Carbohydrate Research, 75 (1979) 191–198

ON THE PHOSPHATE LINKAGES AND THE STRUCTURE OF A DISACCHARIDE UNIT OF THE TYPE-SPECIFIC POLYSACCHARIDE OF PNEUMOCOCCUS TYPE XIX*

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(Received October 16th, 1978; accepted for publication, November 10th, 1978)

ABSTRACT

The structure of the capsular polysaccharide (S-XIX) of Pneumococcus Type XIX, which contains residues of D-glucose, L-rhamnose, 2-acetamido-2-deoxy-D-mannose, and phosphate, has been investigated by acid hydrolysis, treatment with acid phosphatase, mass spectrometry, and ^{13}C-n.m.r. spectroscopy. Phosphoric esters in S-XIX were largely resistant to hydrolysis (4M HCl, 100°, 3 h). With M or 2M HCl at 100° for 3 h, 4-*O*-(2-amino-2-deoxy-β-D-mannopyranosyl)-D-glucose 4′-phosphate was liberated. More-drastic hydrolysis of S-XIX gave 2-amino-2-deoxy-D-mannose 3-, 4-, and 6-phosphates, and 4-*O*-(2-amino-2-deoxy-D-mannopyranosyl)-D-glucose and its 4′-phosphate.

INTRODUCTION

The antigenic, capsular polysaccharide (S-XIX) of Pneumococcus Type XIX is composed of a repeating unit containing residues of D-glucose, L-rhamnose, 2-acetamido-2-deoxy-D-mannose, and phosphoric acid. The partial structure of S-XIX has been reported[1–4]. When S-XIX was treated with mild acid or alkali and then with monophosphatase, it gave an oligosaccharide that was a minimum repeating-unit[4]. However, even drastic conditions of hydrolysis did not completely degrade S-XIX into monosaccharides and phosphoric acid, and sugar phosphates survived. Thus, S-XIX contains acid-labile and acid-resistant moieties, and the position of the phosphate linkages remains to be elucidated.

The chemical properties of sugar phosphates have been documented[5,6]. For teichoic acids, phosphate migration sometimes occurs under acidic conditions[5], and for the meningococcal polysaccharide, 2-acetamido-2-deoxy-D-mannose 6-phosphate is resistant to acid hydrolysis[6]. Thus, determination of the structure of a sugar phosphate may be difficult by chemical means, but mass spectrometry[7] and ^{13}C-n.m.r. spectroscopy[8] can be useful in this connection.

*Polysaccharides of Type XIX Pneumococcus: Part IV. For Part III, see Ref. 4.

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We now report on the preparation of sugar phosphates by acid hydrolysis of S-XIX.

EXPERIMENTAL

The pneumococcal type XIX capsular polysaccharide was generously provided by (the late) Professor J. K. N. Jones and was purified by the method previously described[2].

Quantifications of hexose[9], phosphate[10], and nitrogen[11] were performed by literature procedures. Ascending p.c. and t.l.c. were performed on Toyo Roshi No. 50 filter paper and Merck 5577 cellulose sheet, respectively, at room temperature with *A*, ethyl acetate–pyridine–acetic acid–water (5:5:2:4); *B*, ethyl acetate–pyridine–acetic acid–water (10:10:1:6); *C*, ethyl acetate–pyridine–acetic acid–water (5:5:1:3); and *D*, *tert*-pentyl alcohol–water–toluene-*p*-sulphonic acid (30:15:1). Sugars were detected with alkaline silver nitrate[12] or ninhydrin[13], and sugar phosphates with the Hanes–Isherwood reagent[14]. Radioactive fractions were counted in 5 ml of tT76 emulsion[15] by using a Packard scintillation counter (Model 3330). G.l.c. was performed at 180° with a Shimadzu GC-6A instrument, equipped with a glass column (0.3 × 200 cm) packed with 1% of OV-17 on Gas Chrom Q. Trimethylsilylation of sugar phosphates was performed by the method of Harvey[7]. 2-Amino-2-deoxy-D-mannose 6-phosphate was prepared[16] from 2-amino-2-deoxy-D-mannose. Mass spectrometry (70 eV) was performed on a Hitachi Double Focussing Mass Spectrometer RMU-7L. ^{13}C-N.m.r. spectra were recorded at room temperature on a JEOL-FX 100 spectrometer at 25.0 MHz, in the pulsed Fourier-transform mode with complete proton decoupling. Chemical shifts are reported as p.p.m. downfield from the signal for internal MeOH.

