The Type-Specific Substance from *Pneumococcus* Type 10A(34)

STRUCTURE OF THE DEPHOSPHORYLATED REPEATING UNIT

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1. A hexasaccharide unit was isolated from the specific substance S.10A from *Pneumococcus* type 10A (34) by hydrolysis with alkali followed by enzymic dephosphorylation. 2. The hexasaccharide was shown to be *O*-D-galactopyranosyl-(1 → 3)-*O*-D-galactopyranosyl-(1 → 4)-*O*-[D-galactofuranosyl-(1 → 6)]-*O*-2-acet-amido-2-deoxy-D-galactopyranosyl-(1 → 3)-*O*-D-galactopyranosyl-(1 → 2)-ribitol.

A number of the serologically-specific capsular substances from pneumococci are characterized by the presence of phosphate groups (Brown, 1939; Brown & Robinson, 1943; Shabarova, Buchanan & Baddiley, 1962; How, Brimacombe & Stacey, 1964). Several of these are polymers containing ribitol phosphate residues (Rebers & Heidelberger, 1959, 1961, 1963; Shabarova *et al.* 1962; Roberts, Buchanan & Baddiley, 1963). We now describe the detailed chemistry of the type-specific substance S.10A from *Pneumococcus* type 10A that was made possible by a generous gift from Dr Rachel Brown. This *Pneumococcus* is also called type 34 in the U.S.A.; the problem of nomenclature is discussed by Kauffmann, Lund & Eddy (1960) and by Roberts *et al.* (1963).

Shabarova *et al.* (1962) found that the products of acid hydrolysis of substance S.10A were galactose, galactosamine, ribitol and its phosphates, inorganic phosphate and anhydriitol. A dephosphorylated repeating unit can be obtained by hydrolysis with alkali and enzymic dephosphorylation of the resulting products. The present paper describes the chemistry of this unit; the way in which the units are joined by phosphodiester linkages to form the type-specific substance is discussed in the next paper (Rao, Buchanan & Baddiley, 1966).

EXPERIMENTAL

Materials

The preparation of the specific substance S.10A from *Pneumococcus* type 10A given to us by Dr Rachel Brown is described by Brown & Robinson (1943) as the soluble specific substance of type 10A (strain no. 3868) preparation no. 1a. Intestinal alkaline phosphomonoesterase was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and almond β -glucosidase from L. Light and Co. Ltd., Colnbrook, Bucks.

Chromatography

Paper chromatography was carried out on Whatman no. 1, 3 or 4 paper. The following solvent systems were used: *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-water (2:1:2, by vol.; organic phase) (Jermyn & Isherwood, 1949); *D*, ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer & Nebel, 1955); *E*, butan-1-ol-ethanol-water-aq. NH_3 (sp. gr. 0.88) (40:10:49:1, by vol.; organic phase) (Hirst, Hough & Jones, 1949); *F*, butan-1-ol-pyridine-water-benzene (5:3:3:1, by vol.; organic phase) (Bull, 1962). 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 aminodeoxy sugars (Consdon & Gordon, 1948); alkaline AgNO_3 reagent for sugars and polyols (Trevelyan, Procter & Harrison, 1950); Elson-Morgan reagent for free hexosamines (Partridge, 1948); Morgan-Elson reagent for *N*-acetylhexosamines (Partridge, 1948); benzidine-trichloroacetic acid reagent for reducing sugars and disaccharides (Bacon & Edelman, 1951).

Adsorption chromatography was carried out on silica gel (Hopkin and Williams Ltd., Chadwell Heath, Essex), and thin-layer chromatography on Kieselgel G (E. Merck A.-G., Darmstadt, Germany).

Analytical methods

Total phosphorus was determined by the method of Chen, Toribara & Warner (1956). Galactose and arabinose, before or after acid hydrolysis, were determined by the phenol- H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). After acid hydrolysis total sugar was determined by the ferricyanide method (Park & Johnson, 1949), and galactosamine by a modification of the Elson-Morgan method (Rondle & Morgan, 1955). Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954; Aspinall & Ferrier, 1957), a correction factor 0.14 being used to allow for the extinction of the IO_3^- ion, and formaldehyde by a

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modification of the chromotropic acid method (Roberts *et al.* 1963).

Characterization of substance S.10A

Immunological study. Dr G. T. Mills kindly examined the substance by the Ouchterlony technique with absorbed type 10A antiserum, a gift from Dr E. Lund of the Statens Seruminstitut, Copenhagen. It was highly reactive and gave only one line of precipitation.

Dr Jean M. Tyler kindly tested the S.10A by the same method for the presence of C-substance (How *et al.* 1964). By using two anti-pneumococcal horse antisera (types I and VII) with a high anti-C content, and purified C-substance from a rough type II *Pneumococcus* as standard, it was shown that S.10A contained 10–15% of C-substance.

Electrophoresis. With an apparatus similar to that described by Markham & Smith (1952) paper electrophoresis was carried out for 7 hr. in 0.05M-veronal-HCl buffer, pH 8.5, at a voltage gradient of 8.5V/cm. The substance moved 10 cm. towards the anode and gave a purple colour rapidly with the periodate-Schiff reagents and a blue colour with the phosphate reagent. The latter reagent also showed a faint blue spot at 14 cm. towards the anode.

Extinction at 260 m μ . The E_{260} of a 0.1% solution of S.10A (measured against distilled water) was 0.05.

Infrared spectrum. In a KBr disk S.10A showed characteristic bands at 1639 and 1558 cm.⁻¹ (—NH·CO·CH₃ group).

Optical rotation. S.10A had $[\alpha]_D^{25} + 12.3^\circ$ (c 1.63 in water). Brown & Robinson (1943) give $+12.8^\circ$ in water.

