The Type-Specific Substance from *Pneumococcus* Type 11A(43)

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1. The type-specific substance from *Pneumococcus* type 11A(43) is a polymer containing D-glucose, D-galactose, glycerol, phosphate and O-acetyl in the approximate molecular proportions 2:2:1:1:2. 2. Removal of the O-acetyl groups with ammonia gave a compound no longer active towards type 11A antiserum. 3. Treatment of S.11A with sodium borohydride, followed by hydrolysis with alkali yielded a phosphorus-free polysaccharide, whose structure was studied by methylation and by degradation with periodate. 4. Examination of S.11A and its de-O-acetyl derivative by periodate oxidation led to the partial structure (XI) for the type-specific substance, which thus has several features in common with S.18.

Among the serologically-specific capsular substances from pneumococci are several that contain glycerol phosphate residues (Shabarova, Buchanan & Baddiley, 1962), and much is known of the structure of the capsules of type 18 (Markowitz & Heidelberger, 1954; Estrada-Parra, Rebers & Heidelberger, 1962; Estrada-Parra & Heidelberger, 1963) and type 18A (Heidelberger, Estrada-Parra & Brown, 1964). As an example of such a compound we have examined the soluble specific polysaccharide from *Pneumococcus* type 11A that had been isolated by Dr Rachel Brown (Brown & Robinson, 1943). The *Pneumococcus* type 11A(43) was independently distinguished and classified by Kauffmann, Mørch & Schmith (1940) in Denmark and by Walter, Guevin, Beattie, Colter & Bucca (1941) in the U.S.A. Both groups demonstrated its close immunological relationship with type 11, and its less pronounced relationship with type 16. At present it is known in Denmark as type 11A and in the U.S.A. as type 43 (Kauffmann, Lund & Eddy, 1960). We shall use the Danish nomenclature in this paper.

EXPERIMENTAL

Materials. The specific substance (S.11A) from *Pneumococcus* type 11A was given to us by Dr Rachel Brown. It is described as the soluble specific substance of type 11A, preparation no. 1 (Brown & Robinson, 1943). Intestinal phosphomonoesterase (alkaline phosphatase) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., almond β -glucosidase (Manners & Taylor, 1968) from L. Light and Co. Ltd., Colnbrook, Bucks., and Glucostat (D-glucose oxidase) and Galactostat (D-galactose oxidase) reagents from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Chromatography. Paper chromatography was carried out

on Whatman no. 1 or 4 paper, with the following solvent systems: *A*, butan-1-ol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951); *B*, propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); *C*, butan-1-ol-pyridine-water-benzene (5:5:3:1, by vol.; organic phase) (Bull, 1962); *D*, 2-methylpropan-2-ol-picric acid-water (40:1:10, v/w/v) (Mitchell & Moyle, 1951); *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-acetic acid-water (4:1:5, by vol.; organic phase). Ascending irrigation was used with solvents *B* and *F* and descending irrigation with the remainder. 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), alkaline AgNO_3 reagent for sugars and polyols (Trevelyan, Procter & Harrison, 1950) and the ferric chloride reagent for hydroxamic acids (Thompson, 1951).

Analytical methods. Total phosphorus was determined by the method of Chen, Toribara & Warner (1956). Hexoses were determined by the phenol- H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954; Aspinall & Ferrier, 1957), formaldehyde by means of chromotropic acid (Hanahan & Olley, 1958) and formic acid by the method of Goldstein & Whelan (1962) as modified by Wicken & Baddiley (1963). O-Acetyl groups were determined according to Hestrin (1949).

Characterization of S.11A

Immunological study. The preparation was examined by Professor G. T. Mills by the double-diffusion technique of Ouchterlony (1948) with type 11A antiserum, a gift from Dr E. Lund of the Statens Seruminstitut, Copenhagen. It was highly reactive and gave only one line of precipitation. S.11A that had been deacetylated with NH_3 (see below)

showed little or no precipitin line when compared with S.11A.

Dr Jean M. Tyler tested the S.11A preparation by the same method for the presence of C-substance (Brundish & Baddiley, 1968). By using two horse pneumococci 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 the S.11A preparation contained 5% of C-substance.