Acid hydrolysis of S-XIX. — Samples (0.5 mg) of S-XIX were hydrolysed with M, 2M, or 4M HCl (1 ml) at 100° for 3 h. The acid was removed *in vacuo* at <40°. The hydrolysate was made up to 1 ml with water, and the phosphoric acid in 50 μl was determined[10].

Reduction with [^3H]-NaBH$_4$. — Each hydrolysate was concentrated to dryness, made up to 50 μl, and reduced by the same volume of 0.2M [^{3}H]-NaBH$_4$ (67.1 mCi/mmol) containing 0.1M NaOH at 4° for 24 h. The excess of NaBH$_4$ was decomposed with M HCl.

Fractionation of the acid hydrolysate of S-XIX. — Each reduced hydrolysate was applied to a column (2 × 22 cm) of Dowex-50 X2 (H^+) resin (200–400 mesh), which was eluted with water (200 ml) and then with 0.5M HCl. Portions (25 μl) of each fraction were counted with 5 ml of tT76 emulsion[15].

The non-reduced, acid hydrolysate of S-XIX (120 mg) was applied to a column (2.5 × 30 cm) of Dowex-50 (H^+) resin (200–400 mesh), which was eluted with water (600 ml) and then with 0.5M HCl. Aliquots (6 ml) from the fractions were assayed by the anthrone–H_2SO_4 method[17] and the Elson–Morgan method[18].

Acid-phosphatase treatment of the hydrolysates. — Phosphate-containing frag-

ments were treated[19] with wheat-germ acid phosphatase. Portions (10 μl) of each fraction (~10,000 c.p.m.) were mixed with 0.1M acetate buffer (pH 5.7, 75 μl), 0.2M magnesium chloride (10 μl), and 2 units of wheat-germ acid phosphatase (70 μl). After incubation overnight at 37°, more enzyme solution (2 units) was added. After 8-h incubation, the mixture was dialysed against distilled water (300 ml) for 12 h, and the dialysable fraction was concentrated and subjected to p.c. (solvent *B*).

RESULTS

Acid hydrolysates of S-XIX. — On acid hydrolysis of S-XIX (4M HCl, 100°, 3 h), only 11% of the total phosphate was liberated as phosphoric acid. P.c. (solvent *A*) of the hydrolysate revealed rhamnose, glucose, 2-amino-2-deoxymannose, and four unidentified saccharides (R_F 0.14–0.27) that reacted positively to ninhydrin[13] and the Hanes–Isherwood reagent[14] (except the saccharide of highest R_F).

The fractionation pattern of the [^{3}H]-$NaBH_4$-reduced, acid hydrolysate on Dowex-50(H^+) resin is shown in Fig. 1c. Radioactive peaks eluted with water were designated as *A*–F_{OT}, respectively, and 2 peaks eluted with 0.5M HCl were designated as G_{OT} and H_{OT}. Peak A_{OT} was [^{3}H]-L-rhamnitol and [^{3}H]-D-glucitol, peak H_{OT} was [^{3}H]-2-amino-2-deoxy-D-mannitol, and peak C_{OT} showed $R_{Glucitol}$ 0.18 (p.c., solvent *B*).

The elution patterns of the reduced, milder acid hydrolysates (M and 2M HCl, 100°, 3 h) on Dowex-50(H^+) resin are shown in Fig. 1a,b.

The reduced and non-reduced, acid hydrolysates gave similar elution patterns. The peaks eluted with water were designated *A*–*F*, and those with 0.5M HCl, *G* and *H*.

Characterisation of peak B. — When peak B_{OT} ($R_{Glucitol}$ 0.24) was treated with acid phosphatase, the radioactivity was detected only in the area corresponding to 2-amino-2-deoxymannitol. Thus, *B* was a 2-amino-2-deoxy-D-mannose phosphate.

