The Specific Substance from *Pneumococcus* Type 34(41)

THE STRUCTURE OF A PHOSPHORUS-FREE REPEATING UNIT

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The serologically-specific capsular substances have been prepared from a number of pneumococci by Brown (1939) and by Brown & Robinson (1943), using mild methods of isolation. The substances were shown to be carbohydrates, in agreement with the classical discovery by Heidelberger & Avery (1923). The field has been reviewed by Stacey & Barker (1960) and by Heidelberger (1960).

We were particularly interested in those substances containing phosphorus, because of the discovery that many bacteria contain in their walls polymers of glycerol phosphate or ribitol phosphate to which are attached labile D-alanine ester groups and sugars or aminodeoxy sugars (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958; Baddiley, 1962). These wall substances, the teichoic acids, possess immunological properties (Baddiley & Davison, 1961; Haukenes, Ellwood, Baddiley & Oeding, 1961). The chemical relationship between teichoic acids and the pneumococcal capsule polymers became clearer with the demonstration that the specific substance from type 6 is a polymer in which galactosylglucosylrhamnosylribitol units are joined together through phosphodiester linkages (Rebers & Heidelberger, 1959, 1961). A preliminary survey of some 21 phosphorus-containing substances from pneumococci showed that eight of them contained ribitol and seven contained glycerol, while the remainder appeared to be polysugar phosphates (Shabarova, Buchanan & Baddiley, 1962). It was thought that the micromethods that had been used successfully for the chemical investigation of the teichoic acids were ideally suited to the structural determination of the small quantities of pneumococcal antigens available.

One of these compounds has now been examined in more detail. The preparation was described by Brown & Robinson (1943) as from type 34. This number corresponds to the joint Danish–American nomenclature in use at that time (Sutliff, 1941; Walter, Guevin, Beattie, Cotler & Bucca, 1941; Kauffmann, Mørch & Schmith, 1940). The *Pneumococcus* was also called type 41 in the U.S.A. before 1941 (Sutliff, 1941; Mørch, 1942) and after 1944 (Eddy, 1944). At present it is known as type 34 (Danish system) and type 41 (American system) (Kauffmann, Lund & Eddy, 1960). In future publications on pneumococcal capsular antigens we propose to use the Danish system of nomenclature; this should reduce the possibility of confusion with descriptions in the earlier literature.

Shabarova *et al.* (1962) found that the substance from type 34 (S. 34) gave on acid hydrolysis galactose, glucose, ribitol and its phosphates, inorganic phosphate and anhydroribitol. Substance S. 34 has now been examined in more detail; in particular the products of alkali hydrolysis, and most of the structural features of the dephosphorylated repeating unit, are established by experiments described below.

EXPERIMENTAL

Materials. The preparation of the specific substance from type 34 *Pneumococcus* was kindly supplied by Dr Rachel Brown. It is described by Brown & Robinson (1943) as the soluble specific substance of type 34 (Buckley strain), preparation no. 1. Intestinal phosphomonoesterase was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., almond β-glucosidase from L. Light and Co. Ltd., Colnbrook, Bucks., and Glucostat reagent (D-glucose oxidase) from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Paper chromatography. Paper chromatography was carried out on Whatman paper no. 1, 3 or 4. The following solvent systems were used: *A*, propan-1-ol–aq. ammonia (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*, butan-1-ol–ethanol–water–aq. ammonia (sp.gr. 0·88) (40:10:49:1, by vol.) (organic phase) (Hirst, Hough & Jones, 1949). The products were detected by: the periodate–Schiff reagents for α-glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956); molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949); alkaline silver nitrate reagent for sugars and polyols (Trevelyan, Procter & Harrison, 1950); aniline phthalate reagents for reducing sugars (Partridge, 1949); alkaline triphenyltetrazolium chloride reagent for reducing sugars (Feingold, Avigad & Hestrin, 1956). When quantitative measurements were carried out on materials eluted from paper chromatograms appropriate determinations on equivalent paper extracts were made.

Analytical methods. Total phosphorus was determined by the method of Chen, Toribara & Warner (1956).

Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954) and formaldehyde by a modification of the

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chromotropic acid method (Hanahan & Olley, 1958). The oxidation was carried out at the pH of sodium periodate to avoid hydrolysis of furanosyl linkages. A sample of solution expected to yield 5–20 μg. of formaldehyde was made to 0·5 ml. with water. 0·1 M-Sodium periodate (0·5 ml.) was added and the solution was kept in the dark at room temperature for 2 hr. Sodium hydrogen sulphite (10% w/v, 0·5 ml.) was added, followed by the chromotropic acid solution (5·0 ml.). The tubes were stoppered tightly, heated at 100° for 30 min. and allowed to cool during 1 hr. To them was added water (1·0 ml.) and half-saturated thiourea solution (0·5 ml.); the tubes were shaken and the extinction at 570 mμ was measured after 5 min.

Hexoses and pentoses, before or after acid hydrolysis, were determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

Isolation and composition of the pentasaccharide

Alkali hydrolysis of the specific substance. Substance S. 34 (60 mg.) was hydrolysed in a sealed tube with N-sodium hydroxide (4·0 ml.) at 100° for 3 hr. The hydrolysate was passed through a column of Dowex 50 (NH_4^+ form) resin (30 ml.) and evaporated to dryness *in vacuo*; the residue was then dissolved in water (4·0 ml.). Ascending paper chromatography in solvent *A* revealed one major product ($R_{Ribitol}$ 0·30) which contained phosphorus and gave a purple colour rapidly with the periodate–Schiff reagents.

Enzymic dephosphorylation of the alkali hydrolysate. The neutral alkali hydrolysate (4·0 ml.) was mixed with intestinal phosphomonoesterase (12 mg.) and ammonium carbonate (84 mg.) dissolved in water (16 ml.); the solution was kept under toluene for 16 hr. at 37° and the enzymic hydrolysate was examined by chromatography in solvent *A*. Hydrolysis of the phosphate ester was essentially complete, yielding a major product ($R_{Ribitol}$ 0·59) which also gave a purple colour rapidly with the periodate–Schiff reagents.

Isolation of the pentasaccharide. The enzymic hydrolysate was passed through a column of Dowex 50 (H^+ form) resin (6 ml.), followed by a column of Dowex 2 (CO_3^{2-} form) resin (3 ml.). The eluate was evaporated under reduced pressure to a small volume and freeze-dried. The resulting white solid was dissolved in the minimum amount of cold methanol, and propan-2-ol was added to turbidity. After 12 hr. at 2°, more propan-2-ol was added and the process was repeated, finally with ether, until all the material had been precipitated; the amorphous solid (30 mg.) was recovered by centrifuging. It was not hygroscopic and was homogeneous when examined by descending paper chromatography in solvent *B* ($R_{Lactose}$ 0·67) and solvent *C* ($R_{Lactose}$ 0·44); detection was by periodate–Schiff reagents and silver nitrate. It had $[\alpha]_D^{20}$ +48° (*c* 1·0 in water) (Found: C, 40·6; H, 7·1; N, 0·0. $C_{29}H_{52}O_{25},3H_2O$ requires C, 40·8; H, 6·9%). In the phenol–sulphuric acid procedure the pentasaccharide (103 μg.) contained 84 μg. of hexose (calc. 87 μg.).

Acid hydrolysis. The pentasaccharide (2 mg.) was hydrolysed in a sealed tube with 2N-hydrochloric acid (0·2 ml.) at 100° for 3 hr. The acid was removed *in vacuo* and the residue chromatographed in solvents *A* and *B*. The products were ribitol, anhydroribitol, glucose and galactose.

Ratio and configuration of sugars. A sample of the pentasaccharide (2 mg.) was hydrolysed with 2N-hydrochloric acid (0·2 ml.) at 100° for 6 hr. The acid was removed by passing the diluted hydrolysate through a column of Dowex 2 (CO_3^{2-} form) resin (2 ml.). The eluate was evaporated to a small volume *in vacuo* and chromatographed on Whatman no. 1 paper as a band in solvent *B* (descending). Glucose and galactose were determined by the phenol–sulphuric acid method after elution with water. The ratio glucose:galactose was 1:2·7.

The configuration of the glucose was determined in a similar acid hydrolysate by comparing the value for total sugar (phenol–sulphuric acid method) and that for D-glucose (Glucostat reagent); 26% of the total sugar was D-glucose.

