Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

POLYSACCHARIDES OF TYPE XIX *Pneumococcus*
PART II. THE TYPE SPECIFIC POLYSACCHARIDE AND ITS CHEMICAL BEHAVIOUR*

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(Received May 26th, 1970; accepted for publication, June 9th, 1970)

ABSTRACT

Type XIX *Pneumococcus* specific polysaccharide (S-XIX) is composed of residues of L-rhamnose, D-glucose, 2-acetamido-2-deoxy-D-mannose, and phosphate in the approximate proportions of 2:1:1:1.

Acid phosphatase liberated 7.5% of the total phosphate. After hydrolysis with dilute alkali of the phosphatase-treated polysaccharide, the serological activity disappeared and two main oligosaccharides *F1* and *F2* were isolated. Both oligosaccharides had approximately the same carbohydrate composition as the original polysaccharide but only *F2* contained phosphate residues. Acid phosphatase treatment of *F2* liberated 64.1% of its phosphate residues. Further treatment of this product with acidic and basic ion-exchange resins resulted in its conversion into *F1*.

INTRODUCTION

A previous publication[1] illustrated the difficulty in obtaining a pure sample of the serologically specific substance of Type XIX *Pneumococcus*. It was shown that the crude product** could be purified by zone electrophoresis in the cold, using glass powder and 50mM acetate buffer (pH 5.5). The type-specific material thus obtained had $[\alpha]_D$ +37°, contained 4.1% of phosphorus, 1.64% of nitrogen, and possessed acid- or alkali-labile linkages together with the 2-acetamido-2-deoxy-D-mannose residue.

Preliminary analytical data of the type-specific material S-XIX showed similarity to those of types VI, VII, XVIII, XXXI, and XXXIV[2].

In this communication, aspects of the chemical behaviour of S-XIX polysaccharide, particularly the relation between phosphate content and acid or alkali treatment, and determination of the component sugars, including the recognition of acetamido groups and the absence of alditols, will be discussed.

EXPERIMENTAL

The samples of *Pneumococcus* type-specific polysaccharide were prepared by

*Part of this work was presented at the 88th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1968.

**We thank E. R. Squibb & Sons, Dr. Rachael Brown, and Professor M. Heidelberger for generous supplies of this material.

the procedure outlined in Part I[1]. Pronase (a protease from *Streptomyces griseus*) and trypsin were purchased from Kakenkagaku Co. Ltd. (Tokyo) and from Merck Co. Ltd. (U. S. A.), respectively. Wheat-germ acid phosphatase was purchased from Seikagakukogyo Co. Ltd. (Tokyo).

Phosphorus was estimated by the method of Fiske–Subbarow[3], and total sugar content by the procedure of Dubois *et al.*[4]. Paper chromatography was performed by the ascending or descending methods on Whatman No.1 paper or on Toyo Roshi No. 50 paper with the following solvent systems (v/v): (*A*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (*B*) ethyl acetate–pyridine–water (10:4:3), (*C*) ethyl acetate–pyridine–acetic acid–water (5:5:1:3), (*D*) ethyl acetate–acetic acid–water (3:1:3), (*E*) butyl alcohol–acetic acid–water (4:1:1), (*F*) butyl alcohol–ethanol–water (3:1:1), (*G*) propyl alcohol–ammonia–water (6:3:1), (*H*) phenol–water (7:3). Thin-layer chromatography (t.l.c.) was performed by the ascending method on silica gel plates (Wako gel B-5) with the solvent systems (v/v): (*I*) methanol–acetic acid–water (5:1:2), (*J*) butyl alcohol–ethanol–formic acid–water (5:5:1:1). Sugars were detected with alkaline silver nitrate[5], *p*-anisidine hydrochloride[6], ninhydrin[7], and the Elson–Morgan[8] and Molisch[9] reagents. Phosphate was detected by the Hanes–Isherwood procedure[10].

Protease digestion of S-XIX. — The S-XIX preparation (15.2 mg) was dissolved in 50mM ammonium carbonate–hydrochloric acid buffer (pH 6.7, 5 ml), and to the mixture contained in Visking cellophane tubing, pronase (5.5 mg) was added. After 68 h at 37°, the solution was dialyzed against distilled water (500 ml). This procedure was carried out five times. The aqueous solution (2.5 l) was concentrated and examined. Tests for the presence of sugars were negative. The non-dialyzable portion of the digest was concentrated to *ca.* 6 ml and freed from pronase by shaking with portions (2 ml) of chloroform–butyl alcohol (4:1) until no further gelatinous precipitate separated at the interface of the solutions After centrifugation, the aqueous solution was concentrated, and the product was precipitated by the addition of ethanol. The precipitate (12 8 mg, 80%, after drying *in vacuo*) had a sugar content of 58%. The above treatment did not affect the serological activity of the material as established by agar-gel diffusion.