Characterisation of peaks D–F. — When peak *D* was hydrolysed with 4M HCl at 100° for 8 h, D-glucose, 2-amino-2-deoxy-D-mannose, and phosphoric acid were detected (p.c., solvent *C*). After treatment of peak D_{OT} ($R_{Glucitol}$ 0.1) with acid phosphatase, radioactivity was detected only in the area corresponding to peak G_{OT}. Peak *F* contained[10,11] equimolar amounts of P and N (0.98:1.0), and showed R_F and *T* values similar to those of 2-amino-2-deoxy-D-mannose 6-phosphate[19].

The ^{13}C-n.m.r. spectrum of *D* is shown in Fig. 2, and data for peaks *D*–*F* and authentic sugars are shown in Table I. The downfield shifts of resonances due to carbons attached to the phosphate groups and/or another sugar ring, and also 2- or 3-bond coupling between ^{31}P and ^{13}C, can be used to determine the position of the linkage[20].

In the spectrum of *D*, three ^{31}P, ^{13}C-couplings (70.2, 71.2, and 76.3 p.p.m.) were characteristic. In the spectra of 2-amino-2-deoxy-D-mannose 6-phosphate and 2-amino-2-deoxy-D-mannose, 2-bond coupling of C-6 and 3-bond coupling of α- and β-C-5 were characteristic. For *F* and 2-amino-2-deoxy-D-mannose 6-phosphate, quite similar chemical shifts and ^{31}P, ^{13}C-couplings were observed (Table II)[20]. Similarly,