Another sample of the pentasaccharide (20 mg.) was hydrolysed with 2N-hydrochloric acid (1·3 ml.) at 100° for 3 hr. The acid was removed with a column of Dowex 2 (CO_3^{2-} form) resin (10 ml.) and the eluate was chromatographed as a band on Whatman no. 3 paper in solvent *B* (descending). The galactose was eluted with water, and the total galactose and rotation were determined on measured portions (phenol–sulphuric acid method); it had $[\alpha]_D^{20}$ +83° (*c* 1·0 in water). The equilibrium value for D-galactose is +80·2° (in water) (Pigman & Goepp, 1948).

Products of partial acid hydrolysis

The pentasaccharide (15 mg.) was hydrolysed in a sealed tube with 66% (v/v) formic acid (1·0 ml.) at 100° for 30 min. The hydrolysate was evaporated to dryness *in vacuo*, a few drops of dilute aq. ammonia were added to hydrolyse formyl esters and the solution was evaporated to dryness. Small samples of the hydrolysate were chromatographed (descending) in solvent *B* and developed with the periodate–Schiff and silver nitrate reagents. In addition to unchanged material, products were small amounts of glucose and ribitol, a large amount of galactose, and two compounds in the disaccharide region of the chromatogram. (Hydrolysis of the pentasaccharide with 0·1N-hydrochloric acid at 100° for 30 min. gave the same results.) The disaccharide with the lower mobility (D_1; $R_{Galactose}$ 0·60) gave a yellow colour with the periodate–Schiff reagents, whereas the other (D_2; $R_{Galactose}$ 0·73) gave a purple colour rapidly with the same reagents. The remaining hydrolysate was chromatographed as a band on Whatman no. 3 paper in solvent *B*, and D_1 and D_2 were eluted with water (1 ml.).

Identification of D_1 *as* 2-O-(α-D-*glucopyranosyl*)-D-*galactose.* A small sample of D_1 was hydrolysed with 2N-hydrochloric acid at 100° for 3 hr. The acid was removed *in vacuo* and the hydrolysate examined by chromatography in solvent *B*, the silver nitrate reagent being used. The products were glucose and galactose in approximately equal amounts.

To a sample (0·6 ml. containing about 2 mg. of sugar) was added sodium borohydride (5 mg.) and the solution was kept at 20° for 16 hr. A few drops of acetic acid were added to decompose borohydride and the solution was then passed through a column of Dowex 50 (H^+ form) resin (2 ml.). The eluate was evaporated to dryness *in vacuo* and methanol (1 ml.) added to the residue. This was evaporated to dryness on a warm plate and the addition of methanol and evaporation was repeated twice. The residue was dissolved in water (0·6 ml.) and sugars were determined (phenol–sulphuric acid) on a sample; the hexose value was 40% of that found originally. The reduced material was examined, after chromatography in solvent *A*, with the

periodate–Schiff reagents. Reduction was complete and the product had a slightly higher mobility ($R_{Galactose}$ 0·92) and gave a purple colour rapidly with these reagents. Acid hydrolysis of a sample (0·1 ml.) of the solution containing reduced D_1, followed by chromatography in solvent *B* with aniline phthalate for detection, showed that glucose was the only reducing sugar present. Another sample of the solution of reduced D_1 (0·1 ml.) was mixed with water (0·4 ml.) and 0·1 M-sodium metaperiodate (0·5 ml.). The solution was kept at 20° for 90 min. and formaldehyde was determined; the hexose:formaldehyde ratio was 1·0:1·0.

Colour reactions and enzymic hydrolysis of D_1. Disaccharide D_1 reacted strongly with the periodate–Schiff reagents, weakly with silver nitrate–sodium hydroxide and very weakly with aniline phthalate and alkaline triphenyltetrazolium chloride. Kojibiose, 2-*O*-methylgalactose and D_1 gave a yellow colour with the periodate–Schiff reagents, whereas 3-*O*-methylgalactose, lactose and melibiose (from partial hydrolysis of raffinose) gave a purple colour. In addition, 2-*O*-methylgalactitol and reduced D_1 gave a purple colour rapidly with the periodate–Schiff reagents, whereas cellobi-itol, lactitol and 3-*O*-methylgalactitol gave yellow colours.

Solutions of D_1 (0·1 ml.), reduced D_1 (0·1 ml.) and cellobi-itol (0·1 ml., 0·5%) were each mixed with 0·025 ml. of a 2% (w/v) solution of β-glucosidase and the mixtures were kept at 37° for 24 hr., then chromatographed in solvent *C*. None of the D_1 or reduced D_1 had been hydrolysed, but the hydrolysis of cellobi-itol was almost complete.

