STRUCTURAL STUDIES ON THE SPECIFIC TYPE VII* PNEUMOCOCCAL POLYSACCHARIDE†

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ABSTRACT

The specific type VII pneumococcal polysaccharide was isolated from the crude capsular material by precipitative and chromatographic methods. It contained D-galactose, D-glucose, L-rhamnose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-galactose in the molar ratio of 3.5:2.3:3.0:2.1:1.0. Some of its structural features were revealed by methylation studies, time-lapse hydrolysis, periodate oxidation, and enzymic hydrolysis. The polysaccharide is branched at residues of D-galactose and 2-acetamido-2-deoxy-D-galactose. Non-reducing end groups consisted of D-galactopyranose and 2-acetamido-2-deoxy-D-glucopyranose residues, with the former predominating. Major components of the linear chains were (1→3)-linked L-rhamnose and (1→4)-linked D-glucose; the minor ones were (1→2)-linked L-rhamnose, (1→6)-linked D-galactose, and (1→6)-linked 2-acetamido-2-deoxy-D-glucopyranose. The (1→4)-linked D-glucose components may be present as cellobiose residues. The results are in accord with structural features deduced from the serological cross-reactivity of this polysaccharide.

INTRODUCTION

The capsular polysaccharides of the pneumococci contain the type-specific immunological determinants of those microorganisms; studies of their structures and serological cross-reactions have contributed greatly to our knowledge about the chemistry of immunological specificity[1].

Because the type VII pneumococcal antiserum shows a variety of cross-reactions[1], and because this type has been one of six serotypes that cause most cases of

*Since this work was undertaken, it appears probable that the polysaccharide and antisera used in both the present and earlier studies to which reference is made were derived from pneumococcal type VII (Danish nomenclature), now type LI (F. Kauffman, E. Lund, and B. E. Eddy, *Intl. Bull. Bact. Nomencl. Taxonomy*, 10 (1960) 31, as most of the early isolates from patients were of this type. Until this matter is finally resolved (currently under study by R. Austrian, G. Schiffman, and M. Heidelberger) we retain the designation VII.
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pneumonia[2], structural studies of the type VII polysaccharide were of interest. Tyler and Heidelberger[3,4] described the characterization of the constituent sugars and predicted some structural features on the basis of quantitative precipitin cross-reactions. The present paper describes the results of methylation studies, time-lapse hydrolysis, periodate oxidation, and enzymic hydrolysis of the pneumococcal type VII polysaccharide and relates these to the predictions based upon serology.

RESULTS AND DISCUSSION

A crude preparation of the type VII pneumococcal polysaccharide was purified as follows: the bulk of the impurities were removed by precipitation with cetyltrimethylammonium bromide (Cetavlon), the specific polysaccharide was precipitated