Quantitative analysis of S.10A. S.10A (10.7 mg.) was dissolved in water (10 ml.) and total phosphorus determined on samples (Found: P, 2.41%). Brown & Robinson (1943) found P, 2.57%.

The above solution (1 ml.) was diluted to 10 ml. and samples were used for galactose determination (Found: galactose, 59.2%; P/galactose ratio, 1:4.2).

S.10A (6.9 mg.) was hydrolysed with 4N-HCl (1 ml.) at 100° for 3 hr. The hydrolysate was diluted, neutralized to pH 6 and made to 10 ml. with water (Found: galactosamine, 15; P, 2.5%; P/galactosamine ratio, 1:1.04).

S.10A (8.2 mg.) was hydrolysed with 2N-HCl (0.9 ml.) at 100° for 3 hr., the solution neutralized to pH 6 and diluted to 10 ml. with water [Found: total sugar (calculated as D-galactose), 70.8; P, 2.4%; P/total sugar ratio, 1:5.1].

Configuration of the sugars in S.10A. S.10A (30 mg.) was hydrolysed with 2N-HCl (3 ml.) for 3 hr. at 100°. The hydrolysate was passed through a small column of Dowex 2 (CO₃²⁻ form) resin and the column washed with water. The eluate was evaporated to a small volume and passed through a column of Dowex 50 (H⁺ form) resin (3 ml.). The neutral components were eluted with water and recovered by freeze-drying the solution. The galactosamine was eluted with 0.3N-HCl and the acid eluate evaporated under reduced pressure, finally over solid KOH.

A small portion of the neutral residue was chromatographed in solvent B; galactose, ribitol and anhydriitol were the only compounds detected. The remainder of the residue was dissolved in water, the solution made to 2.0 ml. and the optical rotation measured. The galactose content was determined by the phenol-H₂SO₄ method [Found: $[\alpha]_D^{25} + 74^\circ$ (c 0.82 in water)]. Pigman (1957) reports $[\alpha]_D + 80.2^\circ$ for D-galactose in water at equilibrium. The contribution of the 1,4-anhydriitol to the rotation was

ignored, since it should be largely racemic (Baddiley, Buchanan & Carss, 1957).

The residue from the acid eluate mentioned above was chromatographed as a band on Whatman no. 4 paper in solvent B. A guide strip was sprayed with ninhydrin and the corresponding galactosamine portion was eluted with water. The solution was passed through a small column of Dowex 50 (H⁺ form) resin and after removal of neutral impurities with water the galactosamine was eluted with 0.3N-HCl. The acid eluate was evaporated to dryness and traces of HCl were removed by treatment with ethanol followed by evaporation. The residue was dissolved in water, made to 2 ml. and the optical rotation measured. The galactosamine hydrochloride content was measured by the Elson-Morgan method [Found: $[\alpha]_D^{25} + 86.4^\circ$ (c 0.11 in water)]. Foster & Stacey (1952) give $[\alpha]_D + 80^\circ$ for D-galactosamine hydrochloride at equilibrium in water.

Isolation and composition of the neutral and basic hexasaccharides

Hydrolysis of the specific substance with alkali. S.10A (200 mg.) was dissolved in N-NaOH solution (10 ml.) and heated in a sealed tube at 100° for 1 hr. The hydrolysate was passed through a column of Dowex 50 (NH₄⁺ form) resin (16 ml.) and the eluate (200 ml.) was evaporated under reduced pressure to a small volume and then freeze-dried. The residue was dissolved in water (10 ml.) and the insoluble matter removed by centrifuging. The solution was evaporated *in vacuo* to 3 ml. and a small portion was chromatographed in solvent A. Three groups of products with R_{ribitol} 0.12, 0.22 and 0.43 were detected; all gave a purple colour rapidly with periodate-Schiff reagents but only the first two contained phosphate. No inorganic phosphate was detected.

Enzymic dephosphorylation of the alkali hydrolysate. The remaining neutral alkali hydrolysate (about 3 ml.) was mixed with intestinal phosphomonoesterase (15 mg.) and (NH₄)₂CO₃ (20 mg.) dissolved in water (15 ml.); the solution was kept under a few drops of toluene at 37° for 20 hr. The enzymic hydrolysate was passed through a column of Dowex 2 (CO₃²⁻ form) resin (20 ml.). The eluate (300 ml.) was evaporated under reduced pressure to a small volume (10 ml.) and passed through a column of Dowex 50 (H⁺ form) resin (15 ml.) and the column irrigated with water followed by aq. N-NH₃. The neutral eluate (150 ml.) and the basic eluate (150 ml.) were each evaporated under reduced pressure. The neutral residue weighed 113 mg. and the basic residue 35 mg. The neutral and basic residues were examined chromatographically in solvent A and found to be homogeneous.

Hydrolysis of S.10A with N-NaOH at 100° for 3 hr. or 6 hr. also gave rise to three groups of products, identical with those obtained from hydrolysis for 1 hr. Enzymic dephosphorylation of the products gave two fractions, neutral and basic, the yield of the latter fraction increasing with the duration of hydrolysis.

Neutral hexasaccharide. The above neutral residue (100 mg.) was dissolved in the minimum amount of water and ethanol was added until a slight precipitate was formed. After 6 hr. at 2°, the solid (80 mg.) was removed by centrifugation and washed with ethanol and then ether. The microcrystalline solid, m.p. 195–200°, $[\alpha]_D^{25} + 11.3^\circ$ (c 1.16 in water), was not hygroscopic. The R_{ribitol} values in solvents

A, *C* and *D* were 0.48, 0.39 and 0.42 respectively; it gave a purple colour rapidly with periodate-Schiff reagents (Found: C, 41.3, H, 6.8; N, 1.2. $C_{37}H_{65}NO_{30}$ requires C, 41.3; H, 6.8; N, 1.3%).