Identification of D_2 *as* O-α-D-*galactopyranosylribitol*. A sample of D_2 was hydrolysed in 2 N-hydrochloric acid at 100° for 3 hr., the acid removed *in vacuo*, and the residue chromatographed in solvent *B*. Products (silver nitrate reagent) were galactose and ribitol in approximately equal amounts.

The solution of D_2 (1·0 ml.) was concentrated to 0·4 ml. *in vacuo*. Samples of D_2 (0·1 ml.), lactose (0·1 ml., 0·5%) and methyl α-D-galactoside (0·1 ml., 0·5%) were each mixed with 0·025 ml. of 2% (w/v) almond β-glucosidase solution (containing α- and β-galactosidase). To these was added saturated silver oxide solution (0·01 ml.) to inhibit α-galactosidase activity (cf. Hestrin, Feingold & Schramm, 1955) and the mixtures, together with similar ones without silver oxide, were kept at 37° for 18 hr. and chromatographed on paper in solvent *C*. All the glycosides were hydrolysed in the absence of silver oxide, but in its presence only lactose was hydrolysed.

Oxidation of the pentasaccharide with periodate

A sample (1·0 ml.) of a solution of the pentasaccharide was diluted with water to give a 0·1% solution which was mixed with an equal volume of 20 mM-sodium metaperiodate. The solution was kept at 20° in the dark; samples were removed from time to time, and diluted, and periodate was determined spectrophotometrically. After 80 hr., 7·4 mol.prop. of periodate had been used and no further oxidation was observed.

Formaldehyde production. A sample of S.34 (6 mg.) was hydrolysed with alkali and Na^+ ions were removed with Dowex 50 (NH_4^+ form) as described above. The neutral solution (0·4 ml.) of ammonium salts was chromatographed on Whatman no. 3 paper (ascending) in solvent *A*, and the pentasaccharide phosphate was eluted with water (5·0 ml.) at 20° for 16 hr. Phosphorus, hexose and the formaldehyde produced by oxidation with periodate were determined on samples of eluate. Another sample (0·6 ml.) was mixed with phosphatase solution (0·2 ml.) and the mixture was kept in a sealed tube at 37° for 16 hr., when paper chromatography in solvent *A* showed hydrolysis to phosphate and pentasaccharide to be complete. This pentasaccharide was oxidized with periodate and formaldehyde was determined. The proportions of phosphorus, formaldehyde and hexose (pentasaccharide phosphate) and formaldehyde (pentasaccharide) were 1·0:1·9:3·9:3·1.

Acid hydrolysis of oxidized pentasaccharide. The pentasaccharide (2 mg.) was dissolved in water (0·4 ml.), 0·1 M-sodium metaperiodate (0·4 ml.) added, and the mixture kept at 20° in the dark for 48 hr. To this solution was added sodium borohydride (10 mg.) and the mixture was kept at 20° for 16 hr. Excess of borohydride was decomposed with a few drops of acetic acid, the solution was passed through a column of Dowex 50 (H^+ form) resin (3 ml.) and evaporated to dryness *in vacuo*, and the boric acid removed by evaporation with methanol several times on a warm plate. The residue was hydrolysed in 2 N-hydrochloric acid (0·2 ml.) at 100° for 3 hr. and the acid removed by passing the hydrolysate through a column of Dowex 2 (CO_3^{2-} form) resin (3 ml.). The eluate was examined by chromatography in solvent *B*. Glycerol, glucose and galactose were detected, together with a product with the chromatographic properties of a pentose, giving a pink colour with aniline phthalate. It was identified as arabinose by co-chromatography with the four pentoses in solvent *B*. R_{Ribose} values were: lyxose, 0·97; xylose, 0·90; arabinose and the degradation product, 0·82. Separation of the products on paper, followed by quantitative determination, gave glucose:galactose:arabinose as 1·0:0·9:0·9.