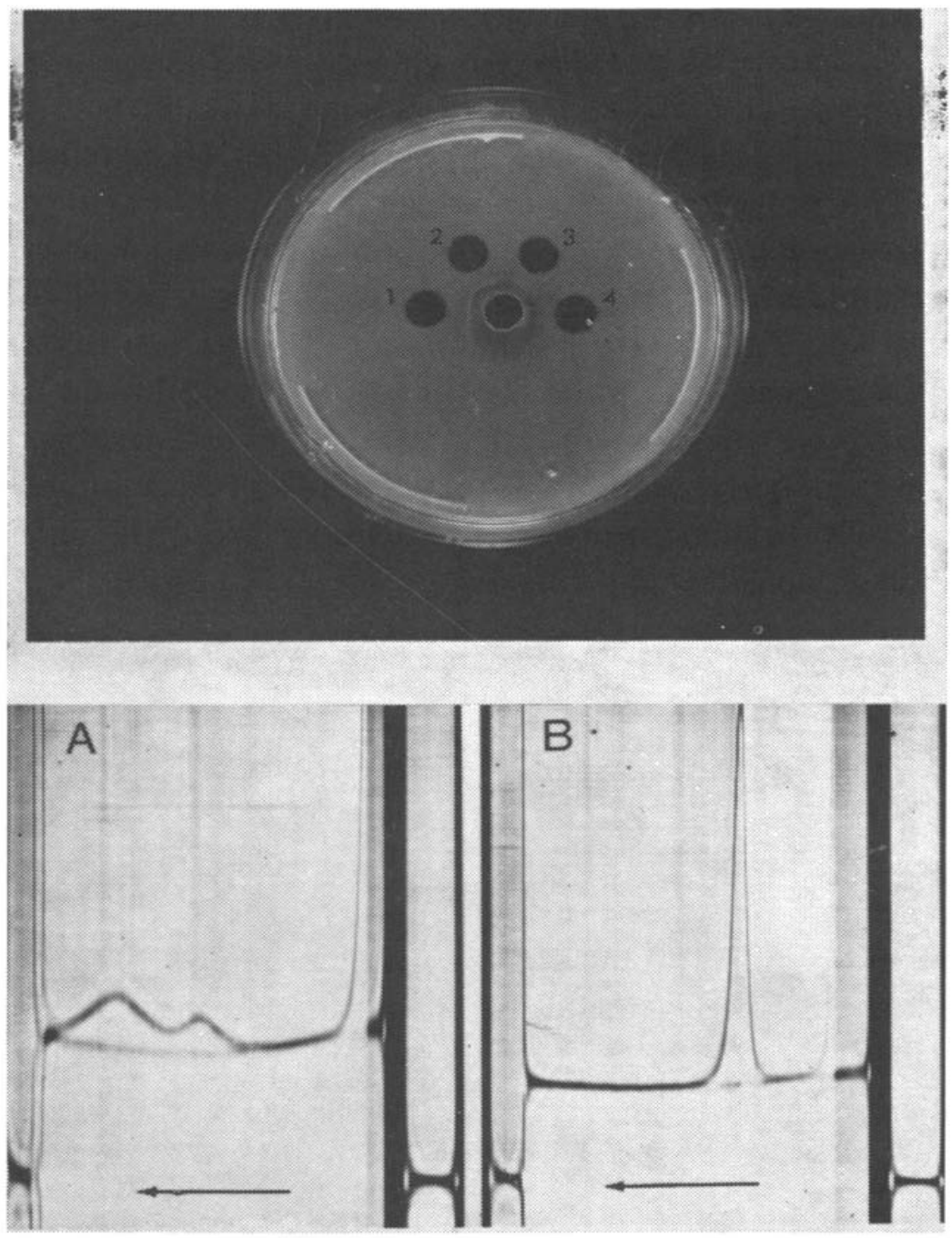

Fig. 1. Immunodiffusion: Center well: type VII antipneumococcal horse serum. 1. Purified type VII polysaccharide. 2. Crude preparation of type VII polysaccharide. 3. Fraction precipitated by Cetavlon. 4. Fraction in supernatant from ammonium sulfate precipitation. Ultracentrifugation: A. Material in supernatant from Cetavlon precipitation. B. Purified type VII polysaccharide.

by ammonium sulfate, and was then freed from smaller amounts of impurities by chromatography on diethylaminoethyl (DEAE)–Sephadex. The fractionation was monitored by immunodiffusion and fractions were examined by ultracentrifugation and electrophoresis. The final product was homogeneous by each of these criteria. Fig. 1 shows the results of immunodiffusion and ultracentrifugation of the crude material and the purified product. The purified polysaccharide, well 1 in the immunodiffusion and B in the ultracentrifugation, showed only a single component. The fractions that were relatively inactive serologically (wells 3 and 4, Fig. 1) represented 69% of the crude preparation.

Analysis of the polysaccharide is shown in Table I. The quantitative values for the constituent sugars are relative, but confidence in the results was provided by the independent checks of *N*-acetyl content and the ratio of *N*-acetylated amino sugars to rhamnose in the intact polysaccharide, as determined by integration of the methyl proton signals in its 100-MHz n.m.r. spectrum. The nitrogen value was 1.1% higher

TABLE I

ANALYSIS OF TYPE VII PNEUMOCOCCAL POLYSACCHARIDE[a]

	Native polysaccharide	*By methylation*
$[\alpha]_D^{20}$	+70°	
N	2.4	
P	0.4	
L-Rhamnose	26.0[b]	27.4
D-Galactose	32.5[b]	30.6
D-Glucose	21.5[b]	21.8
2-Acetamido-2-deoxy-D-glucose	11.0[b]	11.3
2-Acetamido-2-deoxy-D-galactose	9.0[b]	8.9
N-Acetyl	4.0[c] (corresponds to 20.5% of 2-acetamido-2-deoxyhexose)	
2-Acetamido-2-deoxyhexose:rhamnose	1:1.2[c]	
2-Acetamido-2-deoxy-D-galactose:2-acetamido-2-deoxy-D-glucose	1:1.1[d]	

