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

THE PHOSPHODIESTER LINKAGES

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1. The phosphate groups in the type-specific substance S. 34 from *Pneumococcus* type 34 (U.S. type 41) were shown to join the hydroxyl group at position 1 or 5 of ribitol and the hydroxyl group at position 3 of a D-galactofuranosyl residue in the next repeating unit. 2. A partial structure of the type-specific substance was derived. 3. New syntheses of D-galactose 2-phosphate and D-galactose 3-phosphate are described.

In previous papers (Roberts, Buchanan & Baddiley, 1963; Dixon, Buchanan & Baddiley, 1966) the structure of the dephosphorylated repeating unit of the type-specific substance from *Pneumococcus* type 34 (S. 34) was shown to be *O*- β -D-galactofuranosyl-(1 $\rightarrow$ 3)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-galactofuranosyl-(1 $\rightarrow$ 3)-*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-ribitol (I). [We have used Danish nomenclature in our papers on capsular substances from pneumococci. Type 34 (Danish) is the same as type 41 (U.S.). The problem of the nomenclature of S. 34 has been discussed by Roberts *et al.* (1963).] Experiments quoted briefly by Rao, Roberts, Buchanan & Baddiley (1964) had indicated that the phosphodiester linkages between each repeating unit were probably situated on the C-5 hydroxyl group of ribitol and the hydroxyl group on either C-2 or C-3 of a galactofuranosyl moiety in the next repeating unit.

We now describe experiments that have located these linkages more precisely. The location of *O*-acetyl groups in S. 34 has been discussed in separate publications (Dixon, Roberts, Mills, Buchanan & Baddiley, 1968; Roy & Glaudemans, 1968).

EXPERIMENTAL

Materials

The S. 34 preparation was that described by Brown & Robinson (1943) as the soluble specific substance of type 34 (Buckley strain), preparation no. 1, and was given to us by Dr Rachel Brown. It contained approx. 20% of C substance (Dixon *et al.* 1966) and was used without further purification. Intestinal phosphomonoesterase was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Paper chromatography

This was carried out on Whatman paper no. 1, 2 or 4. The following solvent systems were used: *A*, propan-1-ol-aq. NH₃ (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. NH₃ (sp.gr. 0.88) (40:10:49:1, by vol.) (organic phase) (Hirst, Hough & Jones, 1949); *D*, ethanol-m-ammonium acetate (pH 3.8) (5:2, v/v) (Paladini & Leloir, 1952). 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 AgNO₃ reagent for sugars and polyols (Trevelyan, Procter & Harrison, 1950); aniline phthalate reagent for reducing sugars (Partridge, 1949).

Analytical methods

Total phosphorus was determined by the method of Chen, Toribara & Warner (1956). Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954; Aspinall & Ferrier, 1957) and formaldehyde as described by Roberts *et al.* (1963). Hexoses were determined by the phenol-H₂SO₄ method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

Phosphate-containing hydrolysis products

Acid hydrolysis of the specific substance. S. 34 (18 mg.) was hydrolysed in 0.1N-HCl (1.2 ml.) at 100° for 3 hr. The hydrolysate was evaporated to dryness *in vacuo*, dissolved in a little water and chromatographed as a band on Whatman no. 3 paper in solvent *A*. The molybdate reagent showed a number of compounds in the sugar phosphate region of the chromatogram, the faster-running compounds giving a yellow or purple colour rapidly with periodate and Schiff reagent, and the slower compound giving a brown colour with aniline phthalate. The slower compound was eluted with water, and the solution evaporated to 0.4 ml. *in vacuo* and mixed with a solution (0.2 ml.) of alkaline phosphatase [enzyme (1 mg.), (NH₄)₂CO₃ (7 mg.) in water (1 ml.)]. The mixture was kept at 37° for 18 hr., passed through a column of Dowex 50 (H⁺ form) resin (1 ml.), evaporated to dryness *in vacuo* and chromatographed in solvent *B*. Detection of products with AgNO₃ showed only galactose. The faster-running phosphates were eluted and treated with phosphatase in a similar manner to give ribitol as the only product.

