galactosamine, ribitol and anhydribose, with traces of disaccharides. The basic pentasaccharide gave galactose, ribitol, anhydribose, galactosamine (small amount) and disaccharides.

Quantitative analysis of the neutral and the basic pentasaccharide. The neutral pentasaccharide (11.722 mg.) was dissolved in water (2.0 ml.), 0.2 ml. of this solution diluted to 10 ml. and galactose determined on 0.4 ml. and 0.7 ml. samples by the phenol– H_2SO_4 method [Found: galactose, 60.9. $C_{31}H_{55}NO_{25} \cdot 3H_2O$ requires galactose (three units), 60.3%].

The basic pentasaccharide (15.167 mg.) was dissolved in water (2.0 ml.), 0.2 ml. of this solution diluted to 10 ml. and galactose determined on 0.3 ml. and 0.5 ml. samples [Found: galactose, 57.7. $C_{29}H_{53}NO_{24} \cdot 3H_2O$ requires galactose (3 units), 63.3% (57.7% corresponds to 2.73 units of galactose)].

Partial degradations of the basic and the neutral pentasaccharide

Action of nitrous acid on the basic pentasaccharide. The basic pentasaccharide (12 mg.) was treated with 0.02*N*-HCl (0.25 ml.) and $NaNO_2$ (80 mg.) in water (1 ml.) and the mixture heated at 50° in a sealed tube for 4 hr. The solution was passed through a column of Dowex 50 (NH_4^+ form) resin (10 ml.), and the eluate (100 ml.) was boiled for 10 min., evaporated *in vacuo* to small volume and then freeze-dried. A small sample of the residue was chromatographed in solvent *B* and the products were detected with the periodate–Schiff reagents. There were two major products, $R_{\text{galactose}}$ 0.31 and 1.66, and two minor products, $R_{\text{galactose}}$ 0.04 and 0.41.

The compound with $R_{\text{galactose}}$ 0.31 (R_{ribitol} 0.22) gave a strong purple colour rapidly with the periodate–Schiff re-

agents. A slight yellow colour was observed after 45 min. and this finally changed to grey–purple. Further studies on this compound, a tetrasaccharide, are described below.

The compound with $R_{\text{galactose}}$ 1.66 (R_{ribitol} 1.24) gave a yellow colour with the periodate–Schiff reagents and appeared as a long streak indistinguishable from the product of deamination of *D*-galactosamine hydrochloride (2,5-anhydrotalose).

A small sample of the deamination residue (about 1.5 mg.) was dissolved in water (0.4 ml.), $NaBH_4$ (3 mg.) added and the mixture kept at 21° overnight. Excess of borohydride was decomposed with a drop of acetic acid and the solution passed through a column of Dowex 50 (H^+ form) resin. Boric acid was removed by evaporation to dryness with methanol (three times) and the residue was hydrolysed with 2*N*-HCl at 100° for 3 hr. The acid was removed by evaporation *in vacuo* and the hydrolysate examined by paper chromatography in solvent *B*. The products, detected by the periodate–Schiff reagents, were galactose, ribitol, anhydribose and 2,5-anhydrotalitol. With ninhydrin galactosamine could not be detected.

The remainder of the deamination product was chromatographed as a band in solvent *B* and the two major products were eluted. The compound with $R_{\text{galactose}}$ 1.66 was reduced with $NaBH_4$ as described above and the product, without acid hydrolysis, was chromatographed in solvents *B* and *D*. The product, detected with the periodate–Schiff reagents, behaved in a manner identical with that of authentic 2,5-anhydro-*D*-talitol (Rao *et al.* 1966a).

Partial acid hydrolysis of the neutral pentasaccharide. The neutral pentasaccharide (5 mg.) was hydrolysed with 0.02*N*-HCl (0.5 ml.) at 100° for 4 hr. in a sealed tube. The acid was removed by a small column of Dowex 2 (CO_3^{2-} form) resin and the eluate was freeze-dried. Chromatography in solvent *B* showed the presence of galactose, *N*-acetylgalactosamine, galactosamine (trace), ribitol, anhydribose and disaccharide D_1 .