[a]All quantitative data are expressed as percent by weight. [b]Determined by g.l.c. of alditol acetates[5], column *A*. [c]Determined by 100-MHz n.m.r. spectroscopy of protons; *N*-acetyl = percent of acetyl protons in total protons of polysaccharide, 2-acetamido-2-deoxyhexose:rhamnose = ratio of acetyl protons to *C*-methyl protons. [d]As determined by an amino acid autoanalyser.

than that calculated for the amino sugar content, and amino acids were detected during the ion-exchange analysis of amino sugars. It would thus appear that small amounts of protein were still present, despite the rigorous purification. However, the analysis shown accounts for at least 92–93% of the material.

Hydrolysis of the fully methylated polysaccharide yielded the methylated sugars shown in Table II. The products were first separated into neutral and amino sugars by preparative t.l.c. and the components in each of those two fractions were then analysed by the methods shown. The methyl ethers of the neutral sugars were identified by their retention times in g.l.c. on two liquid phases and by the primary fragments

TABLE II

METHYL ETHERS OF SUGARS FROM HYDROLYSIS OF METHYLATED TYPE VII PNEUMOCOCCAL POLYSACCHARIDE

Sugars	*Neutral sugars*[a]			
	T[b] *Columns*		*Primary fragments in mass spectra* m/e	*Mole %*
	B	C		
3,4-Di-*O*-methyl-L-rhamnose	0.88	0.71	131, 189	5.9
2,4-Di-*O*-methyl-L-rhamnose	1.00	0.85	117, 131, 233	21.5
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.23	1.16	45, 117, 161, 205	12.3
2,3,6-Tri-*O*-methyl-D-glucose	2.48	1.78	45, 117, 233	21.8
2,3,4-Tri-*O*-methyl-D-galactose	3.40	2.52	117, 161, 189, 233	9.4
3,4-Di-*O*-methyl-D-galactose	7.05		189	8.9
	Amino sugars[a]			
	$R^{c}_{Rhamnose}$	*T*[d](*Alditols*)		*Mole %*
		Acetates	*Me_3Si ethers*	
2-Deoxy-3,4,6-tri-*O*-methyl-2-methylamino-D-glucopyranose	1.25	1.67	2.60	4.6
2-Deoxy-3,4-di-*O*-methyl-2-methylamino-D-glucopyranose	0.86	2.52	4.00	6.7
2-Deoxy-3-*O*-methyl-2-methylamino-D-galactopyranose	0.55	3.42	6.50	8.9

[a]Separation of amino sugars from neutral sugars was obtained by preparative t.l.c. prior to analyses. [b]Retention times on g.l.c. of the alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; temperature, 180°. [c]Paper chromatography in 40:11:2:19 butyl alcohol–ethanol–pyridine–water. [d]G.l.c. on column D; 210° for alditol acetates, 160° for Me_3Si ethers. Retention times (*T*) in min under conditions described previously[6].

obtained in the mass spectra of each component[7,8]. The methyl ethers of the amino sugars were characterized by their chromatographic behaviour on paper[9,10] and by g.l.c. of two different derivatives[6]. These methods provided proof of the identities of the methylation fragments. The quantitative data were obtained by integration of the peaks obtained by g.l.c. Confidence in their reliability was provided by the good agreement with the analysis of the native polysaccharide (Table I).

The main structural features of the polysaccharide are therefore: (*a*) non-reducing end-groups of D-galactose and 2-acetamido-2-deoxy-D-glucopyranose, (*b*) a variety of components in the linear chains, the predominant ones being (1→3)-linked L-rhamnose and (1→4)-linked D-glucose, the minor ones, (1→2)-linked L-rhamnose, (1→6)-linked D-galactose, and (1→6)-linked 2-acetamido-2-deoxy-D--glucose, (*c*) branches occurring through D-galactose moieties at the C-2 and C-6 positions and through 2-acetamido-2-deoxy-D-galactose at the C-4 and C-6 positions. The results of periodate oxidation were in accord with those from methylation in that L-rhamnose and 2-acetamido-2-deoxy-D-galactose were the only sugars to survive the oxidation.