Qualitative detection of O-acetyl groups. S.11A (2mg.) in aq. 50% (v/v) ethanol (2 drops) was treated with methanolic hydroxylamine [5% (w/v) hydroxylamine hydrochloride in methanol, 12.5% (w/v) NaOH added and NaCl removed by filtration] (1 ml.) and diethyl ether (5 ml.). After 30 min. at 25° acetic acid (0.01 ml.) was added, sodium acetate removed by centrifugation and the resulting product compared with samples of formyl-, acetyl- and propionyl-hydroxamate, prepared by the method of Thompson (1951), by paper chromatography with solvent *F*. The hydroxamate from S.11A was indistinguishable from acetylhydroxamate.

Quantitative analysis of S.11A. Phosphorus determinations were carried out on samples of an aqueous solution containing 10mg. of S.11A/100ml. (Found: P, 3.35%). Brown & Robinson (1943) found P, 3.37%.

Standard solutions of S.11A (10mg. in 10ml. of water) were prepared and samples analysed by the phenol-H₂SO₄ method (Found: hexose, 64.5%). By using a different method Brown & Robinson (1943) found hexose, 63.08%. Other samples were hydrolysed with 2M-HCl at 100° for 3hr. The hydrolysates were passed through columns of Dowex 2 (CO₃²⁻ form) resin and evaporated to dryness. The residues were dissolved in water (2ml.) and D-galactose and D-glucose determined enzymically (Found: D-galactose, 31.4; D-glucose, 31.4%).

S.11A (2mg.) was hydrolysed with 2M-HCl at 100° for 3hr., the diluted solution was passed through a column of Dowex 2 (CO₃²⁻ form) resin, and the combined eluate and washings were freeze-dried and chromatographed in solvent *A*. The bands of filter paper corresponding to galactose and glucose were each extracted with water (10ml.) and the sugar content of these solutions was measured by phenol-H₂SO₄ and enzymically (Found: molecular ratio galactose:glucose, 1:1.0; D-galactose:D-glucose, 1:1.0).

The O-acetyl groups in S.11A were determined [Found: CH₃·CO, 10.4%; molecular ratio O-acetyl:phosphate, 2:2.1. Brown & Robinson (1943) reported total acetyl, 11.98%].

Quantitative periodate oxidation of S.11A and some derivatives. Deacetylated S.11A and the phosphorus-free polysaccharide were prepared as described below. Aqueous solutions of S.11A (46.4mg. in 5ml.), deacetylated S.11A (41.75mg. in 5ml.) and the phosphorus-free polysaccharide (32.5mg. in 5ml.) were each treated with 0.1M-NaIO₄ (5ml., 10mol.prop.) so as to give oxidation solutions that were initially 0.05M in NaIO₄. The solutions were kept in the dark at 4° and samples were periodically withdrawn and diluted, and the periodate concentration was measured spectrophotometrically. S.11A reduced 2.92mol.prop. of periodate/phosphate during 3 days (constant value) whereas deacetylated S.11A reduced 3.8mol.prop. of periodate/phosphate and the phosphorus-free polysaccharide reduced 3.9mol.prop. of periodate/4 hexose units. From S.11A 0.92mol.prop., and from deacetylated S.11A 0.90mol.prop., of formaldehyde was produced.

Hexose determinations after periodate oxidation of S.11A. Samples (1.0ml.) were withdrawn from the above oxidation (i) after 2mol.prop. of periodate had been reduced and (ii) after 3 days (oxidation complete). Excess of periodate was destroyed by addition of ethylene glycol (1ml.). After dilution to 5ml. estimations of phosphate and total hexose were made on each sample [Found: molecular ratio phosphate:hexose, 1:3 for (i), 1:2 for (ii)]. A 2ml. sample of each solution was freeze-dried, hydrolysed with 2M-HCl at 100° for 3hr., deionized with Dowex 2 (CO₃²⁻ form) resin and chromatographed in solvent *A*. The bands corresponding to glucose and galactose were eluted and the hexoses determined enzymically [Found: molecular ratio D-galactose:D-glucose, 1:2, for (i), 1:1 for (ii)].

Determination of formic acid produced during periodate oxidation of S.11A and some derivatives. Samples (2ml.) were removed from each of the above oxidations after 3 days, and excess of periodate was destroyed by addition of ethylene glycol (2ml.). After 30 min. a 10% (w/v) solution of KI (2ml.) was added and liberated iodine titrated with 5mm-Na₂S₂O₃ (Found: 0.08mol.prop. of formic acid/phosphate produced from S.11A, 0.10mol.prop./phosphate from deacetylated S.11A and 1.20mol.prop./4 hexose units from the phosphorus-free polysaccharide).