On paper chromatography in solvent *B*, disaccharide D_1 had $R_{\text{galactose}}$ 0.26, R_{ribose} 0.54, $R_{\text{melibiose}}$ 0.71 and gave strong reactions with alkaline $AgNO_3$ and aniline phthalate (brown colour). With the periodate–Schiff reagents disaccharide D_1 gave initially a very weak purple colour that slowly became intense. Disaccharide D_1 was therefore not a (1→2)-linked disaccharide (Hardy & Buchanan, 1963). A larger sample of the neutral pentasaccharide was hydrolysed with 0.02*N*-HCl as before, neutralized with Dowex 2 (CO_3^{2-} form) and chromatographed as a band in solvent *B*. Disaccharide D_1 was eluted and samples were used for the following experiments.

Acid hydrolysis of disaccharide D_1 with 2*N*-HCl at 100° for 3 hr. followed by paper chromatography in solvent *B* gave only galactose.

Disaccharide D_1 was indistinguishable from authentic 6-*O*-β-*D*-galactopyranosyl-*D*-galactose when subjected to paper chromatography in solvent *B* (detection with aniline phthalate, the periodate–Schiff reagents and alkaline $AgNO_3$).

Disaccharide D_1 was dissolved in a few drops of water, $NaBH_4$ (5 mg.) added and the solution kept at 21° overnight. A few drops of acetic acid were added and the solution was passed through a small column of Dowex 50 (H^+ form) resin. The eluate was evaporated to dryness *in vacuo* and boric acid removed by distillation (three times) with methanol. An identical series of operations was carried out on authentic

6-*O*- β -D-galactopyranosyl-D-galactose. The products were examined by paper chromatography in solvent *B*. Both gave a purple spot, $R_{\text{galactose}}$ 0.29, rapidly with the periodate-Schiff reagents. No yellow colour was observed, showing that disaccharide D_1 was not a 3- or 4-*O*-substituted hexitol (Hardy & Buchanan, 1963). When the reduced disaccharides were each hydrolysed with 2*N*-HCl, the acid was removed *in vacuo* over KOH and the products were chromatographed in solvents *B* and *D*, galactose and a hexitol, presumably galactitol, were produced in each case.

Partial acid hydrolysis of the basic pentasaccharide. The basic pentasaccharide (5mg.) was hydrolysed with 0.02*N*-HCl in the same way as was the neutral pentasaccharide. Paper chromatography (solvent *B*) showed the presence of galactose (small amount), ribitol, anhydrosorbitol, disaccharides D_1 and D_2 and an oligosaccharide mixture.

Disaccharide D_2 had $R_{\text{galactose}}$ 0.14 and R_{lactose} 0.3 in solvent *B*, and reacted strongly with alkaline AgNO_3 and with aniline phthalate (brown colour). It gave a purple colour slowly with the periodate-Schiff reagents and a weak purple colour with ninhydrin. Another sample (8mg.) of the basic pentasaccharide was hydrolysed with 0.02*N*-HCl as before and the product chromatographed as a band in solvent *B*. Disaccharide D_2 was eluted with water and used in the following experiments.

Disaccharide D_2 was hydrolysed with 2*N*-HCl at 100° for 3 hr. and the solution evaporated to dryness *in vacuo* over KOH. Paper chromatography in solvent *B*, with alkaline AgNO_3 being used for detection, showed that partial hydrolysis to galactosamine and galactose had taken place.

Disaccharide D_2 was dissolved in a few drops of water, NaBH_4 (5mg.) added and the solution kept at 21° overnight. A few drops of acetic acid were added and the solution was passed through a small column of Dowex 50 (H^+ form) resin. The column was washed with water and the reduced disaccharide then eluted with aq. N-NH_3 . The eluate was evaporated to dryness and subjected to evaporation with methanol (three times). Acid hydrolysis of the product (2*N*-HCl at 100° for 3 hr.), removal of the acid *in vacuo* over KOH and paper chromatography in solvent *B* showed that the reduced disaccharide D_2 was partially hydrolysed into galactosamine and a hexitol, presumably galactitol. The reduced disaccharide D_2 gave a purple colour rapidly with the periodate-Schiff reagents and had $R_{\text{galactose}}$ 0.17.