Some information on sequencing of the components in the polysaccharide was obtained by time-lapse hydrolysis and by mild hydrolysis of the reduced, periodate-oxidized polysaccharide[11]. Time-lapse hydrolysis revealed that D-galactose was the first sugar to be released, thus supporting its location at the non-reducing ends of the chains. The next sugar released was L-rhamnose, indicating that it is probably the next sugar to D-galactose. Further hydrolysis yielded D-galactose, L-rhamnose, and small proportions of D-glucose, and hexosamines. This hydrolysate also contained small amounts of oligosaccharides that were resolved into four fractions by chromatography and electrophoresis. Hydrolysis of these oligosaccharide fractions yielded: (*1*) L-rhamnose, D-glucose, and 2-acetamido-2-deoxy-D-glucose, (*2*) L-rhamnose, D-galactose, and 2-acetamido-2-deoxy-D-galactose, (*3*) D-glucose, L-rhamnose, and 2-acetamido-2-deoxy-D-galactose, and (*4*) D-glucose, L-rhamnose, D-galactose, and 2-acetamido-2-deoxy-D-galactose. The small quantities obtained precluded further investigation. However, the absence of any rhamnose or glucose disaccharides indicated that the major linear components of (1→3)-linked L-rhamnose residues and (1→4)-linked D-glucose residues were probably not present as rhamnan or glucan chains. Further evidence on the sequencing was obtained by mild acid hydrolysis of the reduced, periodate-oxidized polysaccharide, which yielded no detectable oligosaccharide fragments and from which no polymeric material could be recovered. These results indicated that the periodate-resistant components in the polysaccharide must have been separated from each other by at least one oxidizable residue.

The extent of hydrolysis of the type VII pneumococcal polysaccharide by various enzymes was monitored by identification of the sugars released and by changes in the quantitative precipitation of antibody from homologous antiserum. The results, shown in Table III, indicate that the most effective enzyme was cellulase, followed by β-galactosidase and chitinase in decreasing order of activity. Dextranase and α- and β-amylase were not active towards this polysaccharide. The cellulase must have acted on 4-*O*-substituted β-D-glucose residues and it is likely that those residues would

TABLE III

ENZYMIC HYDROLYSIS OF TYPE VII PNEUMOCOCCAL POLYSACCHARIDE

Enzyme	*Sugars released*[a]	*Titre at equivalence*[b] *μg of antibody N/ml serum*
α-Amylase	nil	530
β-Amylase	nil	530
Dextranase	nil	500
Chitinase	2-Amino-2-deoxy-D-glucose (trace)	420
β-Galactosidase	D-Galactose (trace)	350
Cellulase	D-Glucose	150

[a]Detectable by paper chromatography. [b]Amount of antobody N precipitated at equivalence by enzyme-treated Type VII polysaccharide. The native Type VII polysaccharide 530 μg of antibody N/ml of serum at equivalence.

be in the more exposed parts of the molecule. It is also possible that some of the β-D-glucose components could be present as cellobiose groups. If so, the type VII polysaccharide should give a cross-reaction with type VIII antiserum[1]. When this was investigated it was found that 50 μg of type VII polysaccharide precipitated 62 μg of antibody nitrogen from 1.0 ml of type VIII antiserum. Oat glucan precipitated 89 μg of antibody N from the same type VIII antiserum at equivalence*. The results indicated that serologically active cellobiose residues were very likely present in the type VII pneumococcal polysaccharide.

On the basis of the present information it is not possible to propose a unique structure for the type VII pneumococcal polysaccharide. The results of the structural investigation support in general the conclusions that were drawn from serological studies[4]. Thus, the presence of D-galactose non-reducing end-groups as the most important serological determinant was confirmed as was also the occurrence of branching through 2-acetamido-2-deoxy-D-galactopyranose residues. New information is that L-rhamnose is probably the sugar to which the D-galactose end-groups are linked and that at least some if not all of the D-glucose residues are present as cellobiose groups. The serological studies indicated that the D-glucose was present as (1→6)-linked units because of the cross-reaction of type VII antiserum with a dextran that was rich in those linkages[4]. However, the maximum cross-reaction of a dextran with type VII antiserum was only 5.2% of the homologous reaction. Furthermore, it may be noted that the cross-reaction between tamarind seed polysaccharide and type VII antipneumococcal sera is inhibited significantly (13–22%) and to the same extent by both cellobiose and maltose in three of the four antisera tested[4]. The tamarind seed polysaccharide has been shown to consist of a β-(1→4)-linked chain of D-glucose residues to which side chains of D-xylose, L-arabinose, and D-galactose are attached through the C-6 hydroxyl groups of some of the glucose residues. It would therefore seem that type VII antipneumococcal serum contains antibodies directed towards α- or β-(1→4)-linked D-glucose units, and that the presence of a small number of 1→4 linkages in the dextrans may account for their limited cross-reactivity. Certainly, in the present investigation, all of the D-glucose in the type VII [redacted]