Alkali hydrolysis of S.11A. S.11A (5mg.) was treated with M-NaOH (0.5ml.) at 100° in a sealed tube for 3hr. The yellow solution was passed through a short column of Dowex 50 (H⁺ form) resin and the eluate and aqueous washings were evaporated to dryness *in vacuo*. Paper chromatography in solvent *B* showed the presence of glycerol phosphates, saccharinic acids and material with *R_F* 0.0, all detected with the periodate-Schiff reagents. The appropriate areas from an unsprayed chromatogram were extracted with water and examined as follows.

The compound corresponding to glycerol phosphate was treated with a 1% solution of intestinal phosphomonoesterase (0.5ml.) for 16hr. at 37°. Examination of the evaporated product by paper chromatography in solvents *B* and *E* showed the presence of glycerol, inorganic phosphate and a small amount of unhydrolysed glycerol phosphates.

The material with *R_F* 0.0 was hydrolysed with 2M-HCl at 100° for 3hr., the acid removed by a small column of Dowex 2 (CO₃²⁻ form) resin and the evaporated eluate examined by paper chromatography in solvent *A*. Glucose and galactose were the only compounds detected.

Action of alkali on some disaccharides. Laminaribiose, cellobiose, maltose and lactose (0.5mg. of each) were each treated with M-NaOH (0.2ml.) at 100° for 1hr. in sealed tubes. The solutions were passed through Dowex 50 (H⁺ form) resin to remove Na⁺ ions and evaporated to dryness, and the residues were dissolved in 1 drop of water and chromatographed in solvent *B*. The product from laminaribiose appeared as a rapidly developing purple spot with the periodate-Schiff reagents, whereas each of the others appeared rapidly as yellow spots, which later became purple. This latter behaviour was identical with that observed with the alkali hydrolysis of S.11A and is assumed to be due to the formation of isosaccharinic acids.

Reduction of S.11A with borohydride and alkali hydrolysis of the product. S.11A (2mg.) was treated with NaBH₄ (5mg.) in water (1ml.) at room temperature overnight. Excess of borohydride was destroyed by the addition of acetic acid (1 drop). The solution was made 1M with respect

to NaOH and heated at 100° for 3 hr. in a sealed tube. The colourless solution was passed through a column of Dowex 50 (H⁺ form) resin and the eluate evaporated to dryness. Boric acid was removed by distillation with methanol and the residue was subjected to paper chromatography in solvent *B*. Glycerol phosphates and a product with *R_F* 0.0 were detected by the periodate-Schiff reagents, but no saccharinic acids were present. The glycerol phosphate area was extracted and treated with the intestinal phosphomonoesterase as before; the products were glycerol and inorganic phosphate. The material with *R_F* 0.0 was eluted with water and subjected to hydrolysis with 2M-HCl (100°, 3 hr.); galactose and glucose were the only products detected by paper chromatography in solvent *A*.

Preparation of the phosphorus-free polysaccharide. S.11A (100mg.) was dissolved in water (5ml.), a solution of NaBH₄ (100mg.) in water (5ml.) added, and the mixture kept overnight at room temperature. Excess of NaBH₄ was destroyed with acetic acid (2-3 drops) and the solution freeze-dried. The residue was treated with M-NaOH (2ml.) in a sealed tube at 100° for 3 hr., and the hydrolysate passed through a column of Dowex 50 (NH₄⁺ form) resin, followed by a column of Dowex 2 (CO₃²⁻ form) resin. The eluate was freeze-dried and boric acid removed from the residue as methyl borate. A little water was added and the solution freeze-dried, leaving the phosphorus-free polysaccharide as a white powder (Found: P, 0.0; hexose, 97.6; D-glucose, 47.6; D-galactose, 49.0%).

Methylation of the phosphorus-free polysaccharide. The method of Isbell, Frush, Bruckner, Kowkabany & Wampler (1957) was used, with minor alterations to the design of the apparatus suggested by Dr Elizabeth Percival. The phosphorus-free polysaccharide (10mg.), mixed with Celite (50mg.), was treated with K in liquid NH₃ for 6 hr., the NH₃ removed by evaporation and the residue treated with

methyl iodide for 18 hr. After evaporation of the methanol the cycle was repeated seven times. The residue was extracted with ethanol (4 × 5ml.), the extract filtered to remove Celite, and the combined ethanol extracts were evaporated to dryness. The residue, which contained KI, was extracted with chloroform, and the methylated polysaccharide obtained by evaporation of the extract. The methylated product was treated with 4% (w/v) HCl in methanol at 100° in a sealed tube for 18 hr. (Aspinall, 1963). The product of methanolysis was examined by g.l.c. by Professor G. O. Aspinall (see Table 1).