Basic hexasaccharide. This was obtained from the basic residue above as a white microcrystalline powder in a similar manner to that used for the neutral hexasaccharide. It had m.p. 240–250° (decomp.), $[\alpha]_D^{18} + 7.9^\circ$ (c 1.14 in water). When examined by paper chromatography it had R_{ribitol} values 0.41, 0.32 and 0.36 in solvents *A*, *C* and *D* respectively. It gave a purple colour rapidly with periodate-Schiff reagents.

Hydrolysis of the neutral hexasaccharide and basic hexasaccharide with acid. The hexasaccharides (4mg. of each) were separately treated with 2N-HCl (0.4 ml.) at 100° for 3 hr. The acid was removed by passing the hydrolysates through columns of Dowex 2 (CO_3^{2-} form) resin and the eluates were freeze-dried. The residues were chromatographed on paper in solvents *B* and *D*. Galactose, galactosamine, ribitol and anhydriitol were obtained in both cases. The amount of galactosamine formed from the basic hexasaccharide was much less than that from the neutral hexasaccharide, but there was a correspondingly large amount of disaccharide D_2 (see below).

N-Acetylation of the basic hexasaccharide (Roseman & Ludowieg, 1954). The basic hexasaccharide (18mg.) was dissolved in water (0.6 ml.), and the solution mixed with methanol (0.1 ml.), Dowex 2 (CO_3^{2-} form) resin (0.5 ml.) and acetic anhydride (0.05 ml.). The mixture was stirred at 4° for 90 min. and then filtered. The filtrate and aqueous washings were passed through a small column of Dowex 50 (H^+ form) resin, the eluate heated to boiling point and then evaporated under reduced pressure. The product was obtained as a microcrystalline solid, m.p. 196–200°, by dissolving the residue in a few drops of water and adding ethanol. It was examined chromatographically in solvents *A* and *C* and found to be homogeneous and identical in all respects with the neutral hexasaccharide.

De-N-acetylation of the neutral hexasaccharide. The neutral hexasaccharide (10mg.) was hydrolysed with N-NaOH (1 ml.) at 100° in a sealed tube for 5 hr. The hydrolysate was passed through a column of Dowex 50 (NH_4^+ form) resin (3 ml.) and the eluate evaporated to dryness under reduced pressure. The residue was dissolved in water (1 ml.), the solution was passed through a column of Dowex 50 (H^+ form) resin (2 ml.) and products were eluted with water followed by aq. N-NH₃. The residues obtained by evaporation of the neutral and basic eluates weighed 6mg. and 4mg. respectively. They were examined chromatographically in solvent *A* and found to be identical with the neutral hexasaccharide and basic hexasaccharide respectively.

Quantitative analysis of the neutral hexasaccharide. The neutral hexasaccharide (3.348 mg.) was dissolved in water (10 ml.) and galactose determined on 0.2 ml. and 0.3 ml. samples by the phenol- H_2SO_4 method (Found: galactose, 71.1. $C_{37}H_{65}NO_{30}, 4H_2O$ requires galactose (4 units), 67.0%). A similar analysis on the basic hexasaccharide gave a value of 71.0% ($C_{35}H_{63}NO_{29}, 4H_2O$ requires galactose, 69.6%).

The neutral hexasaccharide (3.65 mg.) was heated with 4N-HCl (0.4 ml.) at 100° for 3 hr., the hydrolysate was diluted to 10 ml. and the galactosamine was determined on 0.5 ml. and 1.0 ml. samples of the solution [Found: galactos-

amine, 14.8. $C_{37}H_{65}NO_{30}, 4H_2O$ requires galactosamine (1 unit), 16.7%].

The neutral hexasaccharide (3.72 mg.) was hydrolysed with 2N-HCl (0.4 ml.) at 100° for 3 hr., the hydrolysate was neutralized to pH 6 and diluted to 100 ml., and total sugar was determined on 0.2 ml. and 0.3 ml. samples. D-Galactose was used as standard (Found: total sugar, 86.1. $C_{37}H_{65}NO_{30}, 4H_2O$ requires 83.6%, calculated as galactose).

A sample containing 3.2 μ moles of the neutral hexasaccharide in water (4.5 ml.) was oxidized with 0.02 M-sodium metaperiodate (4.5 ml., 90 μ moles). In 1 hr. 5.0 mol.prop. and in 192 hr. 14.5 mol.prop. of periodate were reduced and no further reduction was observed. Under similar conditions methyl α -D-galactofuranoside reduced 5.9 mol.prop. of sodium metaperiodate. A compound of formula (VIII) should reduce 4 mol.prop. of periodate rapidly (2 mol.prop. by the ribitol and 1 mol.prop. by each of the exocyclic glycol systems of the galactofuranosyl groups). The C-3-C-4 bond of both galactofuranose groups should then be destroyed (2 mol.prop.) followed by overoxidation of both residues (8 mol.prop.). The loss of the galactofuranose group attached to C-3 of galactopyranose should render the latter susceptible to oxidation (2 mol.prop.; 16 mol.prop. in all).

The neutral hexasaccharide (2.348 mg.) was dissolved in water (10 ml.). Samples (0.3 ml. and 0.5 ml.) of this solution were diluted to 2 ml., mixed with 0.1 M-sodium metaperiodate and kept at room temperature in the dark for 2 hr.; 2.6 mol.prop. of formaldehyde was produced. The theoretical value for formula (VIII) is 3 mol.prop.