Acid hydrolysis of S. 34 degraded by periodate, followed by borohydride. S. 34 (12 mg.) was dissolved in 0.05 M-NaIO₄ (2 ml.) and kept in the dark for 24 hr. Next NaBH₄ (20 mg.) was added, the solution was left at 20° for 16 hr. and a few drops of acetic acid were then added to decompose the excess of borohydride. The solution was passed through a column of Dowex 50 (H⁺ form) resin (3 ml.), the eluate evaporated to dryness and the residue re-evaporated several times with portions of methanol to remove boric acid. The product was hydrolysed with 0.1 N-HCl (0.8 ml.) at 100° for 3 hr. and the products were chromatographed in solvent A. The material reacting with the molybdate reagent, and which also gave a pink colour with aniline phthalate, was eluted with water, dephosphorylated as described above and examined by paper chromatography in solvent B. Arabinose was detected with AgNO₃ and aniline phthalate, but no ribitol or galactose was present.

Pentasaccharide monophosphates

Isolation of the pentasaccharide phosphates. S. 34 (30 mg.) was hydrolysed in a sealed tube with N-NaOH (20 ml.) at 100° for 3 hr. The hydrolysate was passed through a column of Dowex 50 (NH₄⁺ form) resin (10 ml.), evaporated to dryness *in vacuo* and chromatographed as a band on Whatman no. 3 papers in solvent A. The major product, reacting with the molybdate reagent, was eluted from the paper. Analysis on this material (Roberts *et al.* 1963) gave phosphate:hexose:formaldehyde (produced by periodate oxidation) molar proportions 1.0:3.9:1.9. A quantitative periodate determination, carried out with 0.01 M-NaIO₄ at 20°, showed uptake to be complete after 80 hr., with a reduction of 6.5 mol.prop.

Acid hydrolysis of the pentasaccharide phosphates. A sample was hydrolysed with 0.1 N-HCl at 100° for 3 hr. The products were chromatographed as a band in solvent A and the phosphates eluted with water. After dephosphorylation with intestinal phosphomonoesterase the mixture was chromatographed in solvent A (sprayed with periodate and Schiff reagent) and solvent B (alkaline AgNO₃). Ribitol was the sole product detected by these reagents.

Degradation of the pentasaccharide monophosphates. A sample (6 mg.) was dissolved in 0.1 M-NaIO₄ (1 ml.) and the solution kept at 20° in the dark for 24 hr. The reaction products were then reduced with borohydride, and the borate was removed in the usual manner. The residue was dissolved in 2 N-HCl (0.4 ml.) and hydrolysed at 100° for 3 hr. The hydrolysate was evaporated to dryness *in vacuo* and chromatographed in solvent A, and the material reacting with molybdate reagent was eluted from the paper. The eluate was dephosphorylated with intestinal phosphatase as above, and the product chromatographed in solvent C. Detection of the products with periodate and Schiff reagent showed the presence of ribitol, anhydribose and ethylene glycol.

Synthesis of D-galactose 2-phosphate and of D-galactose 3-phosphate

D-Galactose 2-phosphate. 1,3,4,6-Tetra-O-acetyl- α -D-galactopyranose (Helferich & Zirner, 1962) (4.0 g.) in dry pyridine (15 ml.) was treated with redistilled diphenyl phosphorochloridate (4.0 g.) at room temperature for 4 days. The mixture was cooled to 0° and ice-cold water (3.0 ml.)

added. The sticky product was dissolved by the addition of benzene (150 ml.) and successively extracted with water (30 ml.), N-H₂SO₄ (80 ml.), saturated NaHCO₃ solution (100 ml.) and finally water (100 ml.). Evaporation of the dried (Na₂SO₄) solution, *in vacuo* at 40°, gave a syrup, which crystallized from aqueous ethanol. Recrystallization from the same solvent gave *diphenyl 1,3,4,6-tetra-O-acetyl- α -D-galactopyranos-2-yl phosphate* (XII) (5.15 g., 77%), m.p. 73–75°, [α]_D²⁵ + 79.4° (c 0.63 in CHCl₃) (Found: C, 54.3; H, 5.1; P, 5.7. C₂₆H₂₈O₁₃P requires C, 53.8; H, 5.0; P, 5.3%).

The ester (XII) (3.5 g.) and Adams catalyst (0.3 g.) were suspended in dry methanol and shaken in the presence of hydrogen at room temperature and pressure. The calculated 8 mol.prop. of hydrogen (approx. 950 ml.) was taken up in 2 hr. 50 min. The catalyst was removed by filtration and the filtrate evaporated, *in vacuo*, to give a clear glass (2.35 g., 91%), which did not crystallize.