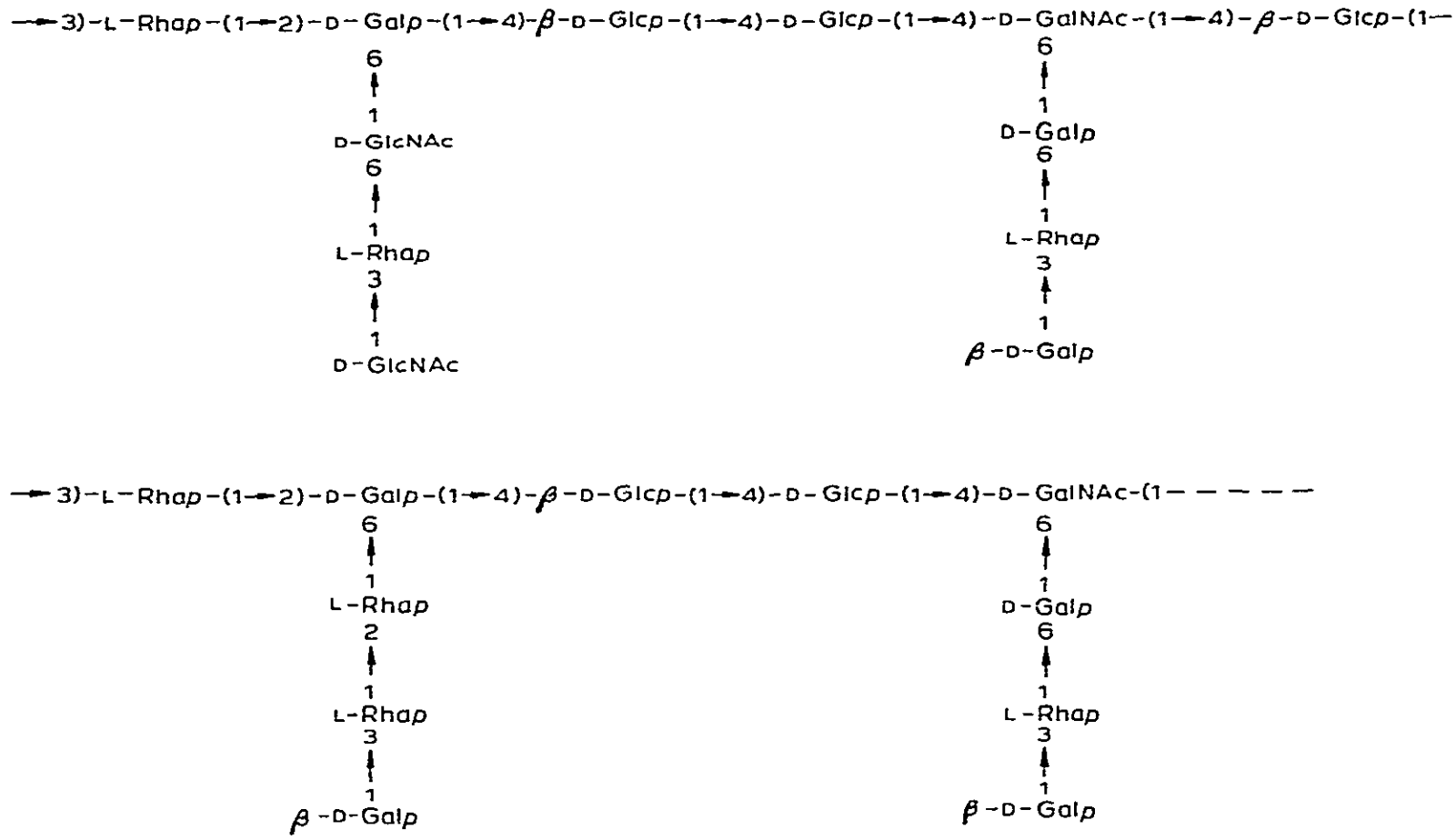

Fig. 2. A possible, tentative structure of pneumoccoccus type VII (SVII).