Disaccharides formed by partial degradation

Action of nitrous acid on the basic hexasaccharide. The basic hexasaccharide (25 mg.) was dissolved in 0.1 N-HCl (0.5 ml.) and the solution was evaporated to dryness *in vacuo*. The hydrochloride thus obtained was dissolved in water (2 ml.) containing NaNO₂ (85 mg.) and the mixture was heated at 50° in a sealed tube for 2 hr. The solution was passed through a column of Dowex 50 (NH_4^+ form) resin (10 ml.); the eluate (120 ml.) was boiled for 10 min. and then evaporated under reduced pressure. A small sample of the residue was chromatographed in solvent *B*. Three products were detected: (1) galactose, giving a brown colour with aniline phthalate; this probably arose by acid hydrolysis of a labile furanosyl linkage; (2) disaccharide D_1 , $R_{\text{ribitol}} 0.53$, giving a purple colour rapidly with periodate-Schiff reagents; (3) a product with $R_{\text{ribitol}} 0.09$, also giving a purple colour rapidly with the periodate-Schiff reagents. The remainder of the residue was chromatographed as a band on Whatman no. 3 paper and products (2) and (3) were eluted for further investigation.

Disaccharide D_1 was indistinguishable from 2(or 4)-O-(α -D-galactopyranosyl)-D-ribitol, a degradation product of S. 34 (Roberts *et al.* 1963), on paper chromatography in solvent *B* with detection by periodate-Schiff reagents. A small sample of disaccharide D_1 was hydrolysed with 2N-HCl at 100° for 3 hr. Paper chromatography in solvent *B* of the evaporated residue showed the formation of galactose, ribitol and anhydriitol.

A solution of disaccharide D_1 (about 200 μ g. in 0.1 ml. of water) was mixed with 2% (w/v) almond β -glucosidase solution (0.025 ml.) containing α - and β -D-galactopyranosidase (cf. Roberts *et al.* 1963). The mixture was kept at 37° for 16 hr. and chromatographed in solvent *B*. The products

were galactose and ribitol in equal amounts together with some unhydrolysed disaccharide D₁.

The product (3) above was reduced with NaBH₄ (15 mg.) at room temperature for 16 hr. A few drops of acetic acid were added and the solution was passed through a column of Dowex 50 (H⁺ form) resin (2 ml.). The eluate was evaporated to dryness *in vacuo* and the boric acid removed by evaporation with warm methanol. A portion of the residue was hydrolysed with 2N-HCl at 100° for 3 hr. and the acid removed by evaporation *in vacuo*. The residue was chromatographed in solvents *B* and *E*. One of the products was galactose; the second product (R_{ribitol} 1.1 in solvent *B* and 1.2 in solvent *E*) gave a blue colour with periodate-Schiff reagents and was identical with 2,5-anhydro-D-talitol, whose preparation is described below.

2,5-Anhydro-D-talitol. This compound was prepared by treatment of D-galactosamine with HNO₂, followed by reduction, either with H₂ over Raney nickel or with NaBH₄. Since the compound has been prepared independently by Defaye (1964), whose structural proof was the same as our own, we quote only physical constants. The anhydro-talitol crystallized from ethanol as prisms, m.p. 106–108°, $[\alpha]_D^{25} +40.2^\circ$ (*c* 1.06 in water). Defaye (1964) gives m.p. 112–113°, $[\alpha]_D +44.5^\circ$ (water). When examined by chromatography in solvents *B* and *E* it gave a single blue spot, R_{ribitol} 1.12 in solvent *B* and 1.22 in solvent *E* (periodate-Schiff reagents).

Partial hydrolysis of the basic hexasaccharide with acid. The basic hexasaccharide (8 mg.) was hydrolysed with N-HCl (1.2 ml.) at 100° for 2 hr. The hydrolysate was passed through a column of Dowex 2 (CO₃²⁻ form) resin and the eluate was freeze-dried. A small sample was chromatographed in solvent *B*. The products identified were galactose, galactosamine (a trace), ribitol and anhydroribitol. Besides these a compound D₂, in the disaccharide region with $R_{\text{galactose}}$ 0.31, reacted with ninhydrin, alkaline AgNO₃ and the Elson-Morgan reagent (cherry-red colour). The remainder of the hydrolysate was chromatographed as a band on Whatman no. 4 paper and disaccharide D₂ was eluted with water.

A sample of disaccharide D₂ was hydrolysed with 2N-HCl at 100° for 3 hr. The acid was removed by evaporation of the hydrolysate *in vacuo* and the residue chromatographed in solvent *B*. The products were galactose, galactosamine and some unchanged disaccharide D₂ ($R_{\text{galactosamine}}$ 0.87 in solvent *D*).

A sample of disaccharide D₂ was reduced with NaBH₄ at room temperature for 16 hr. A few drops of acetic acid were added and the solution was passed through a column of Dowex 50 (H⁺ form) resin and eluted with water followed by aq. N-NH₃. The separate eluates were evaporated to dryness and the residues distilled with methanol three times to remove boric acid. The aqueous eluate left no residue after this treatment. The residue from the aq.-NH₃ eluate was hydrolysed with 2N-HCl at 100° for 3 hr. The acid was removed *in vacuo* and the residue chromatographed in solvent *B*. The products were galactitol ($R_{\text{galactose}}$ 1.08), galactosamine and reduced disaccharide D₂ ($R_{\text{galactose}}$ 0.33). The latter differed from disaccharide D₂ itself in giving a purple colour rapidly with periodate-Schiff reagents; the colour gradually changed to yellow.