Degradation of the phosphorus-free polysaccharide with periodate. The polysaccharide (12mg.) in water (1ml.) was treated with a solution of NaIO₄ (51mg.) in water (2ml.) and kept in the dark at 4° for 72 hr. Inorganic salts were removed by dialysis and the remaining solution was freeze-dried. The residue was dissolved in water (1ml.), NaBH₄ (50mg.) added and the solution kept at room temperature overnight. Excess of NaBH₄ was destroyed by the addition of acetic acid (2-3 drops) and the solution was passed through Dowex 50 (NH₄⁺ form) resin. The eluate was freeze-dried and borate removed as methyl borate. The residue was dissolved in water (2ml.) and freeze-dried to yield the degraded polysaccharide as a white powder.

A portion of the degraded polysaccharide (1mg.) was heated with 2M-HCl at 100° for 1 hr. The hydrolysate was passed through a short column of Dowex 2 (CO₃²⁻ form) resin and the eluate evaporated to dryness. Chromatographic examination of the products in solvents *A* and *E* showed the presence of galactose, threitol, erythritol and glycerol.

The remainder of the degraded polysaccharide was treated with 0.05M-H₂SO₄ (0.5ml.) at 28° for 16 hr., and the solution passed through a short column of Dowex 2 (CO₃²⁻ form) resin. The eluate was freeze-dried and chromatographed in solvents *A* and *E*, showing the presence of glycerol, threitol and a compound shown below to be a β-galactosyl-erythritol. The latter was eluted from an unsprayed area of the chromatogram and the eluate freeze-dried. The residue was dissolved in water (0.5ml.) and passed through a small charcoal-Celite column (Whistler & Durso, 1950). After the column had been washed with 5 bed-vol. of water, the glycoside was eluted with ethanol-water (1:9, v/v). On evaporation crystals were obtained (2mg.), [α]_D +13.4° in water, m.p. 180-181°, undepressed in admixture with 2-O-β-D-galactopyranosyl-D-erythritol, m.p. 179-181°, [α]_D +7.3° in water (Austin, Hardy, Buchanan & Baddiley, 1965); the i.r. spectra (KBr disc) were indistinguishable. Galactose and erythritol were detected chromatographically (solvents *A* and *E*) after hydrolysis with 2M-HCl at 100° for 1 hr.

Deacetylation of S.11A. S.11A (20mg.) was dissolved in water (1ml.), and methanol (0.5ml.) and aq. NH₃ (sp.gr. 0.88, 0.5ml.) were added. The solution was kept at room temperature overnight, the NH₃ removed by aeration and the solution freeze-dried to give a white powder. When tested by the method of Frèrejacque (1955) *O*-acetyl groups were shown to be absent. Acid hydrolysis (2M-HCl) and alkali hydrolysis (M-NaOH) yielded the same products as from S.11A itself.

Degradation of deacetylated S.11A with periodate. Deacetylated S.11A (10mg.) in water (1ml.) was oxidized with NaIO₄ (30mg.) in water (1ml.) at 4° for 72 hr., NaBH₄ (100mg.) was added and the solution left overnight. After dialysis overnight the solution was freeze-dried and the

Table 1. Retention times on g.l.c. of methyl glycosides from methylated phosphorus-free polysaccharide relative to that of methyl 2,3,4,6-tetra-O-methyl-β-D-galactopyranoside

Columns A and B refer to the butane-1,4-diol succinate polyester and polyphenyl ether columns used by Aspinall (1963) under the operating conditions described in that paper. The values in parentheses refer to peaks common to more than one component that are not resolved. (w), Weak; (vw), very weak; n.d., not detected.