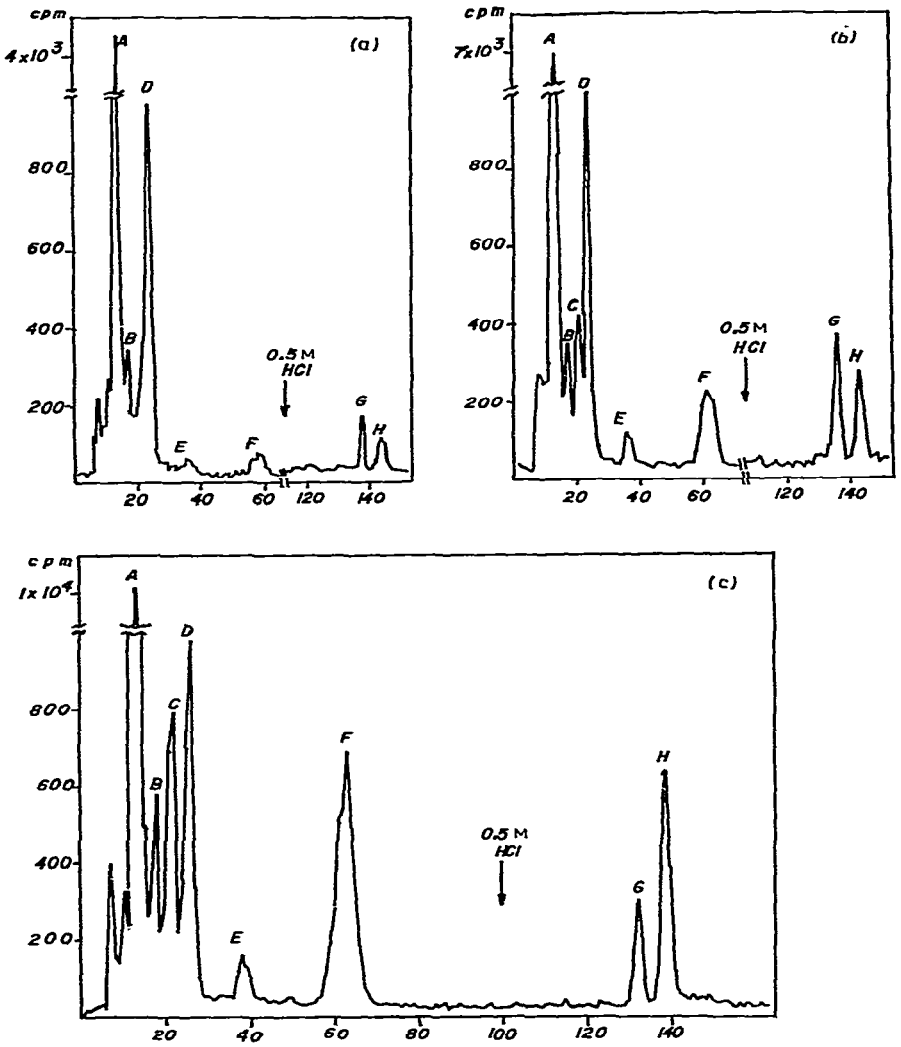

Fig. 1. Elution profile of the reduced, acid hydrolysates of S-XIX on a column of Dowex-50(H^+) resin eluted with water (200 ml) and then 0.5M HCl: (*a*) hydrolysate obtained with M acid, (*b*) 2M acid, and (*c*) 4M acid.

the spectrum of *E* indicated six couplings to ^{31}P for the C-3,4,6-α- and -β-resonances (Table II). These couplings were consistent only with a linkage through position 4, and the chemical shifts were similar to the theoretical values estimated from 2-amino-2-deoxy-D-glucose 4-phosphate[20]. For *D*, three ^{31}P,^{13}C-couplings occurred for resonances of C-3,4,5 of 2-amino-2-deoxy-D-mannose. The spectra of *D* and *E* were similar and consistent with the β configuration, except for the shifts of the C-1 and C-2 resonances. Therefore, the 2-amino-2-deoxy-D-mannose residue was β-linked, and the downfield shift of the D-glucose C-4 signal indicates that 2-amino-2-deoxy-D-mannose was linked to C-4 of D-glucose.

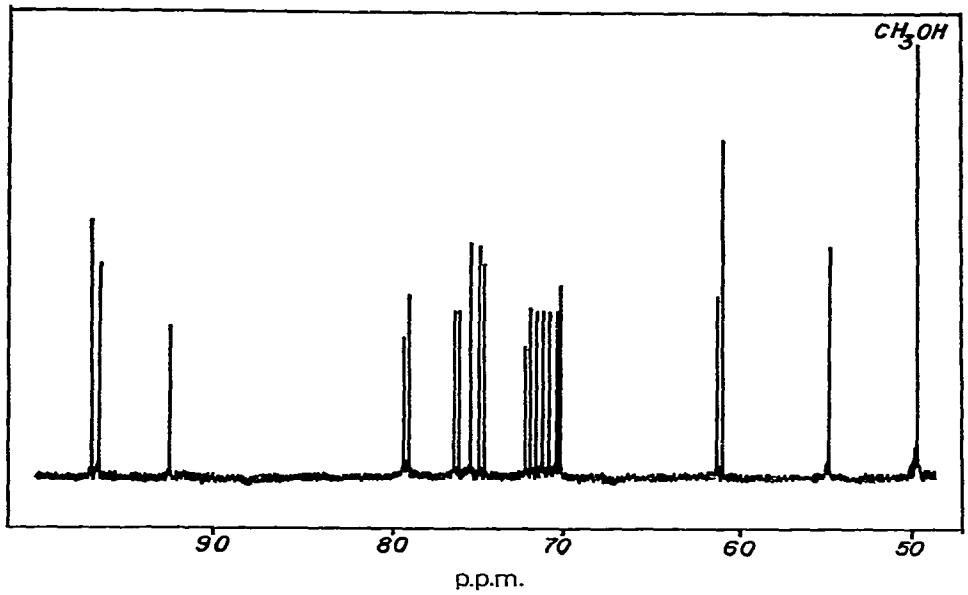

Fig. 2. ^{13}C-N.m.r. spectrum of peak *D* with spectral windows of 2 kHz (65,000 accumulations).