Methylation of oligosaccharides

Methylation of the tetrasaccharide. The tetrasaccharide (20mg.), purified by chromatography in solvent *B*, was dissolved in *NN*-dimethylformamide (2.5ml.) and the solution stirred with methyl iodide (1.1ml.) and freshly prepared Ag_2O (0.7g.) at 30° in the dark for 24 hr. Methyl iodide (1.2ml.) was added and after a further 24 hr. the mixture was filtered. The precipitate was washed with a little dimethylformamide and an excess of chloroform. The filtrate was kept at 4° for 2 hr., filtered from the crystalline solid and the resulting filtrate evaporated to dryness *in vacuo*, finally under high vacuum. The residue was methylated again for another 18 hr. in dimethylformamide (0.3ml.), with methyl iodide (3.0ml.) and Ag_2O (0.5g.), and the product isolated as above. Chromatography with silica gel afforded the permethyl ether (18.5mg.), which was eluted with benzene-ethanol (97:3, v/v). The permethyl ether was homogeneous when examined by thin-layer

chromatography in benzene-ethanol (8:1, v/v). The permethyl ether was methanolysed with 3% (w/v) HCl in methanol at 60° for 24 hr., the solution evaporated to dryness *in vacuo* and the residue dissolved in a few drops of chloroform for examination by gas-liquid chromatography.

Methylation of the neutral pentasaccharide. The neutral pentasaccharide (21mg.) was dissolved in *NN*-dimethylformamide (2.0ml.). Methyl iodide (1.4ml.) and freshly prepared Ag_2O (0.8g.) were added and the mixture was stirred in the dark in a sealed flask at 30°, and samples were examined at intervals by thin-layer chromatography in benzene-ethanol (8:1, v/v). After 24 hr. methyl iodide (1.9ml.) was added and reaction continued for a further 24 hr. The mixture was filtered, and the precipitate washed with dimethylformamide (4ml.) followed by chloroform (about 30ml.). The filtrate was cooled to 4° for 2 hr., filtered from the solid and the resulting filtrate evaporated to dryness *in vacuo*, finally under high vacuum. The residue was methylated again for 24 hr. by using dimethylformamide (0.3ml.), methyl iodide (3.0ml.) and Ag_2O (0.5g.), and the product isolated as before. The permethyl ether was chromatographed on silica gel (0.6cm. $\times$ 14cm.). Elution with benzene-ethanol (97:3, v/v), with 1.5ml. fractions being collected, yielded the permethyl ether (15.2mg.), which was homogeneous, R_F 0.4, when examined by thin-layer chromatography in benzene-ethanol (8:1, v/v).

The permethyl ether was methanolysed with 3% (w/v) HCl in methanol at 60° for 24 hr., the solution evaporated to dryness *in vacuo* and the residue dissolved in a few drops of chloroform for examination by gas-liquid chromatography.

The permethyl ether was hydrolysed with 2*N*-HCl (0.4ml.) for 3 hr. at 100° and the acid removed *in vacuo* over KOH. When the residue was chromatographed in solvent *B* a product was detected, $R_{\text{galactosamine}}$ 3.6, that gave a purple colour with ninhydrin and a red colour with Elson-Morgan reagents. It was identified as 3,4,6-tri-*O*-methylgalactosamine by chromatographic comparison with an acid hydrolysate of methylated methyl 2-acetamido-2-deoxy- α -D-galactopyranoside. The chromatography of methylated products is given in Table 1.

Degradation of the oligosaccharides with periodate

Quantitative oxidation of the neutral pentasaccharide. A sample containing 3.275 μ moles of the neutral pentasaccharide in water (2.5ml.) was oxidized at 21° with 0.02*M*- NaIO_4 (2.5ml., 50 μ moles). In 1 hr. 3.87 mol.prop., in 48 hr. 5.76 mol.prop. and in 96 hr. 6.1 mol.prop. of periodate were reduced and no further reduction was observed.

The neutral pentasaccharide (2.344mg.) was dissolved in water (10ml.). Samples (0.7ml. and 1.0ml.) of this solution were diluted to 2ml., mixed with 0.1*M*- NaIO_4 (0.5ml.) and kept at 21° in the dark for 2 hr.; 0.96 mol.prop. of formaldehyde was produced.

Degradation of the neutral pentasaccharide with periodate, followed by borohydride. The neutral pentasaccharide (3mg.) was treated with 0.1*M*- NaIO_4 (0.7ml.) and the solution was kept at 21° for 72 hr. in the dark. Then NaBH_4 (15mg.) was added and the solution kept overnight at 21°. A few drops of acetic acid were added, the solution was passed through a small column of Dowex 50 (H^+ form)

Table 1. Retention times on gas-liquid chromatography of methyl glycosides from the tetrasaccharide permethyl ether and the neutral pentasaccharide permethyl ether relative to that of methyl 2,3,4,6-tetra-O-methyl- β -D-glucopyranoside

(s), Strong; (m), medium; (w) weak.