residues are shown as cellobiose units, in agreement with the results of serological cross-reactions and hydrolysis by cellulase. The cellobiose fragments were located in the backbone portion of the polysaccharide because D-glucose was not released until late in the time-lapse hydrolysis. The structure shown is also in accordance with the observation that those groups that are resistant to periodate oxidation must be separated from each other. Further information about this structure should be obtainable by examination of the products from hydrolysis by cellulase, by the action of D-galactose oxidase, and by serological inhibition studies with galactosyl-rhamnose oligosaccharides. The small amounts of purified polysaccharide obtained from the limited quantity of crude preparation were not sufficient for further experimentation at this stage and galactosyl-rhamnose oligosaccharides were not available for inhibition studies.

EXPERIMENTAL

General methods. — Solutions were concentrated under diminished pressure at bath temperatures below 40°. Optical rotations were measured with a Perkin–Elmer 141 polarimeter and are equilibrium values unless otherwise stated. Infrared spectra were recorded with a Perkin–Elmer Infracord spectrophotometer. N.m.r. spectra were taken on 1% solutions of type VII polysaccharide in deuterium oxide at 100 MHz by using a Varian XL-100 spectrometer with a Varian C-1024 time-averaging computer. Electrophoresis was performed on cellulose acetate strips (2.5 × 17 cm) at 300 v, 7 mA for 45 min in 0.1M aqueous sodium tetraborate. The polysaccharide was detected on the strips by alcian blue or periodate–Schiff reagent. The Ouchterlony[12]

double-diffusion method was used for qualitative immunoprecipitin tests. A Spinco Model E ultracentrifuge was used for sedimentation studies, which were conducted on 0.5% aqueous solutions of the various fractions in 0.1M sodium tetraborate. Descending paper chromatography was done with Whatman No. 1 paper using the following solvent mixtures (*v/v*) : (*A*) 2:5:5 pyridine–ethyl acetate–water, (*B*) 6:4:3 butyl alcohol–pyridine–water, (*C*) 40:11:2:19 butyl alcohol–ethanol–pyridine–water, (*D*) 2:5:7 pyridine–ethyl acetate–water. Sugars were detected by alkaline silver nitrate[13], *p*-anisidine hydrochloride[14], and 1% ninhydrin in acetone (for amino sugars)[15]. A solvent mixture of 1:1 benzene–acetone (*v/v*)[16] was used for t.l.c., which was performed on glass plates coated with Silica Gel G. G.l.c. was done with a Hewlett-Packard Model 402 gas chromatograph equipped with a hydrogen-flame ionization detector. The columns used were glass U-tubes (150 × 0.3 cm i.d.) filled with the following packings: (*A*) 1% ECNSS-M on Gas-Chrom Q (60–80 mesh), (*B*) 3% ECNSS-M on Gas-Chrom Q (100–120 mesh), (*C*) 15% polyphenyl ether (OS-138) on Chromosorb P, 80–100 mesh, (*D*) 3.8% methylvinyl silicone (UC W-98) on silanized and acid-washed Chromosorb-W, 80–100 mesh. All of these materials were commercial products obtained from Applied Science Laboratories, Inc., State College, Pa., U.S.A. or Chromatographic Specialties Ltd., Brockville, Ont., Canada. A Hewlett–Packard Model 700 gas chromatograph linked to an Atlas CH_4 Mass Spectrometer was used for identification of the methylated alditol acetates. The mass spectrometer was operated with an inlet temperature of 200°; ionizing voltage, 70 eV; ionizing current, 20–40 μA; and ion-source temperature, 250°.

Purification of Type VII pneumococcal polysaccharide. — A solution of a crude preparation of the type VII capsular material* (2.5 g in 40 ml of water) was clarified by centrifugation. An aqueous solution (50 ml of 5%) of Cetavlon (cetyltrimethylammonium bromide) was added to the clear supernatant and the complex that precipitated was removed by centrifugation. The precipitate was dissolved in M sodium chloride (50 ml) and addition of ethanol (150 ml) caused precipitation of a white product that was dialysed and freeze-dried; yield, 1.16 g (46.4%). Examination of this material by immunodiffusion (well 3, Fig. 1) showed a negligible reaction with type VII antipneumococcal serum.