Partial hydrolysis of the neutral hexasaccharide with acid. The neutral hexasaccharide (15 mg.) was hydrolysed with 66% (v/v) formic acid (1.5 ml.) in a sealed tube at 100 for

15 min. The hydrolysate was evaporated to dryness *in vacuo*, a few drops of dilute aq. NH₃ were added to destroy any formyl esters, and the solution was evaporated to dryness. Small samples were chromatographed in solvents *B* and *F*. The products detected were galactose, N-acetylgalactosamine, ribitol, disaccharide D₁, disaccharide D₃ and oligosaccharides. The remainder of the hydrolysate was chromatographed as a band on Whatman no. 3 paper in solvent *B* or *F*, and disaccharide D₃, having $R_{\text{galactose}}$ 0.86 in solvent *B* and 0.82 in solvent *F*, was eluted with water. Hydrolysis of the neutral hexasaccharide with 0.1N-HCl at 100° for 30 min. gave similar results. Disaccharide D₃ gave a weak reaction with alkaline AgNO₃, a yellow colour (becoming reddish-brown) with periodate-Schiff reagents, a faint purple colour with the Morgan-Elson reagents and a brownish-yellow colour with benzidine-trichloroacetic acid.

A sample of disaccharide D₃ was hydrolysed with 2N-HCl at 100° for 3 hr. The acid was removed by evaporation of the hydrolysate *in vacuo* and the residue chromatographed in solvent *B*. The products were galactose and galactosamine in equal amounts.

A sample of disaccharide D₃ was reduced with NaBH₄. The product was isolated in the usual manner and hydrolysed with 2N-HCl at 100° for 3 hr. The residue after evaporation was chromatographed in solvent *B*. One of the products was galactose; the other had $R_{\text{galactose}}$ 0.72 and was identified as galactosaminol by comparison with an authentic sample.

Degradation of the neutral and basic hexasaccharides with periodate

Qualitative detection of galactofuranose units in S.10A, the neutral and the basic hexasaccharides. S.10A (4 mg.) was treated with 0.1M-sodium metaperiodate (0.2 ml., about 4 mol.prop.) and the mixture kept at room temperature in the dark for 4 hr. Then NaBH₄ (10 mg.) in water (0.5 ml.) was added and the solution was kept overnight at room temperature. Excess of borohydride was decomposed 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 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 by evaporation *in vacuo* and the residue was chromatographed in solvent *B*. The pentose formed was identified as arabinose from its R_F value and the red colour it gave with aniline phthalate.

Treatment of the neutral hexasaccharide and basic hexasaccharide with periodate under similar conditions, followed by reduction with NaBH₄ and hydrolysis with acid, gave a pentose that was identified as arabinose by chromatography in solvent *B*.

No arabinose was detected when the oxidation was carried out on S.10A or on the hexasaccharides with 15 mol.prop. of periodate/hexasaccharide unit for 4 hr. or 72 hr.

Unoxidizable galactose units in the neutral hexasaccharide. (a) Study with 0.1M-periodate at room temperature. The neutral hexasaccharide (5.21 mg.) was treated with 0.1M-sodium metaperiodate (1.5 ml., about 30 mol.prop.) and the mixture was kept at room temperature for 6 days in the dark. Then NaBH₄ (30 mg.) was added and the mixture was kept at room temperature overnight. Ions were removed by passing the solution through columns of Dowex 50 (H⁺

form) and Dowex 2 (CO_3^{2-} form) resins. The eluate was freeze-dried and the residue made to 25 ml. with water. The galactose content was 1.05 mol.prop./hexasaccharide unit.

(b) Study with 0.02M-periodate at 2°. The neutral hexasaccharide (5.35 mg.) was treated with 0.02M-sodium periodate (8 ml., about 30 mol.prop.) for 52 hr. at 2°. The oxidation was stopped by the addition of ethylene glycol. The products were reduced with NaBH_4 (20 mg.), the solution was passed successively through a column of Dowex 50 (H^+ form) and one of Dowex 2 (CO_3^{2-} form) resin, and the final eluate was freeze-dried. The residue was hydrolysed with 2N-HCl (0.6 ml.) at 100° for 2 hr. and then made to 50 ml. with water. Galactose was determined on a sample and corresponded to 1.86 mol.prop. for each hexasaccharide unit. Paper chromatography showed that galactose, but no arabinose, was present.

Quantitative degradation of galactofuranose residues in the neutral hexasaccharide to arabinofuranose. The neutral hexasaccharide (about 2.4 μ moles) was dissolved in water (0.2 ml.) and the solution was cooled to 2°. To this was added 0.1M-sodium metaperiodate (0.15 ml.) and the mixture was kept at 2° for 30 min. Ethylene glycol was added to destroy excess of periodate and this was followed by the addition of NaBH_4 (6 mg.) in water (0.5 ml.). The solution was kept at 2° for 3 hr. and then at room temperature overnight. Acetic acid (1 drop) was added and the solution was passed successively through a column of Dowex 50 (H^+ form) and one of Dowex 2 (CO_3^{2-} form) resin. The eluate was freeze-dried and the residue hydrolysed with 2N-HCl (0.3 ml.) at 100° for 3 hr. The hydrolysate was passed through a column of Dowex 2 (CO_3^{2-} form). The eluate was freeze-dried and the residue chromatographed as a band in solvent *B*. The arabinose and galactose were located by means of guide strips; they were eluted with water and controls were run on eluates from appropriate areas of plain paper (Found: galactose/arabinose ratio, 2:2.08).

Comparison of the oxidation of the neutral hexasaccharide and basic hexasaccharide. (a) The neutral hexasaccharide (4 mg.) was treated with 0.1M-sodium metaperiodate (1 ml., about 25 mol.prop.) and the solution was kept at room temperature for 72 hr. in the dark. The product was reduced with NaBH_4 (20 mg.), isolated and hydrolysed with 2N-HCl in the usual manner. The acid was removed *in vacuo* and the residue was chromatographed in solvents *B* and *E*. The main products were glycerol, galactose and galactosamine; traces of threitol and disaccharides were present.