	Relative retention time	Tetra	Penta
1,2,3,4-Tetra-O-methylribitol*	1.14	+	+
2,3,5,6-Tetra-O-methyl- β -galactose*	1.57 (s)	+	—
2,3,5,6-Tetra-O-methyl- α -galactose*	2.04 (w)		
2,4,6-Tri-O-methyl- α - β -galactose†	4.82 (m)	+	+
2,3,5-Tri-O-methyl- α - β -galactose*	5.65 (s)		
	5.25	+	++

*Identified by co-chromatography with the authentic compound.

†Identified by comparison of retention times and relative peak heights with those of an authentic sample.

resin and the eluate was evaporated *in vacuo*. Boric acid was removed by distillation three times with methanol, and the residue was hydrolysed with 2N-HCl at 100° for 3 hr. The acid was removed *in vacuo* and the residue chromatographed in solvents *B* and *D*. The products were galactose, threitol, glycerol and ethylene glycol. The spot corresponding to threitol was stronger than either of those corresponding to glycerol or ethylene glycol (alkaline AgNO₃ and the periodate-Schiff reagents).

In another experiment the neutral pentasaccharide was oxidized with periodate and then reduced with borohydride. The boric acid-free product was subjected to partial acid hydrolysis (0.1 N-H₂SO₄ overnight at 28°), the acid removed with Dowex 2 (CO₃²⁻ form) and the product chromatographed in solvent *B*. The products now included a disaccharide, D₃, that replaced most of the galactose and some of the threitol. Samples of disaccharide D₃, obtained by degradation of the neutral or basic pentasaccharide (see below), were purified by paper chromatography in solvent *B* and used for the following experiments.

Disaccharide D₃ had *R*_{galactose} 0.67 in solvent *B* and gave a purple colour rapidly with the periodate-Schiff reagents. With alkaline AgNO₃ it gave a weak reaction, but the spot was more marked after heating the paper over a steam bath.

A solution of disaccharide D₃ (approx. 200 μ g. in 0.1 ml. of water) was mixed with 2% (w/v) almond β -glucosidase (0.025 ml.). The mixture was kept at 37° for 16 hr. and chromatographed in solvent *B*. The products detected by alkaline AgNO₃ were galactose and threitol in approximately equal amounts as determined by visual comparison.

A solution of disaccharide D₃ (about 250 μ g. in 2 ml. of water) was used for the following determinations. Galactose was determined on 0.1 ml. and 0.2 ml. samples by the phenol-H₂SO₄ method. Formaldehyde was determined after periodate oxidation of 0.2 ml. and 0.3 ml. samples that had been diluted to 2 ml. The galactose/formaldehyde molar ratio was 1:0.87.

Formaldehyde production on periodate oxidation of the basic pentasaccharide. The basic pentasaccharide (3.033 mg.) was dissolved in water (10 ml.). Samples (0.5 ml. and 0.7 ml.) of this solution were diluted to 2.0 ml. and mixed with 0.1 M-NaIO₄ (0.5 ml.), and formaldehyde was determined after 2 hr. (Found: 0.91 mol.prop.).

Degradation of the basic pentasaccharide with periodate followed by borohydride. The basic pentasaccharide (6 mg.)

was treated with 0.1 M-NaIO₄ (1.5 ml.) and kept at 21° for 72 hr. in the dark. The oxidation product was reduced with NaBH₄ (25 mg.) and the polyol isolated as above. The residue was hydrolysed with 0.1 N-H₂SO₄ (0.6 ml.) overnight at 28°, the acid removed with a small column of Dowex 2 (CO₃²⁻ form) and the eluate freeze-dried. Paper chromatography in solvent *B* showed galactose (traces), threitol, glycerol, ethylene glycol and disaccharide D₃.

Degradation of the tetrasaccharide with periodate, followed by borohydride. The tetrasaccharide (0.5 mg.) in water (0.1 ml.) was treated with 0.1 M-NaIO₄ (0.1 ml.) and kept at 2° in the dark for 30 min. Excess of periodate was destroyed by the addition of ethylene glycol. NaBH₄ (3 mg. in 0.2 ml. of water) was added and the mixture kept at 21° overnight. The solution was passed successively through small columns of Dowex 50 (H⁺ form) resin and Dowex 2 (CO₃²⁻ form) resin and the eluate evaporated to dryness *in vacuo*. The residue was hydrolysed with 2N-HCl at 100° for 3 hr., the acid was removed by evaporation *in vacuo* and the products were analysed by paper chromatography in solvent *B*. Galactose and arabinose were detected by aniline phthalate and identified by comparison with authentic samples.