TABLE I

^{13}C CHEMICAL SHIFTS (P.P.M. FROM INTERNAL MeOH) OF THE HYDROLYSATE AND AUTHENTIC SUGARS

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
α-D-ManN HCl	91.1	55.3	67.7	67.1	72.8	61.2
β-D-ManN HCl	91.8	56.4	70.3	67.0	76.9	61.2
α-D-ManN 6-phosphate	91.3	55.2	67.6	66.6	71.7	64.6
β-D-ManN 6-phosphate	92.0	56.4	70.0	66.5	75.7	64.6
α-D-Glc	92.9	72.3	73.6	70.5	72.3	61.6
β-D-Glc	96.7	75.0	76.7	70.5	76.8	61.7
Peak *D*	97.2	55.0	70.2	71.2	76.3	61.1
	92.7	72.0	71.9	79.0	70.8	61.1
	96.7	74.7	74.9	79.1	75.4	61.2
Peak *E*	90.8	54.7	67.8	71.6	72.4	61.0
	91.9	55.9	70.3	71.4	76.1	61.2
Peak *F*	92.0	55.2	67.6	66.6	71.7	64.5
	91.3	56.4	70.1	66.4	75.7	64.5

Characterisation of peak G. — Characterisation of *G* was effected mainly by mass spectrometry. Hydrolysis of *G* with 4M HCl at 100° for 8 h gave D-glucose and 2-amino-2-deoxy-D-mannose in the molar ratio 1.0:1.03. Similarly, acid hydrolysis of peak G_{OT} ($R_{Glucitol}$ 0.4) gave glucitol$_{OT}$ and 2-amino-2-deoxy-D-mannose. The mass spectrum (Fig. 3) of *N*-acetylated[21] and trimethylsilylated[22] *G* contained peaks at *m/e* 204, 217, 330, 420, 451, 492, 508, 521, 637, 638, and 872, which were characteristic of a (1→4)-linked, trimethylsilylated 4-*O*-(2-acetamido-2-deoxyaldohexosyl)aldohexose[23].

TABLE II

2- AND 3-BOND CARBON–PHOSPHORUS COUPLINGS (Hz) FOR THE PHOSPHORYLATED SUGARS

Compound	*C-3*	*C-4*	*C-5*	*C-6*
α-D-ManN 6-phosphate			6.8	4.9
β-D-ManN 6-phosphate			7.8	4.9
Peak *D*	1.4	5.4	6.8	
Peak *E*	2.2	5.2	7.3	
	1.5	3.8	7.3	
Peak *F*			8.1	5.1
			8.1	5.1

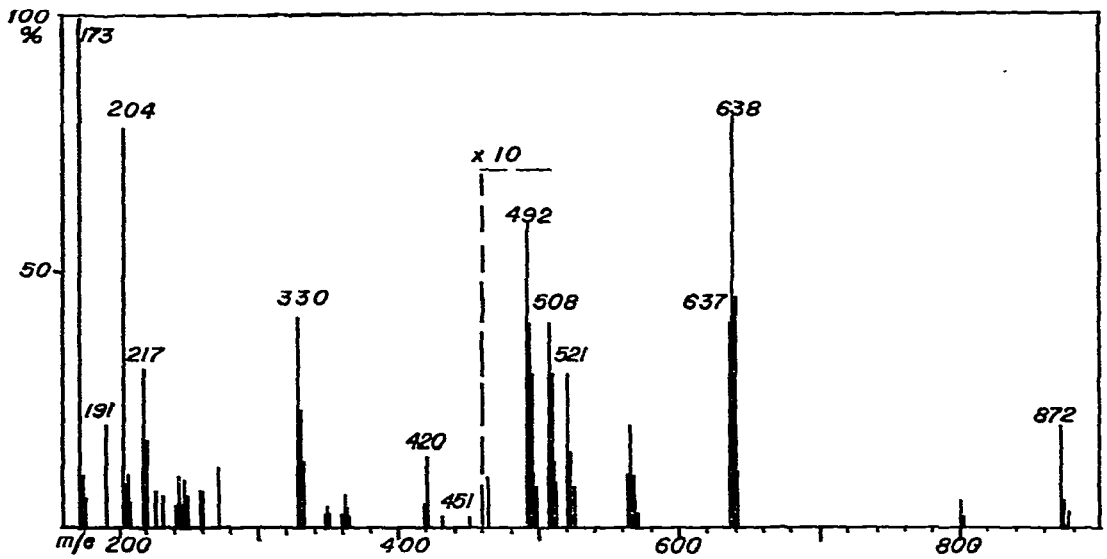

Fig. 3. Mass spectrum (peaks having *m/e* > 173) of *N*-acetylated and pertrimethylsilylated peak *G*.