(b) The basic hexasaccharide (4 mg.) was treated with sodium metaperiodate followed by NaBH_4 and subsequent acid hydrolysis in a similar manner to (a) above. The final products were glycerol, threitol, galactose, galactosamine and disaccharide D_2 .

Methylation of the neutral hexasaccharide

The hexasaccharide (13.6 mg.) was dissolved in *NN*-dimethylformamide (1.2 ml.) and mixed with methyl iodide (0.6 ml.). Freshly prepared Ag_2O (350 mg.) was added and the mixture was stirred at room temperature in the dark for 24 hr. The mixture was filtered through Hyflo Super-Cel and washed with dimethylformamide (2 ml.) followed by several portions (4 ml. each) of chloroform. The filtrate was cooled to 2–5° for 2 hr. and the white substance that separated was removed by filtration. The filtrate was evaporated under

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

Columns A and B refer to the butan-1,4-diol succinate polyester and polyphenyl ether columns used by Aspinall (1963) under the operating conditions described in that paper. For reference purposes authentic 1,2,3,5-tetra-O-methylribitol was prepared by permethylation of 4-O- α -D-glucopyranosyl-D-ribitol (Austin, Hardy, Buchanan & Baddiley, 1964) followed by methanolysis, with the same technique as used for the neutral hexasaccharide. (s), Strong; (m), medium.

	Column A	Column B
1,2,3,5-Tetra-O-methylribitol	0.95	0.62
2,3,5,6-Tetra-O-methylgalactose	1.45 (s)	1.22 (s)
	1.82 (m)	1.60 (m)
2,4,6-Tri-O-methylgalactose	4.15 (m)	2.06 (m)
	4.68 (s)	2.36 (s)

reduced pressure and the dimethylformamide was finally removed by distillation in a high vacuum. The residue was remethylated by using the same procedure. The product after the second methylation was further methylated by heating it with methyl iodide (2 ml.), Ag_2O (250 mg.) and Drierite (50 mg.) under reflux for 5 hr. The product (18.5 mg.) was chromatographed on silica gel (1.4 g., 0.5 cm. $\times$ 10 cm. column), 4 ml. fractions being collected. Most of the substance (17.2 mg.) was eluted with acetone and acetone-methanol (9:1, v/v). When examined by thin-layer chromatography on silica in benzene-ethanol (20:3, v/v) the material moved as a streak near the origin. An infrared spectrum of a thin film of the permethyl ether showed a band at 1653 cm^{-1} but none at 1550 or 3280 cm^{-1} .

The permethyl ether (8 mg.) was treated with a few drops of 3% (w/v) HCl in methanol and heated at 80° in a sealed tube for 8 hr. The solution was kept overnight at room temperature, neutralized with freshly prepared Ag_2CO_3 and filtered, and the precipitate was washed with dry methanol. The filtrate and washings were carefully evaporated under reduced pressure and the methyl ethers were examined by gas-liquid chromatography by Dr G. O. Aspinall (see Table 1).

The permethyl ether was hydrolysed with 2N-HCl for 3 hr. at 100° and the acid removed *in vacuo* over KOH. An aqueous solution of the residue was passed through a column of Dowex 50 (H^+ form) resin and neutral products were eluted with water. The amino sugars were eluted with 0.3N-HCl and the eluate was evaporated *in vacuo*. When examined by paper chromatography in solvents *B* and *D*, two products were detected: a compound, present only in small amounts, that appeared to be galactosamine, and a compound with $R_{\text{galactosamine}}$ 1.37 in solvent *B* and 1.15 in solvent *D*. The latter differed from 3-, 4- and 6-O-methylgalactosamine, which had $R_{\text{galactosamine}}$ values 1.48, 1.4 and 1.58 in solvent *B*. The unknown methyl ether reacted weakly with ninhydrin but strongly with alkaline AgNO_3 ; in the Elson-Morgan reaction it gave a purple colour, unlike the colours given by galactosamine or its 3-, 4- or 6-methyl ethers.

Methyl 4,6-O-benzylidene- α -D-galactosaminide was

methylated in the manner described above for the neutral hexasaccharide (cf. Stoffyn & Jeanloz, 1954). The amorphous product was purified by vacuum sublimation. This methylated product was first hydrolysed with aq. 60% (v/v) acetic acid and then with 2*N*-HCl at 100° for 3 hr. Paper chromatography in solvents *B* and *D* showed that one of the hydrolysis products closely resembled the unknown methyl ether above in R_f values and colour reactions. The other hydrolysis products were a small amount of galactosamine and a major amount of 3-*O*-methylgalactosamine.

DISCUSSION

The method of degradation that has proved most valuable in the study of ribitol teichoic acids and pneumococcal antigens containing ribitol has been hydrolysis with alkali followed by enzymic dephosphorylation of the products (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958; Baddiley, 1962; Rebers & Heidelberger, 1959). The resulting compound, obtained in high yield, has been the dephosphorylated repeating unit of the polymer, linked originally by phosphodiester groups. The ease with which the diesters are hydrolysed is due to the presence of vicinal hydroxyl groups in the polymer. When S.10A was treated with *N*-sodium hydroxide at 100° three groups of products were detected by paper chromatography. The R_f values corresponded to diphosphates, monophosphates and phosphorus-free compounds. Enzymic dephosphorylation of the crude mixture yielded two compounds, one neutral and the other basic, which were separated from each other by means of a cation-exchange resin. The two compounds were distinguishable from each other by paper chromatography. They were obtained as colourless non-hygroscopic microcrystalline powders that were chromatographically homogeneous. The total yield of both the fragments was 70–75%. The individual yields depended on the duration of the hydrolysis in alkali; hydrolysis for 1 hr. yielded more of the neutral fragment, whereas hydrolysis for 6 hr. gave more of the basic fragment.