Degradation of *S. 29* with periodate

Quantitative oxidation of *S. 29*. A solution (3 ml.) of *S. 29* (5.85 mg.) was mixed with 0.02 M-NaIO₄ (3.0 ml.) at 21° and the reduction of periodate was determined spectrophotometrically. After 1 hr. 1.9 mol.prop., after 48 hr. 4.0 mol.prop. and after 120 hr. 4.11 mol.prop. of periodate were reduced for each phosphate group and no further reduction was observed.

S. 29 (8.1 mg.) was dissolved in water (10 ml.), a sample (0.5 ml.) was diluted to 2 ml. and 0.1 M-NaIO₄ (0.5 ml.) was added. After 2 hr. at 21° in the dark no formaldehyde was produced.

Degradation of *S. 29* with periodate followed by borohydride. *S. 29* (4 mg.) was treated with 0.1 M-NaIO₄ (1 ml.) and kept at 21° for 4 days in the dark. Then NaBH₄ (20 mg.) was added and the solution kept at room temperature overnight. Excess of borohydride was destroyed by the addition of a few drops of acetic acid and the solution was passed through a small column of Dowex 50 (H⁺ form) resin. The eluate was evaporated *in vacuo* and boric acid removed by evaporation of the residue with hot methanol (three times),

The residue was hydrolysed with 2*N*-HCl (0.4 ml.) at 100° for 3 hr., the acid was removed by evaporation *in vacuo* over KOH and the products were chromatographed in solvents *A*, *B* and *D*. The products, identified by comparison with authentic substances, were galactose, galactosamine, threitol and ethylene glycol. With molybdate reagent an organic phosphate, $R_{\text{galactose}}$ 0.28 in solvent *A*, was detected that gave a purple colour with ninhydrin. This was eluted from paper and enzymically dephosphorylated in the usual manner to give galactosamine. Glycerol was not present among the degradation products.

Another sample of S.29 (7.4 mg.) was treated with periodate, the product reduced with borohydride and boric acid removed as above. The resultant residue was hydrolysed with 2*N*-HCl (0.7 ml.) at 100° for 3 hr. The hydrolysate was neutralized to pH 6 and the volume adjusted to 10 ml. with water. Phosphorus and galactose (phenol- H_2SO_4) were determined on samples (Found: P, 3.1; galactose, 19.7%; phosphate/galactose molar ratio, 1:1.09).

Preparation of reference compounds

1,2,3,4-Tetra-O-methyl-DL-ribitol. 1-*O*-Triphenylmethyl-DL-ribitol (Bien & Ginsburg, 1958) (3.36 g.) was dissolved in dry *NN*-dimethylformamide (21 ml.), and redistilled methyl iodide (5.6 ml., 10 mol.prop.) and freshly prepared Ag_2O (9.9 g., 5 mol.prop.) were added. The mixture was stirred at room temperature (21°) for 24 hr. in a flask protected from moisture. The mixture was filtered and the precipitate washed with dimethylformamide (10 ml.) and chloroform (25 ml.). The combined filtrate was shaken with aq. 1% (w/v) NaCN (2 × 10 ml.), and the final chloroform layer dried (Na_2SO_4) and evaporated *in vacuo* to remove both chloroform and dimethylformamide. The oily residue was chromatographed on alumina (200 g.). Benzene-ether (2:1, v/v) eluted the tetramethyl ether (3.6 g.), which was homogeneous by thin-layer chromatography in benzene-ether (1:1, v/v). The product was dissolved in aq. 80% (v/v) acetic acid (75 ml.) and heated at 80° for 15 min. The acetic acid was evaporated *in vacuo*, the residue treated with water (50 ml.) and the triphenylmethanol removed by filtration. The filtrate was evaporated *in vacuo* to yield the tetramethyl ether as a pale-yellow oil (1.58 g., 89% yield), which was purified by distillation, b.p. 60° (bath)/0.004 mm. (Found: C, 51.75; H, 9.5. $\text{C}_9\text{H}_{20}\text{O}_5$ requires C, 51.9; H, 9.7%).