Another sample of pentasaccharide (10 mg.) was oxidized with sodium metaperiodate and the product reduced with sodium borohydride in the above manner. The material was hydrolysed under the mild conditions employed in the preparation of D_1 and D_2. The hydrolysate was chromatographed in solvent *B*. A compound D_3 with the chromatographic properties of a disaccharide was observed; it had $R_{Galactose}$ 0·86 and gave a yellow colour with the periodate–Schiff reagents. The hydrolysate was chromatographed as a band in solvent *B*, and D_3 was eluted and rechromatographed in solvent *C* ($R_{Galactose}$ 0·72). A sample of the purified material was hydrolysed with 2 N-hydrochloric acid and the hydrolysate was chromatographed in solvent *B*; glucose and arabinose were the only products. Another sample was reduced with sodium borohydride in the same manner as for D_1; one product was found having $R_{Galactose}$ 1·0 in solvent *B* and giving a purple colour rapidly with the periodate–Schiff reagents. Hydrolysis of reduced D_3 with acid and chromatography in solvent *B* showed glucose to be the only reducing sugar.

Limited oxidation of the pentasaccharide with periodate. The pentasaccharide (2·0 mg.) was dissolved in water (0·1 ml.) and cooled to 2°. To this was added a solution of 85 mM-sodium metaperiodate (0·11 ml.) at the same temperature and the mixture was kept at 2° for 30 min. (cf. Kjølberg, 1960). The oxidation was stopped by passage of sulphur dioxide through the mixture; a stream of air was blown through to remove excess of sulphur dioxide and the solution was immediately neutralized with dilute aq. ammonia. Sodium borohydride (5 mg.) was added and the

solution kept at 20° for 16 hr. Ions were removed by passing through columns of Dowex 50 (H^+ form) resin (1 ml.) and Dowex 2 (CO_3^{2-} form) resin (1 ml.), solvent was evaporated *in vacuo*, and products were hydrolysed with 2N-hydrochloric acid at 100° for 3 hr. The hydrolysate was passed through a column of Dowex 2 (CO_3^{2-} form) resin (3 ml.) and the sugars were determined by chromatography (solvent *B*), elution and estimation in the usual manner. The proportion galactose:glucose:arabinose was 1·0:1·1:1·7.

DISCUSSION

Substance S. 34 was readily hydrolysed by alkali to a mixture of monophosphates from which the phosphate was completely hydrolysed by a phosphatase. In this respect it resembles the ribitol teichoic acids (Armstrong *et al.* 1958), the lability of the phosphodiester linkages being a consequence of the presence of adjacent hydroxyl groups. The major product, isolated in pure form, was a non-reducing compound containing four sugars and ribitol, which for convenience is referred to below as the 'pentasaccharide'. It contained no nitrogen and on acid hydrolysis gave D-glucose (1 mol.prop.), D-galactose (3 mol.prop.), ribitol and anhydroribitol; it is known that under the conditions normally required for hydrolysis of glycosides ribitol is partly converted into its anhydro compound (Baddiley, Buchanan, Carss & Mathias, 1956). Carbon and hydrogen analyses were consistent with a glycoside comprising ribitol and a tetrasaccharide.

When the pentasaccharide was subjected to mild acid hydrolysis with formic acid or hydrochloric acid there were three major products: galactose, a disaccharide of glucose and galactose (D_1), and a galactosylribitol (D_2). The galactose was identified chromatographically; its rapid liberation by mild acid treatment suggested that it might be present as a furanoside in the pentasaccharide. The presence of galactofuranosyl residues was confirmed by experiments discussed below.

Chromatographically homogeneous preparations of the disaccharide D_1 were shown to have structure (I) (Fig. 1). Complete acid hydrolysis gave glucose and galactose in approximately equal amounts. Reduction with sodium borohydride gave a product with the properties of a glucosylhexitol; the hexose