Methyl glycosides	Column A		Column B	
	Authen- tic	Mixture	Authen- tic	Mixture
2,3,4-Tri-O-methylglucose	2.60	2.61	1.35	1.37
	3.70	3.71	1.83	1.82
2,3,6-Tri-O-methylglucose	3.52	3.54	1.71	(1.68)
	4.78	(4.75)	2.18	2.18
2,3,6-Tri-O-methylgalactose	3.21	3.20	1.66	(1.68)
	3.93 (vw)	n.d.	2.55	2.55
	4.30 (w)	n.d.		
	4.70	(4.75)		
2,4,6-Tri-O-methylgalactose	4.12	4.18	2.09	2.07
	4.70	(4.75)	2.37	2.37

residue treated with 0.05 M-H₂SO₄ (0.5 ml.) at 28° for 18 hr. The acid was neutralized by addition of BaCO₃, the solids were removed by centrifugation and the supernatant was evaporated to dryness *in vacuo*. Chromatographic examination in solvents *A* and *B* showed the presence of galactosyl-erythritol, threitol, ethylene glycol and erythritol phosphate. The presence of erythritol phosphate was confirmed by elution from the paper, treatment with intestinal phosphomonoesterase (1%, 0.5 ml.) at pH 9.4, followed by paper chromatography. Erythritol and inorganic phosphate were the sole products.

Degradation of S.11A with periodate. (a) Conditions for complete oxidation. S.11A (45 mg.) in water (2 ml.) was treated with NaIO₄ (102 mg.) in water (3 ml.) at 4° in the dark for 72 hr. After dialysis, the product was treated with NaBH₄ (100 mg.) overnight, excess of borohydride destroyed with acetic acid (2 drops), and then hydrolysed with M-NaOH at 100° for 3 hr. The resulting solution was passed through short columns of Dowex 50 (H⁺ form) and Dowex 1 (CO₃²⁻ form) resins and then freeze-dried. Complete hydrolysis (2 M-HCl at 100° for 3 hr.) of a portion (1 mg.) and paper chromatography in solvents *A* and *E* showed the presence of galactose, glucose, erythritol and threitol, but no glycerol. Partial hydrolysis (0.1 M-H₂SO₄ at 28° for 16 hr.) of the remainder of the residue was followed by paper-chromatographic separation of the products (solvents *A* and *E*). These consisted of threitol, galactosylerythritol, a glucosyl-threitol and a compound with low *R_F* value (compound A, see below). The 2-*O*-β-D-galactopyranosyl-D-threitol (V) was isolated and characterized as described previously.

The glucosylthreitol was eluted from the chromatogram and had $[\alpha]_D + 85^\circ$ in water. A sample (0.5 mg.) was treated with 2 M-HCl at 100° for 1 hr. Glucose and threitol were the only compounds detected after paper chromatography in solvents *A* and *E*. Treatment of the compound with a 1% solution of β-glucosidase was without effect. The compound is probably 2-*O*-α-D-glucopyranosyl-D-threitol (VIII).

The compound A with low *R_F* value was eluted and hydrolysed (2 M-HCl, 100°, 3 hr.) to give threitol, glucose and galactose, shown by chromatography in solvents *A* and *E*.

(b) Conditions for partial oxidation. S.11A (45 mg.) was treated with NaIO₄ (102 mg.) in water (5 ml.) at 4° in the dark. Samples were removed periodically and periodate was determined. When 2 mol.prop. had been reduced (24 hr.) ethylene glycol was added to destroy the remaining periodate and the solution was dialysed. The product was reduced with NaBH₄ (100 mg.) overnight, excess of borohydride destroyed with a few drops of acetic acid, and the solution made 1 M with respect to NaOH. After hydrolysis at 100° for 3 hr. the solution was passed through columns of Dowex 50 (NH₄⁺ form) and Dowex 2 (CO₃²⁻ form) resins and the eluate freeze-dried.

The residue was treated with 0.05 M-H₂SO₄ (0.1 ml.) at 28° for 16 hr., then neutralized with BaCO₃, and the products were separated by paper chromatography in solvent *E*. Threitol, galactosylerythritol, glucosylthreitol and possibly glycolaldehyde were detected, together with compounds A and B, with low *R_F* values (see Table 2).

The galactosylerythritol and glucosylthreitol were eluted and examined as above.