The material in the supernatant from the Cetavlon precipitate was recovered by dialysis and precipitation by ethanol; yield 1.16 g. This product gave the ultracentrifugation pattern shown in A, Fig. 1, indicating the presence of at least two components. The material (1 g) was dissolved in water (6 ml) and a saturated solution of ammonium sulfate containing 5% of sodium acetate was added until precipitation was complete (100 ml). The precipitation with ammonium sulfate was repeated once, and the product recovered by centrifugation was dialysed and freeze-dried; yield 0.45 g (18%) (Found: N, 2.52; P, 0.55%).

The supernatants from the ammonium sulfate precipitations were dialysed and

*Made by E. R. Squibb and Sons, New Brunswick, N. J. and provided by Dr. M. Heidelberger, New York University Medical School, N. Y., U. S. A.

freeze-dried to yield a product (0.560 g, 22.4%) that gave no reaction with type VII antipneumococcal serum (well 4, Fig. 1).

The material precipitated by ammonium sulfate was further purified by chromatography (two runs with 225 mg each) on a DEAE (diethylaminoethyl)-Sephadex (column, 22 × 2.5 cm) in the phosphate form, with 0.01M sodium phosphate (pH 6.8) as eluant. The purified polysaccharide (270 mg, 10.8% of crude material) was recovered from fractions 2–5 inclusive (15-ml fractions at 5 fractions/h). It gave a strong reaction with type VII antipneumococcal serum (well 1, Fig. 1), showed as a single component on ultracentrifugation (B, Fig. 1), and gave a single band on electrophoresis on cellulose acetate.

Analysis of Type VII pneumococcal polysaccharide. — The analytical data are given in Table I. For analysis of the constituent sugars, the polysaccharide (10 mg) was hydrolysed by 2M hydrochloric acid (2 ml) in a sealed tube for 6 h at 100°. The hydrolysate was neutralized (silver carbonate), and the sugars were analysed as their alditol acetates by g.l.c. using dual columns with packing (*A*) and a temperature from 140–230° at 3°/min[5]. Qualitative and quantitative analysis of the amino sugars was also obtained by ion-exchange chromatography with a Model 120C Spinco amino acid analyser[17]. N.m.r. spectroscopy at 100 MHz provided independent checks of the quantitative data for the *N*-acetylated amino sugars and for L-rhamnose by integration of the proton signals from *N*-acetyl (δ, 2.68) and *C*-methyl (δ, 1.96). Tetramethylsilane was used as an external standard for measurement of chemical shifts.

Methylation of Type VII pneumococcal polysaccharide. — The polysaccharide (100 mg) was methylated by the Hakamori[18] procedure with dimethyl sulfoxide (10 ml), methylsulfinyl carbanion[19] (8 ml) and methyl iodide (10 ml). The reaction was diluted with water (50 ml), dialysed, and the non-dialysable product was recovered (118 mg) by evaporation to dryness. This product showed slight hydroxyl absorption in the infrared and was therefore subjected to two, successive, methylations by Purdie's reagents[20]. The methylated polysaccharide (115 mg) showed no hydroxyl absorption in the infrared; $[\alpha]_D^{20}$ +48° (*c* 2%, chloroform), (Found: OMe, 34.11%; Calc. OMe, 36.2%).

Hydrolysis products from methylated Type VII pneumococcal polysaccharide. — The methylated polysaccharide (75 mg) was hydrolysed in M hydrochloric acid (12 ml) for 8 h at 100°. The supernatant solution was decanted away from a gummy residue and was neutralized (silver carbonate) and concentrated. The gummy residue was heated for 8 h under reflux in 2% methanolic hydrogen chloride (10 ml). Methanol was evaporated by a stream of air and the residue was hydrolysed further in M hydrochloric acid (6 ml) for 8 h at 100°. After neutralization as before the two hydrolysates were combined.

The hydrolysis products were resolved into two fractions that contained the amino sugars and neutral sugars, respectively, by t.l.c. Preliminary experiments with standard compounds showed that all methylated 2-methylamino sugars were immobile and that all methylated neutral sugars had some mobility under these conditions.