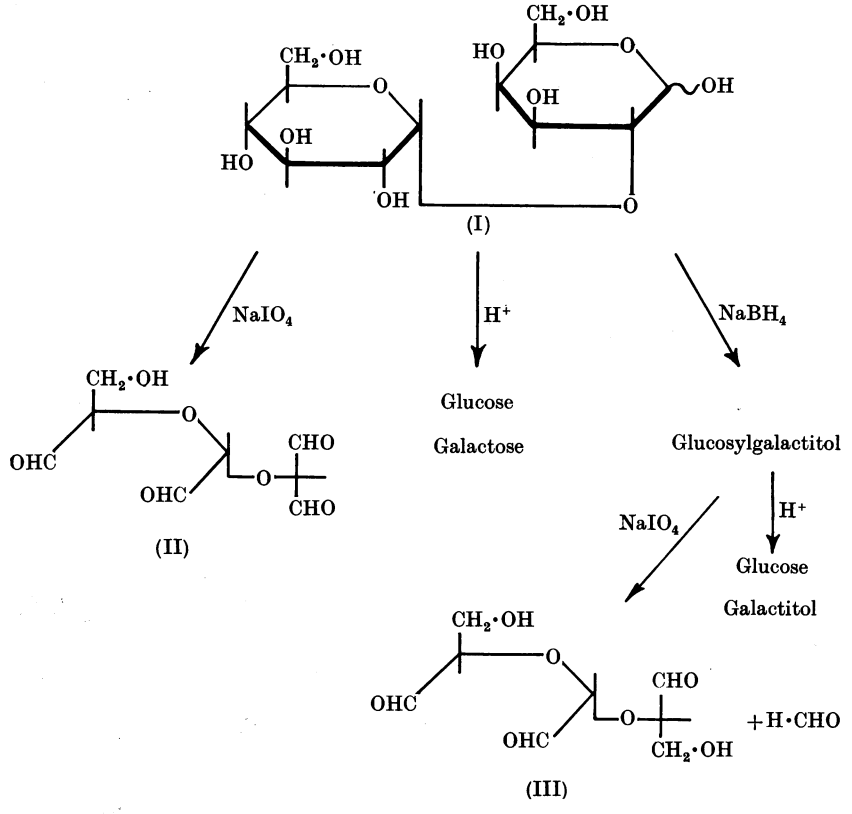

Fig. 1. Structure and degradation of glucosylgalactose (D_1) from partial hydrolysis of the pentasaccharide.

content, as determined by the phenol–sulphuric acid method, was 40% of its former value after reduction. The product was not a reducing sugar and on acid hydrolysis it gave glucose and a hexitol; disaccharide D_1 is therefore a glucosyl-galactose.

The glycosidic linkage in D_1 was shown to involve the C-2 hydroxyl group of galactose. Disaccharide D_1 gave a yellow colour with the periodate–Schiff reagents, as did kojibiose [2-*O*-(α-D-glucopyranosyl)-D-glucose] and 2-*O*-methylgalactose. Lactose, 3-*O*-methylgalactose and melibiose [6-*O*-(α-D-galactopyranosyl)-D-glucose] did not give yellow colours. This is consistent with observations made over several years in this Laboratory that substances giving yellow colours with these reagents are those expected to give a malondialdehyde derivative [e.g. (II)] with periodate. The product of reduction of D_1 with borohydride gave a purple colour rapidly with the same reagents, as did reduced 2-*O*-methylgalactose; cellobi-itol, lactitol and reduced 3-*O*-methylgalactose gave yellow colours. This behaviour is consistent with the formation of the aldehyde (III) and formaldehyde from reduced D_1; compounds which give purple colours rapidly contain a glycol grouping at the end of a chain and are able to give formaldehyde on oxidation with periodate.

A glycosyl linkage to the C-2 hydroxyl group of galactose is also indicated by the insensitivity of other sugar reagents towards D_1; alkaline silver nitrate was relatively insensitive, whereas aniline phthalate and alkaline triphenyltetrazolium were very insensitive in the detection of D_1 on paper chromatograms. When the reduction product of D_1, of known hexose content, was oxidized by an excess of periodate, 1 mol.prop. of formaldehyde was produced. This is not only consistent with the presence of a 2-*O*-glucosyl linkage, but proves that in the glucose moiety the sugar ring is pyranose. Since D_1 and its reduction product are not hydrolysed in the presence of almond emulsin, the complete structure of D_1 is given by (I), 2-*O*-(α-D-glucopyranosyl)-D-galactose.

The galactosylribitol D_2 had the chromatographic properties of a disaccharide and gave approximately equal quantities of galactose and ribitol, together with a little anhydroribitol, on acid hydrolysis. It was hydrolysed in the presence of almond emulsin to galactose and ribitol. The enzyme preparation contained both α- and β-galactosidase, but when the α-galactosidase was inhibited by silver oxide D_2 was not hydrolysed and so must possess an α-D-galactopyranosyl linkage. The galactosylribitol D_2 gave a purple colour in the periodate–Schiff test, and so cannot be a 3-*O*-glycosylribitol; moreover, the oxidation studies with periodate on the pentasaccharide that are described below confirm the pyranose ring size and prove that D_2 is 2 (or 4)-*O*-(α-D-galactopyranosyl)-D-ribitol (V) (Fig. 2).