Tetrasaccharide A had the lowest *R_F* value. It reacted with the periodate-Schiff reagent to give a purple colour rapidly. It was purified by adsorption on a small charcoal-Celite column and elution with ethanol-water (1:9, v/v). A

Table 2. *R_{glucose}* values of some S.11A degradation products in solvent A

Compound	<i>R_{glucose}</i>
Glucose	1.00
Galactose	0.92
Ethylene glycol	1.90
Glycerol	1.54
Erythritol	1.36
Threitol	1.32
2- <i>O</i> -α-D-Glucopyranosylthreitol (VIII)	0.89
2- <i>O</i> -β-D-Galactopyranosylerythritol (V)	0.80
Trisaccharide B ₁	0.42
Trisaccharide A ₁	0.35
Tetrasaccharide B	0.30
Tetrasaccharide A (X)	0.20

sample (0.5 mg.) was treated with 2 M-HCl at 100° for 1 hr., deionized by passage through a column of Dowex 2 (CO₃²⁻ form) resin, and chromatographed in solvents *A* and *E*. Glucose, galactose and threitol were detected, the intensity of the glucose spot being considerably greater than that due to galactose.

Tetrasaccharide A (3 mg.) was treated with a 1% solution of almond β-glucosidase at 37° for 24 hr. Chromatography in solvents *A* and *C* showed that galactose and a trisaccharide A₁, which gave a purple colour rapidly with the periodate-Schiff reagents, were present. When trisaccharide A₁ was eluted from a chromatogram with water and subjected to acid hydrolysis (2 M-HCl, 100°, 3 hr.) glucose and threitol were detected by paper chromatography in solvents *A* and *E*.

Tetrasaccharide A was treated with 0.15 M-H₂SO₄ at 100° for 30 min. and the hydrolysate deionized by passage through Dowex 2 (CO₃²⁻ form) resin. Paper chromatography in solvent *A* showed the presence of galactose, threitol and a little glucose together with a disaccharide, A₂. In solvent *C*, disaccharide A₂ was indistinguishable from isomaltose and differed from gentiobiose, cellobiose, maltose, laminaribiose and sophorose. Disaccharide A₂ was eluted from a chromatogram; complete acid hydrolysis gave only glucose, and disaccharide A₂ was unaffected by β-glucosidase.

Tetrasaccharide B gave a greyish-purple colour slowly with the periodate-Schiff reagents. It was eluted from a chromatogram and acid hydrolysis (2 M-HCl, 100°, 3 hr.) of a sample (1 mg.) yielded glucose, galactose and threitol as shown by chromatography in solvents *A* and *E*. Treatment of a sample of tetrasaccharide B (1 mg.) with 1% β-glucosidase at 37° for 24 hr., followed by chromatography in solvent *A*, gave galactose and trisaccharide B₁ that gave a colour slowly with the periodate-Schiff reagents.

(c) Conditions for oxidation of glycerol residues only. S.11A (10 mg.) was oxidized with 0.04 M-NaIO₄ (2 ml.) at 4° for 1 hr. After addition of ethylene glycol the solution was dialysed and then freeze-dried. The residue was treated with excess of phenylhydrazine formate at pH 6 at 100° for 90 min. (Brown, Hall & Letters, 1959). Excess of phenylhydrazine was removed by extracting the cold solution with chloroform (5 × 20 ml.); the aqueous layer was passed through a column of Dowex 50 (H⁺ form) resin and evaporated to dryness. The product was heated with 2 M-HCl at 100° for 3 hr. The hydrolysate became coloured and a dark-brown solid was deposited. After evaporation

over solid NaOH *in vacuo* the residue was chromatographed in solvents *B* and *D*; a sugar phosphate was detected. It was eluted, treated with intestinal phosphomonoesterase at pH 9.4, and the products were shown by chromatography in solvents *A* and *B* to be only glucose and inorganic phosphate.

RESULTS AND DISCUSSION

Acid hydrolysis of S.11A yielded D-glucose, D-galactose and glycerol phosphates, confirming the earlier work (Shabarova *et al.* 1962). The molecular ratio glucose:galactose was 1:1, whereas in the original preparation of S.11A the hexose:phosphate ratio was 3.7:1. Since the preparation contained 0.75% of nitrogen and gave a positive immunological reaction for C-substance (nitrogen content, 6.0%; Brundish & Baddiley, 1968), it appears that there are four hexose units for each phosphate group in pure S.11A, and this was borne out by subsequent experiments. *O*-Acetyl groups were shown to be present by treatment of S.11A with hydroxylamine and examination of the resulting hydroxamic acid by paper chromatography. Quantitative determination showed that there are at least two *O*-acetyl groups for each phosphate.