Tripsin digestion. — The S-XIX preparation (35 mg) dissolved in sodium hydrogen carbonate buffer (pH 7.2, 10 ml) was treated with trypsin (20 mg) for 41 h at 37°, and the product was isolated as described above. The product did not lose its serological activity.

Electrodialysis of S-XIX. — The S-XIX preparation (13.4 mg) was electrodialysed at 150 volts and 15 mamp for 24 h. The non-dialyzable material was precipitated by ethanol, after concentration. The precipitate was washed with ether and dried, and the carbohydrate content[4] was then 57–59%. The serological activity was unaffected.

Titration curve of S-XIX. — A solution of S-XIX preparation was treated with Amberlite IR-120(H^+) resin at 3° and filtered. The filtrate was concentrated, and the acid material was precipitated with ethanol, collected by centrifugation, washed with

ether, and dried. A solution (51.3 mg) of this substance in water was titrated with 4.78mM barium hydroxide by using a pH meter to record the change in pH (see Fig. 1).

Acid hydrolysis of S-XIX. — (*a*) The S-XIX preparation (*ca.* 3 mg) was heated in 0.5M sulphuric acid (1.5 ml) in a sealed tube at 100° for 12 h. After neutralisation ($BaCO_3$) and filtration, the filtrate was concentrated and examined by paper chromatography (solvents *B* and *C*). Components corresponding to rhamnose, glucose, and 2-amino-2-deoxymannose (weak), together with unidentified materials, were detected.

(*b*) The S-XIX preparation (*ca.* 3 mg) in 4M hydrochloric acid was heated in a sealed tube, and the product was isolated after the solution had been neutralized (see *a*). Paper chromatography of the concentrate showed 2-amino-2-deoxymannose clearly, but much of the rhamnose had been destroyed. Paper electrophoresis in 26mM borate buffer for 2 h (1 mamp/cm) on Toyo Roshi No. 50 paper showed[6] glucose and 2-amino-2-deoxymannose (M_G 0.51); *cf.* 2-amino-2-deoxyglucose (M_G 0.17) and 2-amino-2-deoxygalactose (M_G 0.32).

(*c*) Crude S-XIX and purified S-XIX (2.3 and 2.4 mg, respectively) were heated in 50mM sulphuric acid (1.5 ml) at 80° for 1 h. After cooling, 0.2-ml samples were withdrawn and oxidized with 0.2M sodium metaperiodate (0.1 ml). After 20 min at ambient temperature, 10% sodium arsenite (1 ml) and 0.6% thiobarbituric acid (3 ml) were added. The mixtures were heated at 100° for 15 min and extracted with cyclohexanone. No absorbance of the solution at 549 mμ was detected, indicating the absence of sialic acid[11].

Identification of L-*rhamnose and* D-*glucose.* — Crude S-XIX (40 mg) was hydrolysed with 0.5M sulphuric acid as described above, and the resulting mixture of sugars was separated by preparative paper chromatography. The rhamnose fraction (5 mg) was extracted from the paper chromatogram and boiled under reflux with *p*-nitroaniline in methanolic solution (containing 0.01% of conc. HCl). The deep-yellow crystals which separated from the reaction mixture had $[\alpha]_D$ +300° (*c* 0.2, pyridine), and m.p. 220° undepressed on admixture with *N*-*p*-nitrophenyl-L-rhamnosylamine.

The glucose area on the chromatogram was sprayed with D-glucose oxidase (Glucostat) solution. The paper was kept in moist air in a closed chamber for 30 min and then air-dried. When it was sprayed with 0.04% Bromphenol Blue in ethanolic solution, a yellow spot appeared on the chromatogram showing that the sugar had been oxidised and was D-glucose.

Quantitative estimation of the component sugars of the S-XIX preparation. — (*a*) A solution of the S-XIX preparation (13.7 mg) in M sulphuric acid was sealed in a tube and heated in a boiling-water bath for 10 h. After neutralisation ($BaCO_3$) and filtration, the concentrated hydrolysate was subjected to paper chromatography (Toyo Roshi No. 50, 20 × 40 cm) by two developments with solvent (*B*). After air drying, the areas containing the sugars were quantitatively extracted with distilled water. The extract was filtered through sintered glass, and the amount of sugar in a 5-ml portion of the filtrate was estimated colorimetrically[4]. The molar ratio of rhamnose

and glucose was 2.69:1. The third and fourth compounds, which moved slower than glucose, were isolated in the same way. The ratio of rhamnose and glucose in these fractions was 1:5.5, as determined by the Dische method[12] (see *b*). Thus, the final ratio of rhamnose and glucose in the hydrolysate was 1.78:1.