When the pentasaccharide was treated with an excess of sodium periodate, the product reduced with borohydride and then hydrolysed with acid, glucose, galactose and arabinose were formed in equal amounts; no ribitol, anhydroribitol or ethylene glycol was detected in the hydrolysate, but glycerol was present. Since only one of the four sugars in the pentasaccharide does not appear as a sugar residue in the final products, the pentasaccharide must consist of an unbranched tetrasaccharide linked glycosidically to ribitol. The arabinose arises by oxidation of a galactofuranose residue to an aldehyde, which is then reduced with borohydride to a glycoside of arabinofuranose (cf. Kjølberg, 1960; Bourne, Hartigan & Weigel, 1961). When mild acid was used for the final hydrolysis a new disaccharide D_3 was produced; it gave a yellow colour with the periodate–Schiff reagents and a pink colour with aniline phthalate. It was shown by methods similar to those used in the study of D_1 that D_3 is 2-*O*-glucosylarabinose. This must arise from a 2-*O*-(α-D-glucopyranosyl)-D-galactofuranosyl moiety in the pentasaccharide. The glucose and one of the galactose units in the pentasaccharide are unaffected even by prolonged treatment with periodate. Since the pentasaccharide is unbranched, both of these units must have pyranose rings and possess substituents on the C-3 hydroxyl group.

The presence of a second galactofuranose unit was indicated by a brief treatment of the pentasaccharide with periodate. When the product was reduced and hydrolysed the galactose : glucose : arabinose proportion was 1 : 1·1 : 1·7. This second galactofuranose unit was oxidized by prolonged treatment with periodate, giving fragments which were not detected after reduction and hydrolysis. Since the pentasaccharide was unbranched, this galactofuranose must be terminal and its destruction presumably occurs through over-oxidation. This was confirmed by quantitative oxidation; the pentasaccharide consumed 7·4 mol.prop. of periodate and yielded 3 mol.prop. of formaldehyde (cf. Bourne *et al.* 1961).

Oxidation of the pentasaccharide with periodate provides information on the position of the glycosidic linkage on ribitol. The glycerol produced by reduction and hydrolysis of the oxidation product represents three of the carbon atoms originally present in the ribitol. The galactopyranosyl unit linked to ribitol in the pentasaccharide (and represented by the partial hydrolysis product D_2) cannot therefore be linked to the C-1 (or C-5) hydroxyl group; such an arrangement would lead to formation of ethylene glycol in this reaction sequence.