When S.11A was hydrolysed with hot aqueous alkali, paper chromatography of the products showed the presence of glycerol phosphates and a compound that had R_F 0.0 and that gave only glucose and galactose on acid hydrolysis. Together with these compounds there were three others that reacted with the periodate-Schiff reagents and that were also present in alkali hydrolysates of maltose, cellobiose and lactose, but not of laminaribiose. It therefore seems that S.11A

consists of a polysaccharide composed of equimolar amounts of glucose and galactose, to which are attached side chains of glycerol phosphate on statistically every fourth hexose residue. The formation of saccharinic acids, presumably isosaccharinic acids, implies that (1→4)-linkages are present in S.11A. It was therefore decided to convert S.11A into a phosphorus-free compound by treatment first with sodium borohydride and then with hot aqueous alkali. As expected, reduction of the terminal sugar to a polyol prevented formation of any saccharinic acid on subsequent alkali treatment. Glycerol phosphate was formed quantitatively, showing that the phosphate cyclization occurred exclusively on to the glycerol. The phosphorus-free product was separated from the glycerol phosphates by using ion-exchange resins and is referred to below as the phosphorus-free polysaccharide. On acid hydrolysis it yielded only glucose and galactose in equal amounts, the quantity of polyol from the end group being too small for detection. Detailed studies on this polysaccharide are now described.

Methylation of the phosphorus-free polysaccharide and examination of the products of subsequent methanolysis by g.l.c. showed the presence of the methyl glycosides of 2,3,4- and 2,3,6-tri-*O*-methylglucose and of 2,3,6- and 2,4,6-tri-*O*-methylgalactose in approximately equal quantities. Assuming that the sugars were originally present as pyranosides, the polysaccharide consists of the units (I–IV).

Justification and extension of these ideas came from the behaviour of the phosphorus-free polysaccharide towards the degradation sequence periodate oxidation, reduction with sodium borohydride and acid hydrolysis (Smith degradation;

Goldstein, Hay, Lewis & Smith, 1965). When this sequence was carried out with a final strong acid hydrolysis, the products were glycerol (from I), erythritol (from II), threitol (from III) and galactose (from IV). When the final acid hydrolysis was a mild one, the products were glycerol, threitol and a galactosylerythritol (V). The last-named was obtained in crystalline form, yielded galactose and erythritol on acid hydrolysis and was indistinguishable from a synthetic sample of 2-*O*- β -D-galactopyranosyl-D-erythritol (Austin *et al.* 1965). The phosphorus-free polysaccharide therefore consists of tetrasaccharide units composed of the monosaccharides (I-IV) of which the monosaccharides (IV) and (II) are represented in the disaccharide unit (VI). In agreement with this, quantitative periodate oxidation showed the reduction of 3.9 mol.prop. of periodate for every tetrasaccharide unit, with the formation of 1.2 mol.prop. of formic acid and no formaldehyde.

In S.11A itself 2.9 mol.prop. of periodate was reduced for each tetrasaccharide unit, with the formation of 0.92 mol.prop. of formaldehyde and negligible amounts of formic acid. By treatment with ammonia S.11A was deacetylated without loss of the glycerol phosphate groups, and the resulting compound reduced 3.8 mol.prop. of periodate with the formation of 0.90 mol.prop. of formaldehyde and negligible amounts of formic acid. The *O*-acetyl groups are thus in such a position as to prevent oxidation of one α -glycol system present in the deacetylated polymer. As is shown below, the formaldehyde arose from oxidation of glycerol 1-phosphate side chains. Since the amount of periodate reduced by deacetylated S.11A was the same as that reduced by the phosphorus-free polysaccharide, the glycerol phosphate residue must occupy a potential oxidation site on the polysaccharide, i.e.

it must be adjacent to a free hydroxyl group in the deacetylated S.11A.

When deacetylated S.11A was treated with periodate, then reduced with borohydride and the inorganic ions removed by dialysis, mild acid hydrolysis of the product yielded galactosylerythritol (V), threitol, ethylene glycol and erythritol phosphate. In the similar degradation of the phosphorus-free polysaccharide glycerol is formed from the glucose residue (I). In deacetylated S.11A the partial structure (VII) accounts for the formation of ethylene glycol and erythritol phosphate in place of glycerol in the Smith degradation (see Scheme 1). Hydrolysis of the phosphodiester is unidirectional, giving no erythritol or ethylene glycol phosphate.