Acid hydrolysis of both neutral and basic components gave galactose, galactosamine, ribitol and anhydribose. The amount of galactosamine produced from the basic component was less than that from the neutral component, but there was formed a correspondingly greater amount of a disaccharide containing galactosamine. The relative stability towards acid of glycosides of 2-amino-2-deoxy sugars compared with their *N*-acetyl derivatives is well known (Moggridge & Neuberger, 1938; Foster, Horton & Stacey, 1957; Johansen, Marshall & Neuberger, 1960). Thus the neutral component was identical with the *N*-acetylated basic component; the neutral component can be considered as the phosphorus-free repeating unit of

the polymer. Elementary analysis, together with other analytical data on the neutral component and on S.10A itself, showed that it contained one nitrogen atom (as *N*-acetyl-*D*-galactosamine), four or five *D*-galactose units and one ribitol unit. Subsequent analytical work showed that the number of *D*-galactose units was four, and we shall refer to the two fragments of degradation in alkali as the neutral hexasaccharide and the basic hexasaccharide.

In studies on the problem of the linkages of the component sugars attention was first directed to the basic hexasaccharide. The galactosaminyl linkage is relatively stable towards mineral acid but is very easily destroyed by nitrous acid. It was therefore possible to establish part of the structure in the galactosamine region of the molecule. The destruction of glycosaminide linkages by nitrous acid was first described by Foster, Martlew & Stacey (1953) and was used in studies on teichoic acids by Baddiley, Buchanan, RajBhandary & Sanderson (1962) and by Ellwood, Kelemen & Baddiley (1963). When the hydrochloride of the basic hexasaccharide was treated with sodium nitrite there were two major products. One was a disaccharide, *D*₁, galactopyranosyl-2-*O*-methyl-β-*D*-ribofuranose (I), whose properties were the same as the 2(or 4)-*O*-α-*D*-galactopyranosyl-*D*-ribose, first isolated by Roberts *et al.* (1963) as a partial hydrolysis product of the repeating unit of S.34. Disaccharide *D*₁ could be hydrolysed enzymically or by acid to give galactose and ribitol. The methylation evidence (see below) showed that in the neutral hexasaccharide a single substituent was present on ribitol at position 2 or 4. The other product, of lower R_f value, was treated with sodium borohydride, followed by hydrolysis in acid. Together with galactose there was present a compound with the same chromatographic properties as 2,5-anhydro-*D*-talitol, an authentic sample of which had been prepared by deamination of *D*-galactosamine followed by reduction with borohydride. The 2,5-anhydrotalitol is derived from the galactosamine unit in the basic hexasaccharide and its presence indicates that the galactosamine possesses a pyranose ring.

When the basic hexasaccharide was hydrolysed with *N*-hydrochloric acid a disaccharide, D_2 , was produced. Prolonged treatment with acid gave galactose and galactosamine together with some unhydrolysed disaccharide D_2 . The resistance to hydrolysis by acid suggested that disaccharide D_2 was a galactosaminylgalactose. This was confirmed by reduction with borohydride followed by hydrolysis with acid to give a hexitol (presumably galactitol) and galactosamine. In the Elson-Morgan reaction (Partridge, 1948) disaccharide D_2 gave a cherry-red colour, similar to that given by galactosamine, despite the fact that the reducing end group was galactose. It was concluded that the galactosamine was attached to the C-3 or C-4 hydroxyl group of galactose, and so was eliminated during the preliminary treatment with alkali in the Elson-Morgan method (Kuhn, Gauhe & Baer, 1956). The same conclusion was drawn from the colour reaction of reduced disaccharide D_2 with periodate-Schiff reagents (Hardy & Buchanan, 1963); a purple colour, gradually changing to yellow, indicated the formation of formaldehyde and a malonaldehyde structure that could be derived from a 3- or 4-substituted galactitol. A decision in favour of the structure 3-*O*-galactosaminyl-*D*-galactose (II) for disaccharide D_2 was possible as a result of the periodate oxidation and methylation studies described below. These two degradations of the basic hexasaccharide lead to the partial structure (III) for the neutral hexasaccharide.

When the neutral hexasaccharide was subjected

to partial acid hydrolysis with 0.1 *N*-hydrochloric acid or 66% formic acid the products were galactose, ribitol, *N*-acetylgalactosamine, disaccharide D_1 and another disaccharide, D_3 , whose structure was shown to be 4-*O*-*D*-galactopyranosyl-*N*-acetylgalactosamine (IV). Acid hydrolysis of disaccharide D_3 gave galactose and galactosamine in equal amounts. Reduction with borohydride followed by hydrolysis with acid yielded galactose and galactosaminol, showing that disaccharide D_3 was a galactosylgalactosamine; the mobility of disaccharide D_3 on paper chromatography in various solvents indicated that the galactosamine was present as its *N*-acetyl derivative. Disaccharide D_3 reacted with periodate-Schiff reagents and relatively weakly with alkaline silver nitrate; it gave with benzidine-trichloroacetic acid a brownish-yellow colour similar to that reported for 4-*O*- β -*D*-galactopyranosyl-*N*-acetyl-*D*-glucosamine (Côté & Morgan, 1956). In the Morgan-Elson reaction (Partridge, 1948) it gave a barely perceptible colour, indicating that the linkage was 1 $\rightarrow$ 4 (Kuhn,

Gauhe & Baer, 1954). This linkage was confirmed by the periodate oxidation studies on the basic hexasaccharide discussed below.