The glass (2.0 g.) was dissolved in ethanol (35 ml.) and treated with 0.2 N-potassium ethoxide solution until neutral (phenolphthalein; approx. 15 ml.) and then 1 ml. extra added. The mixture was kept at 0° for 4 hr., and the precipitated white solid collected by centrifugation, washed twice with ethanol and finally with dry ether. The product (1.07 g., 68%), presumably the dipotassium salt, was extremely hygroscopic. The barium salt was prepared by treating the potassium salt (1.0 g.) in water (7 ml.) with barium acetate (0.76 g.). A slight turbidity was removed by centrifugation, 5–6 vol. of ethanol added and the flocculent white precipitate collected by centrifugation. The product was washed several times with ethanol, once with dry ether and air-dried to give *D-galactose 2-phosphate barium salt dihydrate* (0.415 g., 32.5%), [α]_D²⁵ + 18.5° (3 min.) $\rightarrow$ + 27.7° (20 min., const.) (c 0.86 in water) (Found: C, 17.1; H, 3.3; P, 7.75. C₆H₁₁BAO₅P₂H₂O requires C, 17.4; H, 3.2; P, 7.5%). Paper chromatography of the product as the ammonium salt, prepared by double decomposition of the barium salt with (NH₄)₂SO₄, in solvent D gave a sandy-yellow spot with aniline phthalate, *R*_{Gal} 0.49; with periodate and Schiff reagent a yellow-green colour developed in about 7 min. (Hardy & Buchanan, 1963). In solvent A the phosphate had *R*_{Gal} 0.35.

D-Galactose 3-phosphate. 1,2,4,6-Tetra-O-acetyl-D-galactopyranose, prepared by the method of Flowers (1966) from 4,6-O-ethylidene-1,2-O-isopropylidene- α -D-galactopyranose (Ball, 1966) (3.0 g.), was treated with diphenyl phosphorochloridate in dry pyridine as described above to give *diphenyl 1,2,4,6-tetra-O-acetyl-D-galactopyranos-3-yl phosphate* (XIII) (3.85 g., 77%), [α]_D²⁵ + 21° (c 0.84 in CHCl₃). Hydrogenation in the same manner as described above (uptake complete in 3½ hr.) gave the product as a clear glass (1.98 g., 89%). The phosphate was dissolved in dry methanol (200 ml.) and treated with 0.5 M-barium methoxide (10 ml.) overnight at 0–5°. The pale-yellow precipitated solid was collected by centrifugation, dissolved in water (6 ml.), turbidity removed by centrifugation and the product reprecipitated by the addition of 6–7 vol. of ethanol. This was repeated a further four times, the final product washed several times with ethanol and finally ether and the purified product was air-dried to give *D-galactose 3-phosphate barium salt dihydrate* (0.32 g., 21%), [α]_D²⁵ + 43.3° (c 0.46 in water) (Found: C, 16.6; H, 3.7; P, 7.3. C₆H₁₁BAO₅P₂H₂O requires C, 17.4; H, 3.2; P, 7.5%). Paper chromatography of the product as the diammonium salt, prepared by double decomposition of the barium salt

with $(\text{NH}_4)_2\text{SO}_4$, in solvent *D*, gave a brownish-grey spot with aniline phthalate, R_{Gal} 0.52. With periodate and Schiff reagent, a green-blue colour developed in about 35–40 min. In solvent *A*, the phosphate had R_{Gal} 0.28.

Acid hydrolysis of D-galactose 2-phosphate, D-galactose 3-phosphate and S.34

D-Galactose 2-phosphate. The barium salt (15 mg.) was hydrolysed in 0.1 N-HCl (5 ml.) at 100° for 2 hr. The hydrolysate was evaporated to dryness *in vacuo* and subjected to paper chromatography in solvent *A*. Aniline phthalate showed only unchanged starting material, R_{Gal} 0.35, and galactose. The molybdate reagent showed only starting material and inorganic phosphate.