S.11A itself was oxidized with periodate, then reduced with borohydride and the product hydrolysed with alkali to cleave phosphate diesters. When the deionized product was hydrolysed carefully with acid the products were the galactosylerythritol (V), a glucosylthreitol, a tetritol and a compound with low R_F value that, on elution and acid hydrolysis, gave glucose, galactose and threitol. The glucosylthreitol was hydrolysed with acid to give glucose and threitol, and had the R_F value expected of a monoglucoside. Since it had a high positive specific rotation, and was not hydrolysed by almond emulsin, we assign it the α -configuration, as in structure (VIII).

On comparing the results of Smith degradation of S.11A with those of a similar degradation on deacetylated S.11A the outstanding feature is the survival of glucose in the former case but not in the latter. Its survival must be attributed to the presence in the polysaccharide chain of the sequence (IX). The location of the glycerol phosphate residue on a glucose unit was confirmed by oxidation of

Scheme 1. Location of the glycerol phosphate residue.

Scheme 2. Structure of tetrasaccharide A.

S.11A with periodate under conditions in which only the glycerol phosphate side chains were oxidized. Subsequent treatment with phenylhydrazine removed the glycolaldehyde residue (Brown *et al.* 1959), and when the carbohydrate product was hydrolysed with acid a glucose phosphate was detected by paper chromatography.

When S.11A was subjected to periodate oxidation under conditions where only 2mol.prop. of reagent was reduced, the product then reduced with borohydride and hydrolysed with alkali to remove glycol phosphate residues, subsequent mild acid treatment gave several compounds separable by paper chromatography. Threitol, galactosylerythritol and glucosylthreitol were detected, together with two compounds with low R_F values that appeared to be tetrasaccharides. The one with the lowest R_F value, compound A, was purified on a charcoal-Celite column. It gave a purple colour

rapidly with the periodate-Schiff reagents, which indicated the formation of formaldehyde. Acid hydrolysis showed that compound A is a reduced tetrasaccharide containing two units of glucose, one unit of galactose and one unit of threitol. By the action of almond β -glucosidase (containing galactosidases) galactose was produced together with a trisaccharide A₁, that gave only glucose and threitol on acid hydrolysis and had an R_F value intermediate between that of tetrasaccharide A and the glucosylthreitol. Trisaccharide A₁ was resistant to further action of almond emulsin and was a diglucosylthreitol. Partial acid hydrolysis of tetrasaccharide A gave galactose, threitol and a little glucose together with a disaccharide A₂ indistinguishable from isomaltose on paper chromatography. Taken in conjunction with the earlier work, compound A is therefore the tetrasaccharide (X). The other tetrasaccharide, compound B, is discussed below.

The isolation of tetrasaccharide A indicates the point of linkage of the two disaccharide units (VI) and (IX) in the intact S.11A, and a tentative structure (XI) can be written for the repeating unit of S.11A. In view of the high positive specific rotation of S.11A and the presence of a β -configuration in one of the galactose units the configuration of the remaining galactose unit is taken to be α .

Eddy (1944) has reported a strong cross-reaction between S.18 antiserum and S.11A (43). This is not surprising in view of the similarities in structure between S.11A and S.18 (Estrada-Parra & Heidelberger, 1963). Although the *O*-acetyl group and glycerol phosphate residue are not assigned to a particular sugar in the repeating unit of S.18, one of the postulated structures contains the sequence

that is present in S.11A. The deacetylation of S.11A with ammonia causes almost complete loss of immunological response to S.11A antiserum, and it is noteworthy that Estrada-Parra & Heidelberger (1963) found *O*-acetyl groups to be important with S.18.

From the Smith degradation of S.11A was isolated a second tetrasaccharide, compound B, which yielded glucose, galactose and threitol on acid hydrolysis. It did not appear to give formaldehyde with the periodate-Schiff reagents. Almond emulsin caused the formation of *D*-galactose and another saccharide, compound B₁, that again did not give formaldehyde on periodate treatment. Tetrasaccharide B may be a 1,3-di-*O*-substituted threitol and would therefore arise from a branch point in the polysaccharide, but the other evidence of periodate oxidation and methylation would seem

to preclude branching in the polysaccharide. It is possible that tetrasaccharide B may be a cyclic acetal of tetrasaccharide A and glycolaldehyde, of the type encountered by Gorin & Spencer (1965) as artifacts from Smith degradations. A solution to this problem must await further work on the structure of tetrasaccharide B.

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