(*b*) Analysis of S-XIX (10.0 mg) for hexose and 6-deoxyhexose by the Dische procedure[12] gave a molar ratio of rhamnose–glucose of 1.66:1.

(*c*) The S-XIX preparation (5.1 mg) was hydrolysed in 2M hydrochloric acid (2 ml) at 100° for 12 h. The amount of hexosamine in 0.1-ml samples was determined by the method of Elson–Morgan as modified by Blix[13], with 2-amino-2-deoxy-D-mannose as a standard. The content of *N*-acetylhexosamine found was 23.8%.

Acid phosphatase treatment of S-XIX preparation. — Wheat-germ acid phosphatase was purified by treating a 10% aqueous solution with ammonium sulphate to 47% saturation. The precipitate was discarded, and the supernatant was brought to 61% saturation by the addition of ammonium sulphate. The precipitate was collected and dialyzed against distilled water at 3° for 24 h. The enzyme was recovered by lyophilisation of this solution.

The S-XIX preparation (22.4 mg) was dissolved in 50mM acetate buffer (pH 5.6, 6 ml), purified phosphatase (5 mg) was added, and the solution was kept at 37°. After 3 days, a further quantity of enzyme (1 mg) was added, and the liberated phosphate was then determined[3]. The total phosphate liberated, compared with that liberated from D-glucopyranosyl phosphate, was as follows: 6.8, 85.6% (1 day); 6.1, 83.1% (3 days); 7.5, 99.4% (9 days, constant). After 11 days, the reaction mixture was dialyzed against distilled water, and the non-dialyzable residue was concentrated under reduced pressure. The residue showed the same serological activity as the original S-XIX preparation.

Effect of alkali on S-XIX preparation. — To 1 ml of acid phosphatase-treated S-XIX solution (phosphorus content, 366.8 μg/ml) was added 0.2M sodium hydroxide (1 ml), and the mixture was kept at 37°. At intervals aliquots were removed, cooled, and neutralized with 0.1M hydrochloric acid, and their serological activities were determined. Similarly, the effect of M sodium hydroxide on S-XIX was examined (Table I).

TABLE I

EFFECT OF ALKALI ON THE SEROLOGICAL ACTIVITY[a] OF S-XIX

Time (h)	*0.5*	*1*	*2*	*3*	*4*	*6*	*9*	*12*	*24*	*48*
0.1M NaOH	+	+	+	+	+	+	+	±	±	−
1M NaOH	+	±	±	−	−	−	−	−	−	−

[a]Determined by the agar-gel diffusion method: +, precipitation band is clearly visible; ±, precipitation band is faintly visible; −, precipitation band is not visible.

Degradation of acid phosphatase-treated S-XIX with alkali. — The S-XIX preparation (22.4 mg) which had been treated with acid phosphatase was dissolved in 0.2M sodium hydroxide (5 ml), and the solution was diluted with an equal volume of

water and kept at 37° for 48 h. The solution was then cooled, neutralized (dilute HCl), and made up to 15 ml.

To a portion (3 ml) of the above solution (phosphorus content, 111.6 μg/ml), acid phosphatase (0.5 mg in 0.5 ml of water) was added, and the solution was made up to 10 ml. The mixture was kept at 37° for 5 days and then a further quantity of the enzyme (0.5 mg) was added. The total phosphate liberated[3], compared with that liberated from D-glucopyranosyl phosphate, was 44.5, 74.8% (1 day); 48.6, 88.9% (2 days); 53.8, 92.5% (4 days); 68.2, 96.3% (6 days); 75.2, 96.3% (7 days); 71.2, 96.3% (9 days).

The remainder (12 ml) of the solution after alkali-treatment was dialyzed against distilled water (400 ml) for 6 h. The external solution was renewed three times. The external solution was concentrated and examined by t.l.c. with solvents (*I*) and (*J*). The faster- (*F1*) and slower-moving component (*F2*) were eluted with water from the appropriate sections of the chromatogram.

Analysis[3] of *F1* showed that this component oligosaccharide was free of phosphorus and contained D-glucose[12], L-rhamnose[12], and 2-acetamido-2-deoxy-D-mannose[13] in the proportions 1:1.99:1.1. Analysis of *F2* indicated that it contained phosphate, D-glucose, L-rhamnose, and 2-acetamido-2-deoxy-D-mannose in the proportions 1.43:1.0:2.13:1.08.