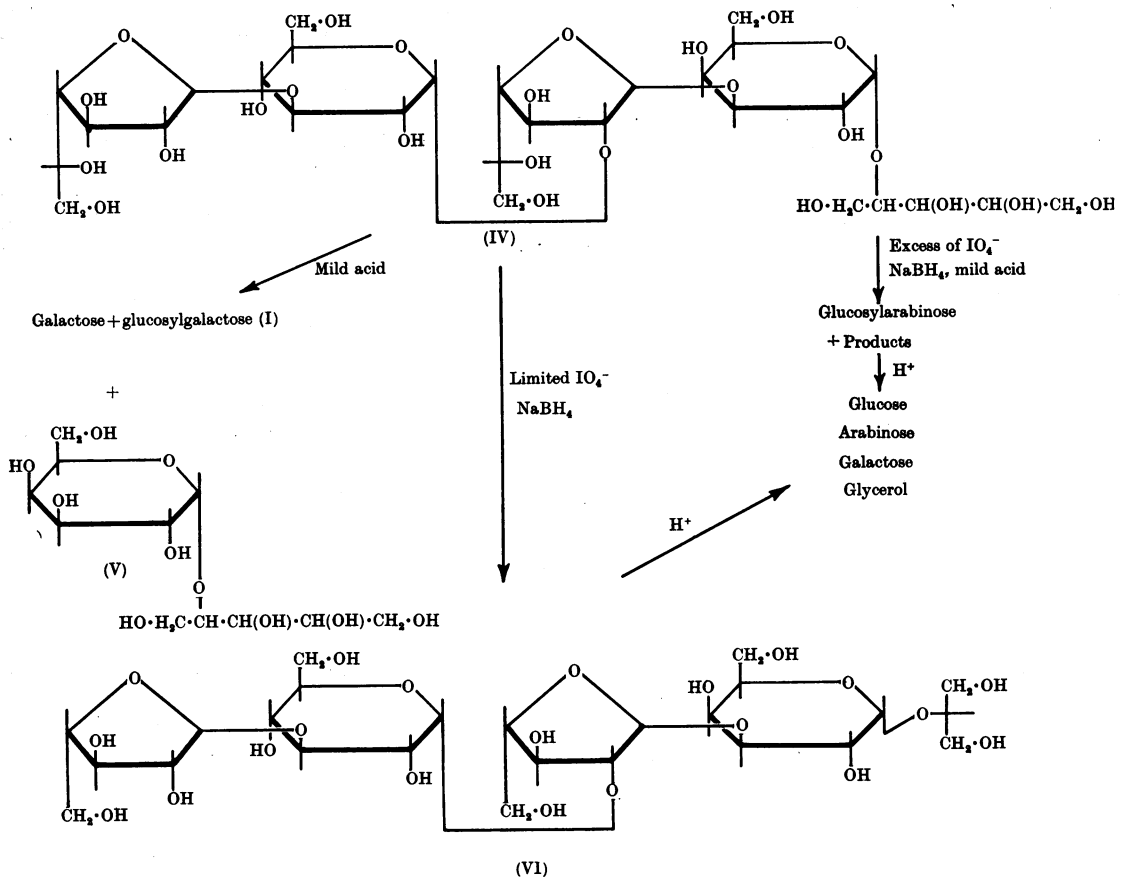

Fig. 2. Structure of the pentasaccharide and its degradation.

It follows that the sugar must be linked to the C-2 (or C-4) hydroxyl group of ribitol.

The pentasaccharide can now be represented by (IV), and the product of limited oxidation with periodate and reduction with borohydride by (VI).

The most interesting feature of this repeating unit is the presence of two D-galactofuranose residues in the molecule. The polysaccharide galactocarolose from *Penicillium charlesii* (Haworth, Raistrick & Stacey, 1937; Gorin & Spencer, 1959), a polysaccharide from *Gibberella fujikuroi* (Siddiqui & Adams, 1961) and a galactomannan from *Trychophyton granulosum* (Bishop, Blank & Hranisavljevic-Jakovljevic, 1962) have all been shown to contain galactofuranose units. This is the first time such units have been reported as part of a pneumococcal antigen and nothing is yet known of their influence on the immunological specificity. E. V. Rao, J. G. Buchanan & J. Baddiley (unpublished work) have found that D-galactofuranose is also part of the repeating unit in S. 10A.

SUMMARY

1. A pentasaccharide repeating unit has been isolated from the specific substance from *Pneumococcus* type 34(41) by alkali hydrolysis followed by enzymic dephosphorylation.

2. The pentasaccharide has been shown to be *O*-D-galactofuranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→2)-*O*-D-galactofuranosyl-(1→3)-*O*-α-D-galactopyranosyl-(1→2)-ribitol.

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The Colorimetric Micro-Determination of Long-Chain Fatty Acids

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The investigation reported here arose from the need for a simple micro-method for the determination of long-chain fatty acids eluted from paper chromatograms. When the acids were labelled with ^{14}C or ^{3}H, measurement of the radioactivity of a portion of each eluate would then permit the specific activities of the individual fatty acids to be calculated.

Titration of weak acids at the required level (a few millilitres of a 10 μM solution) is not simple, and interference might be caused by traces of acetic acid from the chromatographic solvent. A colorimetric method for long-chain fatty acids was devised by Ayers (1956), who measured the extinction of a chloroform solution of their copper soaps, prepared by shaking a solution of the potassium soaps with copper nitrate and chloroform. The useful range was 8–40 mM. In a modification of the method, Iwayama (1959) used a copper nitrate–triethanolamine reagent shaken with a chloroform solution of the free fatty acids, and claimed greater sensitivity. This was still not high enough for the required purpose, however, and some means of increasing it was sought. It seemed possible that this might be achieved by adding to Iwayama's copper-containing chloroform solution one of the reagents used for the colorimetric micro-determination of copper. Sodium diethyldithiocarbamate was chosen for investigation, since it is inexpensive and fairly sensitive, the reaction conditions are not critical and the coloured complex formed with copper is soluble in chloro-