Mild acid treatment of S.10A or of the neutral hexasaccharide or basic hexasaccharide resulted in the release of free galactose, indicating that some of the galactose residues might be present in the furanose form. When the neutral hexasaccharide was oxidized with periodate 2.6mol.prop. of formaldehyde was produced. Since oxidation of a ribitol moiety linked at position 2 or 4 would account for only 1mol.prop. of formaldehyde it seemed that there might be two galactofuranose residues in the repeating unit of S.10A. This was confirmed qualitatively by brief oxidation with periodate, followed by reduction with borohydride and hydrolysis in acid to give arabinose (Goldstein, Hay, Lewis & Smith, 1965; Roberts *et al.* 1963). When the neutral hexasaccharide was oxidized with 0.02M-sodium metaperiodate at 2° for 52hr. and the resulting product reduced with sodium borohydride and then subjected to hydrolysis in acid, 1.86mol.prop. of galactose but no arabinose was produced. This indicated that both galactopyranose units were not oxidized and that both galactofuranose units were present as terminal groups. When a similar experiment was carried out with only a brief oxidation with periodate, galactose and arabinose in the ratio 2:2.08 were produced, confirming the presence of the two unsubstituted galactofuranosyl residues in the neutral hexasaccharide. The neutral hexasaccharide was methylated with methyl iodide and silver oxide in dimethylformamide (Kuhn, Trischmann & Löw, 1955). The permethyl ether was methanolysed and the resulting methyl ethers were examined by gas-liquid chromatography. Glycosides of 2,3,5,6-tetra-*O*-methylgalactose and 2,4,6-tri-*O*-methylgalactose were detected, corresponding to equal quantities of the parent sugars.

Since no other galactose methyl ethers were present, there must be two galactofuranosyl end groups and two 3-*O*-substituted galactopyranosyl groups in the neutral hexasaccharide. 1,2,3,5-Tetra-*O*-methylribitol was present, confirming the structure (III), but no galactosamine methyl ether was detectable by this method. The partial structure (V, where GalNAc represents *N*-acetyl-galactosamine) for the neutral hexasaccharide was consistent with the available evidence, leaving the problem of the positions of branching on the *N*-acetylgalactosamine unit.

When the permethyl ether of the neutral hexasaccharide was hydrolysed with aqueous acid and the products were examined by paper chromatography no spot corresponding to the 3-, 4- or 6-methyl ethers of galactosamine was detected. A sugar was present that reacted strongly with

alkaline silver nitrate, gave a purple colour with the Elson-Morgan reagents and reacted weakly with ninhydrin. The infrared spectrum of the permethyl ether had a band at 1653cm.⁻¹ (amide I), showing that the acetyl group had not been removed, but had no absorption at 1550cm.⁻¹ (amide II) or 3280cm.⁻¹ (NH stretching). This result was consistent with *N*-methylation having occurred. The methylated amino sugar corresponded in chromatographic properties to one of the products from the methylation and hydrolysis of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy- α -D-galactopyranoside under conditions similar to those used for the neutral hexasaccharide (the major product was 3-*O*-methylgalactosamine). It was therefore tentatively identified as 3-*O*-methyl-*N*-methylgalactosamine. The conditions necessary for *N*-methylation, which occurs particularly easily with the neutral hexasaccharide, have not yet been defined.

Other evidence also pointed to 4,6-di-*O*-substitution on the *N*-acetylgalactosamine residue. When the neutral hexasaccharide was oxidized with periodate and the product reduced with borohydride and then hydrolysed with acid, a strong spot corresponding to galactosamine was observed on paper chromatography. This result was obtained even under conditions of complete overoxidation, when only one of the galactopyranose units remained unoxidized. A negligible amount of threitol was found. When the same experiment was carried out on the basic hexasaccharide threitol was a major product and only a small amount of galactosamine (observed as galactosamine and the acid-stable disaccharide D₂) remained unoxidized. The results are readily explained by partial structures (VI) and (VII) for the basic hexasaccharide and neutral hexasaccharide respectively. The basic hexasaccharide (VI) possesses the vicinal amino alcohol system, which is oxidizable by periodate. D-Threitol should arise from the C₄ unit C-3-C-6 of the

galactosamine and cannot be formed when an *N*-acetyl group is present, as in structure (VII). The fact that some of the galactosamine in the basic hexasaccharide survives prolonged oxidation with periodate may possibly be due to Schiff-base formation between the amino group and aldehyde groups produced in other parts of the molecule.

The formation of disaccharide D₃ on partial hydrolysis of the neutral hexasaccharide with acid showed that the disaccharide unit 3-*O*-galactofuranosylgalactopyranose was joined to the hydroxyl group at position 4 of *N*-acetylgalactosamine. This was confirmed by prolonged oxidation of the neutral hexasaccharide with periodate. Under conditions where complete oxidation of both galactofuranosyl units and oxidation of one galactopyranosyl unit occurred the *N*-acetylgalactosamine was unoxidized. These conditions had exposed a 3-substituted galactopyranose residue to oxidation by overoxidation of a galactofuranose end group. If such a galactofuranose had been directly attached to the hydroxyl group at position 4 of the *N*-acetylgalactosamine the latter would have been susceptible to oxidation under these conditions. Quantitative aspects of the periodate oxidation of the neutral hexasaccharide are described in the Experimental section.

These considerations led to structure (VIII) for the neutral hexasaccharide, the dephosphorylated repeating unit of S.10A. The low positive specific rotations of S.10A, the neutral hexasaccharide and the basic hexasaccharide indicate that both α - and β -linkages are present. The mode of linkage of the neutral hexasaccharide by phosphodiester groups is discussed in the next paper (Rao *et al.* 1966).

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