Effect of acid phosphatase on F2. — To a portion of the solution of *F2* (1 ml; total phosphorus, 61.6 μg of phosphorus/ml) was added acid phosphatase (2 mg). The solution was diluted to 10 ml with the acetate buffer and kept at 37°. The total phosphate liberated[3], compared with that liberated from D-glucopyranosyl phosphate, was 48.7, 88.1% (1 day); 64.9, 100% (2 days); 63.3, 100% (3 days).

After acid phosphatase-treatment (48 h) of the remainder of the solution of *F2*, it was passed down columns of acidic (Amberlite IR-120) and basic (Amberlite IR-45) resins, and the eluate was concentrated. T.l.c. (solvent *J*) showed the residue to be indistinguishable from *F1*.

DISCUSSION

Type XIX specific polysaccharide[1], which had $[\alpha]_D$ +37° and contained 4.1% of phosphorus and 1.64% of nitrogen, was not affected by pronase and trypsin. These enzymes and electrodialysis did not affect its serological activity, indicating it to be free of peptides. The titration curve (Fig. 1) indicates that the polymer is a disubstituted phosphate ester. However, a small amount of monosubstituted phosphate (end group?) appears to be present, since 7.5% of the total phosphate is liberated by wheat-germ phosphomonoesterase.

Hydrolysis by acid, followed by paper-chromatographic analysis of the liberated sugars, showed the presence of L-rhamnose, D-glucose, 2-amino-2-deoxy-D-mannose, and phosphate. 2-Amino-2-deoxy-D-mannose was first detected in a natural product (*Pneumococcus* strain 39458 which is related to Type XIX) by Baddiley *et al.*[14]. Quantitative determination of the sugars by paper chromatography, after acidic hydrolysis of the polysaccharide, was complicated by the acid lability of the

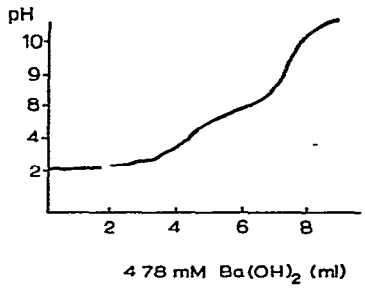

Fig. 1. Titration curve of S-XIX (*c*, 5.13%).

L-rhamnose and the difficulty in liberating the 2-amino-2-deoxy-D-mannose and D-glucose quantitatively. There is a difference in the ratio of L-rhamnose and D-glucose determined by the paper-chromatographic method (1.78:1) and that (1.66:1) using Dische's procedure[12] in which the polysaccharide itself is analysed.

Previously[1], it was shown that the polysaccharide possessed an acetamido group, indicating that 2-amino-2-deoxy-D-mannose is present as its *N*-acetyl derivative; the content[13] is 23.8%. The proportions of L-rhamnose, D-glucose, 2-acetamido-2-deoxy-D-mannose, and phosphate are approximately 2:1:1:1.

Alkali at 37°, even at low concentrations, caused extensive changes in the type S-XIX polysaccharide. In this respect, it has properties similar to those of S-VI and S-XVIII, but such alditols as ribitol and glycerol were not detected in this case. Mild, alkaline treatment caused S-XIX to become serologically inactive (Table I) and rendered it susceptible to attack by acid phosphatase. With M sodium hydroxide, the serological activity disappeared within 3 h. The two fragments which result from the mild treatment with alkali differ in that one (*F1*) contains no phosphate and is composed of L-rhamnose, D-glucose, and 2-acetamido-2-deoxy-D-mannose in the proportions 2:1:1, whereas the other (*F2*) contains the same sugars in the same ratio but also contains a 1.5 molar proportion of phosphate. Approximately two thirds of the total amount of organically bound phosphate was liberated in the phosphomonoesterase treatment.

From the above results, it is suggested that fraction *F1* is the minimum repeating unit of the specific polysaccharide and that the polymer is composed of these units united by means of phosphate ester bonds. Further, when S-XIX polysaccharide is hydrolysed with dilute acid, a small proportion of a phosphate-rich glucose molecule was obtained, indicating the presence of an acid-resistant D-glucose–phosphate linkage such as that present in D-glucose 6-phosphate. The chemical structure of these repeating units will be discussed in a later publication.

ACKNOWLEDGMENT

The authors thank Professor J. K. N. Jones for his assitance and advice.

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