The tetramethyl ether (0.2 g.) was dissolved in dry pyridine (0.3 ml.) and phenyl isocyanate (0.4 ml., about 4 mol.prop.) added. The mixture was kept at room temperature for 24 hr., the excess of reagent decomposed by a few drops of water and the mixture evaporated to dryness *in vacuo*. The residue crystallized from light petroleum (b.p. 80–100°) and had m.p. 73°. Two recrystallizations from light petroleum (b.p. 60–80°) yielded the pure *phenylurethane*, m.p. 75° (Found: C, 58.6; H, 7.6; N, 4.4. $\text{C}_{16}\text{H}_{25}\text{NO}_6$ requires C, 58.7; H, 7.7; N, 4.3%).

Methyl 2,3,5-tri-O-methyl-β-D-galactofuranoside. Methyl β-D-galactofuranoside (Austin, Hardy, Buchanan & Baddiley, 1963) (1.07 g.) in dry pyridine (12 ml.) was treated with triphenylmethyl chloride (1.53 g., 1 mol.prop.) for 5 days at room temperature. The mixture was poured into ice-water and the product isolated by using chloroform. The residue (2–3 g.) was chromatographed on neutral silica

(40 g.). Benzene-ether (9:1, v/v) eluted triphenylmethanol and benzene-ether (1:1, v/v) eluted methyl 6-*O*-triphenylmethyl-β-D-galactofuranoside as a syrup (0.77 g., 32%), $[\alpha]_{\text{D}}^{18} -45.8^\circ$ (c 1.50 in chloroform) (Found: C, 71.5; H, 6.8. Calc. for $\text{C}_{26}\text{H}_{28}\text{O}_6$: C, 71.55; H, 6.5%). Siddiqui & Urbas (1967) give $[\alpha]_{\text{D}} -46.3^\circ$ and m.p. 91–92.5°, and Roy & Glaudemans (1968) give $[\alpha]_{\text{D}} -49.4^\circ$ for this compound.

The triphenylmethyl ether (0.6 g.) was dissolved in dry *NN*-dimethylformamide (5 ml.), and redistilled methyl iodide (0.89 ml., 10 mol.prop.) and freshly prepared Ag_2O (1.6 g., 5 mol.prop.) were added. The mixture was stirred for 18 hr., filtered and the precipitate washed with dimethylformamide (5 ml.) and chloroform (20 ml.). A further 20 ml. of chloroform was added and, after 2 hr. at 4°, the precipitate was removed by filtration and the filtrate dried (Na_2SO_4) and evaporated *in vacuo*, leaving a mobile syrup (0.62 g.). The syrup was chromatographed on neutral silica (10 g.). Benzene-ether (9:1, v/v) eluted the trimethyl ether (0.58 g.), $[\alpha]_{\text{D}}^{18} -50.2^\circ$ (c 1.48 in chloroform), which was dissolved in aq. 80% (v/v) acetic acid (30 ml.) and heated at 70° for 15 min. The acetic acid was evaporated *in vacuo*, the residue treated with water and triphenylmethanol removed by filtration. The filtrate was evaporated *in vacuo* and the resultant residue distilled, b.p. 65° (bath)/0.01 mm., to yield methyl 2,3,5-tri-*O*-methyl-β-D-galactofuranoside, $[\alpha]_{\text{D}}^{18} -95.3^\circ$ (c 1.29 in chloroform) as a colourless oil (Found: C, 51.3; H, 8.8; $\text{C}_{10}\text{H}_{20}\text{O}_6$ requires C, 50.9; H, 8.5%).

Other reference compounds. Methylation of methyl α- and β-D-galactofuranoside (Austin *et al.* 1963) was carried out by the procedure of Kuhn, Trischmann & Löw (1955). The permethyl ethers, after chromatographic purification on silica gel, were homogeneous by thin-layer and gas-liquid chromatography.

2,4,6-Tri-*O*-methyl-D-galactose was a gift from Professor G. O. Aspinall and was treated with methanol and HCl before gas-liquid chromatography.

Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (13 mg.) was methylated twice at room temperature (21°) by the method of Kuhn *et al.* (1955). The product was hydrolysed with 2*N*-HCl at 100° for 3 hr., the acid removed by evaporation *in vacuo* and the residue used as a chromatographic standard.