DISCUSSION

Structural studies of pneumococcal capsular polysaccharides are important for a better understanding of pneumococcal pneumonia and of pneumococcal polysaccharide vaccines[24]. The structures of several pneumococcal capsular polysaccharides[25,26] have been studied and some of these polysaccharides contain sugar phosphates.

Previously, we reported that S-XIX contained acid- and alkali-labile linkages[1–4] attributable to phosphate groups. When S-XIX was treated with wheat-germ acid phosphatase, 7.5% of the total phosphate was liberated as phosphoric acid[3], suggesting that the main phosphate linkage is not monoester. Only 11% of the total phosphate was liberated as phosphoric acid by hydrolysis using 4M HCl (100°, 3 h). In order to identify these phosphate linkages, analysis of the sugar phosphate moiety and the determination of the structure of a disaccharide unit in S-XIX were carried out.

It was proposed[4], from the results of methylation and periodate-oxidation analysis, that the repeating unit involved D-ManNAc-(1→4)-D-Glc or D-Glc-(1→6)-D-ManNAc. The mass spectrum (Fig. 3) of *N*-acetylated and trimethylsilylated *G*, which is a non-phosphorylated oligosaccharide, showed a fragmentation pattern similar to that of a 4-*O*-(2-acetamido-2-deoxyaldohexosyl)aldohexose[23]. The ratio of the intensities of the peaks at *m/e* 217 and 204 was <1, there was no peak at *m/e* 552, and the ratio of the intensities of peaks at *m/e* 638 and 637 was >1. These results are characteristic[23] of the (1→4)-linkage. On the other hand, hydrolysis of G_{OT} gave glucitol$_{OT}$, but not 2-amino-2-deoxymannitol$_{OT}$. Therefore, *G* is 4-*O*-(2-amino-2-deoxy-D-mannopyranosyl)-D-glucose, and this is the disaccharide unit of S-XIX. Similarly, from the results of ^{13}C-n.m.r. spectroscopy and phosphatase treatment, *D* is identified as 4-*O*-(2-amino-2-deoxy-β-D-mannopyranosyl)-D-glucose 4′-phosphate.

Hydrolysis of S-XIX, using 4M HCl at 100° for 3 h, gave, *inter alia*, three 2-amino-2-deoxy-D-mannose phosphates, two of which were identified as 2-amino-2-deoxy-D-mannose 4- (*E*) and 6-phosphate (*F*) by ^{13}C-n.m.r. spectroscopy. Peak *B* was 2-amino-2-deoxy-D-mannose 3-phosphate, since it had an elution volume different from that of the other two phosphates.

2-Acetamido-2-deoxy-D-mannose 6-phosphate has been reported[27] in group A *Neisseria meningitidis* capsular polysaccharide, and 2-acetamido-2-deoxy-D-glucose 4-phosphate in group X *N. meningitidis* capsular polysaccharide[20]. 2-Acetamido-2-deoxy-D-mannose 3- and 4-phosphates were hitherto unknown.

Acid- or base-catalysed migration of phosphate, which is well known for teichoic acids[5] and *H. influenzae* capsular polysaccharides[28], could occur on acid hydrolysis of S-XIX. Phosphorylated oligomer occurred only in peak *D*, and its yield was inversely proportional to the acid concentration, but the yields of other phosphorylated fractions were proportional (Fig. 1a–c). These results suggest that drastic, acid hydrolysis of S-XIX is accompanied by phosphate migration. One location of phosphate groups in S-XIX is at position 4 of 2-acetamido-2-deoxy-D-mannose, and the acid-resistant portion of S-XIX is a (1→4)-linked 2-amino-2-deoxy-β-D-hexose unit as in chitin.

The location of the other phosphate groups in S-XIX is being investigated.

ACKNOWLEDGMENTS

The authors thank Mr. Y. Shida and Miss C. Takagai for the ^{13}C-n.m.r. spectra and the mass-spectral measurements, respectively.

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