D-Galactose 3-phosphate. Hydrolysis as above and paper chromatography in solvent *A* revealed the presence of two closely running spots reacting with aniline phthalate. The slower, R_{Gal} 0.28, was brownish grey and corresponded to the original 3-phosphate. The faster, R_{Gal} 0.36, was greyish brown and is presumably galactose 4-phosphate. There was no trace of galactose in the hydrolysate.

S.34. S.34 (15 mg.) was hydrolysed in the same manner as described above for the D-galactose phosphates. Paper chromatography in solvent *A* and detection with aniline phthalate showed a similar behaviour pattern to the hydrolysate from D-galactose 3-phosphate. In the phosphate region there were two closely associated spots, the slower brownish grey, R_{Gal} 0.28, and the faster greyish brown, R_{Gal} 0.36. There was also a spot corresponding to free galactose, but none of the 2-phosphate.

DISCUSSION

In view of the homogeneity of the pentasaccharide (I) obtained by Roberts *et al.* (1963), by hydrolysis of S.34 with alkali and dephosphorylation of the product, it seemed that S.34 itself was composed of these units, joined through phosphodiester bridges.

Acid hydrolysis of S.34 yielded both galactose phosphates and ribitol phosphates, indicating that a ribitol in one unit was linked through a phospho-

diester to a galactose in the next. When S.34 was subjected to oxidation with periodate, and the product reduced with borohydride (Goldstein, Hay, Lewis & Smith, 1965), subsequent acid hydrolysis yielded arabinose phosphate in place of galactose phosphate; no ribitol phosphate could be detected. In S.34 therefore a phosphate group must be linked to a galactofuranose unit on the C-2 or C-3 hydroxyl group, and to a terminal hydroxyl group of the ribitol.

Prolonged periodate oxidation of the pentasaccharide (I) results in overoxidation of the terminal galactofuranose residue (Roberts *et al.* 1963). On the other hand, similar oxidation of deacetylated S.34 results in the reduction of only 3.18 mol.prop. of periodate for each phosphate group (Dixon *et al.* 1966), corresponding to oxidation of both galactofuranose residues between C-5 and C-6, and cleavage of the ribitol. In S.34 overoxidation of the terminal galactofuranose of the repeating unit must be prevented by a phosphate group, as in the partial structure (II). It was shown (Roberts *et al.* 1963) that hydrolysis of S.34 by alkali gave a high yield of a product behaving as a pentasaccharide phosphate. The phosphate:hexose:formaldehyde (produced by periodate oxidation) molar proportions were 1:3.9:1.9, and we have now shown that acid hydrolysis yields ribitol phosphates but no galactose phosphates. Thus, in the unidirectional hydrolysis of the phosphodiester groups under alkaline conditions, cyclization had occurred exclusively on to ribitol, leading to compounds (III) and (IV), which undergo hydrolysis of the cyclic phosphate group to yield compounds (V) and (VI). The pentasaccharide phosphate reduced 6.5 mol.prop. of periodate, owing to overoxidation of the galactofuranose end group in compounds (V) and (VI). In addition, when the pentasaccharide phosphate was oxidized with periodate, the product reduced with borohydride

and subsequently hydrolysed with acid, phosphates of ribitol (from compound VI) as well as ethylene glycol phosphate (from compound V) were detected, a clear indication of the degradative sequence (II) $\rightarrow$ (IV) $\rightarrow$ (V) + (VI).

The relative positions of sugar and phosphate substitution on the ribitol are now established. Arguments from biosynthesis that were developed for S.10A (Rao, Buchanan & Baddiley, 1966) make it likely that the sugars are linked to the C-2 hydroxyl group, and the phosphodiester to the C-5 hydroxyl group of D-ribitol. The problem of the phosphate linkage to galactose will now be considered in detail.

The exclusive cyclization of the phosphodiester group on to ribitol as represented by the sequence (II) $\rightarrow$ (III) + (IV) may be understood in terms of the geometry of the furanose ring (Rao *et al.* 1964). A cyclic phosphate involving the C-2 and C-3 hydroxyl groups of the galactofuranose would be highly strained, even in the most favourable conformation of the furanose ring (Lemieux, 1964). Hydrolysis of phosphodiester under acid conditions also proceeds, if possible, through a mechanism involving cyclic phosphate formation (Brown, Magrath, Neilson & Todd, 1956; Brown & Higson, 1957). Since S.34 yields some galactose phosphate on acid hydrolysis, cyclization of the phosphodiester