DISCUSSION

In agreement with the earlier work (Shabarova *et al.* 1962) S. 29 was shown to yield, on acid hydrolysis, galactose, galactosamine, ribitol, anhydro-ribitol, ribitol phosphate, a galactosamine phosphate and inorganic phosphate. The quantitative analysis of S. 29 showed the presence of three galactose units for each phosphate group, and the Kjeldahl nitrogen and acetyl values determined by Brown (1939) correspond to one unit of *N*-acetyl galactosamine.

The degradation of S. 29 with alkali was carried out in a manner similar to that of S. 10A (Rao *et al.* 1966a). Three groups of products were detected by paper chromatography, corresponding to diphosphates, monophosphates and phosphorus-free compounds. These arise through hydrolysis of phosphodiester linkages through the usual cyclic

GalN = galactosamine; GalNAc = *N*-acetylgalactosamine; *p* = pyranose.

phosphate mechanism. When the mixture was dephosphorylated enzymically, two compounds resulted, one neutral and the other basic, separable from each other by means of Dowex 50 (H^+ form) resin. They were obtained as non-hygroscopic micro-crystalline solids by precipitation with ethanol from a concentrated aqueous solution. On paper chromatograms each was homogeneous and distinguishable from the other. When the period of alkali hydrolysis was increased from 1 to 3 hr. the relative amount of neutral to basic compound changed from 1:2.5 to 1:5 (w/w). A similar situation occurs in S.10A (Rao *et al.* 1966a) and it is very probable that the neutral compound is the *N*-acetyl derivative of the basic compound. Qualitative and quantitative analyses of the neutral and basic compounds lead to a 'pentasaccharide' structure for the neutral repeating unit, containing three galactose residues, one *N*-acetylgalactosamine and one ribitol, confirming the analytical values on S.29 itself.

When the basic pentasaccharide was treated with nitrous acid (Rao *et al.* 1966a) fission of the galactosaminyl linkage took place, with the formation of 2,5-anhydrotalose and a tetrasaccharide as the major products. The basic pentasaccharide can therefore be expressed as (I), the neutral pentasaccharide as (II) and the tetrasaccharide as (III).

Partial acid hydrolysis of the neutral pentasaccharide (II) yielded *N*-acetylgalactosamine, galactose, ribitol, anhydribose and a disaccharide, D_1 . Disaccharide D_1 yielded only galactose on acid hydrolysis and was indistinguishable from 6-*O*- β -D-galactopyranosyl-D-galactose on paper chromatography. Borohydride reduction of the two disaccharides gave indistinguishable products.

Partial acid hydrolysis of the basic pentasaccharide (I) gave rise to ribitol, anhydribose and a small amount of galactose, together with disaccharide D_1 , another disaccharide, D_2 , and an oligosaccharide mixture. Disaccharide D_2 had the chromatographic properties and colour reactions of an *O*-galactosaminylgalactose. Hydrolysis with acid yielded galactosamine and galactose. When disaccharide D_2 was reduced with borohydride, and the product hydrolysed with acid, galactosamine and a hexitol, presumably galactitol, were formed. The formation of disaccharide D_2 is to be expected because of the stability of the glycosaminide linkage

towards acid (cf. Rao *et al.* 1966a). The basic pentasaccharide may now be represented by (IV), the neutral pentasaccharide by (V) and the tetrasaccharide by (VI).

Thus it appeared that two of the galactosyl linkages were appreciably more acid-labile than the other one. The presence of galactofuranose linkages was therefore suspected and was shown conclusively by methylation and by periodate oxidation. When the tetrasaccharide (VI) was methylated with methyl iodide and silver oxide in dimethylformamide (Kuhn *et al.* 1955), and the resulting permethyl ether subjected to methanolysis and gas-liquid chromatography (Aspinall, 1963), 1,2,3,4-tetra-*O*-methylribitol and the methyl glycosides of 2,3,5,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose and 2,3,5-tri-*O*-methylgalactose were detected in approximately equal amounts. When the same operation was carried out on the neutral pentasaccharide (V) the methyl glycosides of the tetra-*O*-methylgalactose were no longer present and an increased amount of the methyl glycosides of 2,3,5-tri-*O*-methylgalactose appeared. A methylated galactosamine derivative was not detected under these conditions, but aqueous acid hydrolysis of the permethyl ether from pentasaccharide (V) yielded a compound corresponding to

3,4,6-tri-*O*-methylgalactosamine on paper chromatography. The structure (VII) can now be written for the neutral pentasaccharide, in which β -linkages have been assigned throughout because of its strongly negative rotation ($[\alpha]_D -46.9^\circ$).

Partial acid hydrolysis of the neutral pentasaccharide (VII) causes hydrolysis of both labile furanosyl linkages and of that between the *N*-acetylgalactosamine and galactose unit A. Thus disaccharide D₁, 6-*O*- β -D-galactopyranosyl-D-galactose, arises from galactose units B and C. In similar acid treatment of the basic pentasaccharide (VIII) the galactosaminyl linkage is more stable and the disaccharide D₂ can be isolated. The colour reactions of disaccharide D₂, and its reduction product, with the periodate-Schiff reagents are consistent with its formulation as 6-*O*-(2-amino-2-deoxy- β -D-galactopyranosyl)-D-galactose (Hardy & Buchanan, 1963).

Procedures involving periodate oxidation have confirmed fully the structures (VII) and (VIII). The neutral pentasaccharide (VII) reduced 6.1 mol.prop. of periodate; in another oxidation 0.96 mol.prop. of formaldehyde was produced. These results are accounted for by the *N*-acetylgalactosamine (1 mol.prop.), galactose units A and C (each 1 mol.prop.) and ribitol (3 mol.prop., with the formation of 1 mol.prop. of formaldehyde). When the product of periodate oxidation was reduced with borohydride, and the resulting polyol hydrolysed with acid (Goldstein, Hay, Lewis & Smith, 1965), glycerol (from the *N*-acetylgalactosamine), a strong spot of threitol (from galactose units A and C), galactose (unit B) and ethylene glycol (from the ribitol) were detected. When the polyol was hydrolysed under milder conditions a reduced

disaccharide, D₃, was detected. The same product was isolated from similar degradation of the basic pentasaccharide (VIII) (see below). Hydrolysis of disaccharide D₃ by almond β -glucosidase yielded galactose and threitol in equal amount. When disaccharide D₃ was analysed for hexose and for the formaldehyde produced on periodate oxidation, the ratio was 1:0.87, leading to the structure (IX) for disaccharide D₃, which arises from the galactose units B and C.

Formaldehyde (0.91 mol.prop.) was produced by periodate oxidation of the basic pentasaccharide (VIII), and the major products of the sequence periodate oxidation, borohydride reduction and mild acid hydrolysis were glycerol (from the galactosamine), threitol (from galactose unit A), compound D₃ (from galactose units B and C), ethylene glycol (from the ribitol) and a trace of galactose.

When the tetrasaccharide produced by deamination of the pentasaccharide (VIII) was treated briefly with periodate, the product reduced with borohydride and then subjected to acid hydrolysis, arabinose was detected by paper chromatography.

S. 29 itself yielded no formaldehyde on periodate oxidation. These experiments confirm the methylation evidence that removal of the amino sugar moiety exposes a terminal galactofuranose residue.

The initial observation that S. 29 yielded ribitol phosphates and galactosamine phosphates on acid hydrolysis indicated strongly that a ribitol residue in one unit was linked by a phosphodiester group to an *N*-acetylgalactosamine residue in the next. The *N*-acetylgalactosamine residue in the neutral pentasaccharide is oxidizable by periodate, but in S. 29 itself no such oxidation takes place. The phosphate linkage to the *N*-acetylgalactosamine must therefore be on the 3- or the 4-hydroxyl group. In the periodate oxidation of S. 29 4·1 mol. prop. of periodate was reduced and no formaldehyde was formed. These facts are accommodated in structure (X) for S. 29.

When S. 29 was subjected to the sequence periodate oxidation, borohydride reduction and acid hydrolysis, only one galactose residue was unoxidized (B). Galactosamine and its phosphates were detected, together with threitol (from galactose residues A and C) and ethylene glycol (from the ribitol); no glycerol was produced.

In the hydrolysis of the phosphodiester (X), by alkali or by acid, cyclic phosphate formation on either ribitol or *N*-acetylgalactosamine is possible because of the presence of a vicinal hydroxyl group in each case. Hydrolysis is therefore not unidirectional, a situation that has already been discussed in some detail (Rao *et al.* 1966b). In structure (X) the ribitol is assigned the configuration shown on biosynthetic grounds (cf. Rao *et al.* 1966b).

The preparation of 1,2,3,4-tetra-*O*-methyl-DL-ribitol and methyl 2,3,5-tri-*O*-methyl-β-D-galacto-

furanoside, required for chromatographic comparisons, is described in the Experimental section.

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