group on to galactose must be taking place at a rate comparable with that of cyclization on to ribitol. This can only occur if the glycosidic linkage of the galactofuranose unit bearing the phosphate group is first hydrolysed, allowing the formation of a galactopyranose ring, as in compound (VII). It was already known that this linkage is easily hydrolysed under mild acidic conditions (Roberts *et al.* 1963). The formation of a five-membered cyclic phosphate is now much more favourable, since the fusion is to a six-membered ring. Although a *cis* cyclic phosphate is formed more easily than a *trans* (Brown & Higson, 1957; Brown, Hall & Higson, 1958; Pizer & Ballou, 1959; Tomlinson & Ballou, 1961), compounds of the latter type have been prepared (Brown & Higson, 1957; Khorana, Tener, Wright & Moffatt, 1957; Kilgour & Ballou, 1958) and noted as probable hydrolysis intermediates. It is therefore possible to consider either of the structures represented by formula (VII) undergoing hydrolysis to a galactose phosphate. To decide between the C-2 and the C-3 hydroxyl group as the original site of the phosphate group we have treated authentic samples of D-galactose 2-phosphate (VIII) and D-galactose 3-phosphate (IX) with acid. The synthesis of these compounds is described at the end of the Discussion section.

When galactose 2-phosphate (VIII) was heated

Ac = acetyl; Ph = phenyl

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with 0.1N-hydrochloric acid for 2 hr. at 100° small amounts of free galactose and inorganic phosphate were produced, but the original ester was largely unchanged. When galactose 3-phosphate (IX) was treated in the same way, it was partially converted into another galactose phosphate, probably galactose 4-phosphate (X), and no hydrolysis to free galactose took place. In neither experiment was there any evidence of equilibration between the 2-phosphate and 3-phosphate, which would have required the intermediate formation of the less favourable (*trans*) 2,3-cyclic phosphate. The 3-phosphate can form a (*cis*) 3,4-cyclic phosphate whereas the 2-phosphate, as the α -anomer, can form the (*cis*) 1,2-cyclic phosphate, which is capable, through hydrolytic fission of the phosphorus-

oxygen bond, of yielding the acid-labile 1-phosphate (Piras, 1963; Paladini & Leloir, 1952). When S. 34 was treated with acid under the same conditions the galactose phosphates produced were indistinguishable from those arising from the 3-phosphate, there being no trace of the 2-phosphate. We conclude that in S. 34 the phosphodiester group is joined to the C-3 hydroxyl group of the terminal galactofuranose of each repeating unit, leading to the partial structure (XI) for the type-specific substance.

Although Piras (1963) has prepared D-galactose 2-phosphate by acid cleavage of α -D-galactose 1,2-phosphate, no details were given of physical constants. We have synthesized the 2-phosphate by a method analogous to that of Farrar (1949) for

the D-glucose isomer. 1,3,4,6-Tetra-O-acetyl- α -D-galactopyranose (Helferich & Zirner, 1962) yielded the crystalline ester (XII) after treatment with diphenyl phosphorochloridate in pyridine. Hydrogenolysis of the phenyl groups followed by removal of the O-acetyl groups under alkaline conditions afforded D-galactose 2-phosphate, characterized as the barium salt.

An earlier synthesis of D-galactose 3-phosphate (Foster, Overend & Stacey, 1951) is probably in error (Ball & Jones, 1958; Buchanan & Miller, 1958). Although 4,6-O-ethylidene-1,2-O-isopropylidene- α -D-galactose is now well characterized (Ball & Jones, 1958; Ball, 1966; Flowers, 1966) and has the necessary free C-3 hydroxyl group for phosphorylation, acidic conditions, which might cause phosphate migration, are necessary for the removal of the protecting groups. 1,2,4,6-Tetra-O-acetyl-D-galactopyranose (Flowers, 1966) was therefore phosphorylated with diphenyl phosphorochloridate in pyridine, to give the ester (XIII). After hydrogenolysis of the phenyl groups careful removal of the O-acetyl groups with barium methoxide in methanol yielded the 3-phosphate (IX) as the barium salt. The 3-phosphate is very alkali-labile, as expected from the behaviour of D-glucose 3-phosphate (Brown, Hayes & Todd, 1957).

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