The methylated neutral sugars were converted into their alditol acetates and were analysed by g.l.c.–mass spectrometry[7,8]. The results are given in Table II. Lindberg, *et al.*[8] did not provide relative retention times for 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol and it was, therefore, important to know that it was distinguishable from the 3,4-di-*O*-methyl isomer. The mass-spectral analysis indicated that the two were indeed separated and this was confirmed by co-chromatography with authentic samples. Similarly, it was important to confirm the identity of 2,3,6-tri-*O*-methyl-D-glucose and to be sure that no 2,3,4-tri-*O*-methyl-D-glucose was present. It has been shown that g.l.c. on column *C* will distinguish the alditol acetates of these isomers[21] and the identity of the fourth peak was further confirmed by co-chromatography on column *C* with an authentic sample.

The methylated amino sugars were analysed by paper chromatography[9,10]; the corresponding alditol derivatives were analysed as both trimethylsilyl ethers and acetates by g.l.c.[6]. The chromatographic mobilities from these three methods as given in Table II provided unambiguous identifications of the methylated amino sugars; the identities were confirmed by co-chromatography with authentic samples.

Time-lapse hydrolysis. — The type VII pneumococcal polysaccharide (10 mg) was heated in 0.25M sulfuric acid (1 ml) at 100°. Samples were removed after 10, 20, and 40 min for examination by paper chromatography (solvent *A*). In the 10-min hydrolysate, galactose was the only detectable sugar; after 20 min galactose and rhamnose were detected; hydrolysis for 40 min released galactose, rhamnose, traces of glucose, 2-acetamido-2-deoxy-glucose, 2-acetamido-2-deoxy-galactose, and four oligosaccharide components.

A further sample of the polysaccharide (75 mg) was hydrolysed for 40 min as already described and the oligosaccharides were separated by preparative paper chromatography (solvent *B*) and paper electrophoresis in 0.1M sodium tetraborate. Oligosaccharide 1 ($R_{Rhamnose}$, 0.0) behaved as a single component on paper electrophoresis and yielded rhamnose, glucose, and 2-amino-2-deoxyglucose on hydrolysis. Oligosaccharide 2 ($R_{Rhamnose}$, 0.038) gave two components on paper electrophoresis, each of which yielded rhamnose, galactose, and 2-amino-2-deoxygalactose on hydrolysis. Oligosaccharide 3 ($R_{Rhamnose}$, 0.11) was pure by paper electrophoresis and contained rhamnose, glucose, and 2-amino-2-deoxygalactose. Oligosaccharide 4, ($R_{Rhamnose}$, 0.19) was also pure by paper electrophoresis and yielded rhamnose, glucose, galactose, and 2-amino-2-deoxygalactose.

Periodate oxidation of Type VII pneumococcal polysaccharide. — The polysaccharide (50 mg) was oxidized in 0.12M sodium metaperiodate (65 ml) for 72 h at room temperature. After removal of iodate and periodate as insoluble barium salts, the oxidized product was reduced by sodium borohydride (50 mg) for 6 h. The reaction was acidified with acetic acid and boric acid was removed by repeated evaporations with methanol. Hydrolysis of the oxidized, reduced polysaccharide in 2M hydrochloric acid (1 ml) for 6 h at 100° yielded L-rhamnose, 2-acetamido-2-deoxy-D-galactose, erythritol, and glycerol, as shown by paper chromatography (solvent *B*).

When the reduced product was hydrolysed in 0.5M hydrochloric acid for 8 h

at 22°, chromatography (solvent *D*) revealed four components having $R_{Rhamnose}$ values of 0.8, 0.7, 0.35, and 0.05. From their mobilities, the first three of these components must have been erythritol and glycerol glycosides of monosaccharides[11]. The component with $R_{Rhamnose}$ 0.05 could have been an oligosaccharide glycoside but was more likely to be an erythritol or glycerol glycoside of 2-acetamido-2-deoxy-D-galactose. Lack of material prevented any further investigation of these products. No acetone-precipitable material could be obtained from the mild hydrolysis of the periodate oxidized, reduced polysaccharide, showing the absence of any periodate-resistant core.

Enzymic hydrolyses of Type VII pneumococcal polysaccharide. — For each test, the polysaccharide (20 mg) was dissolved in acetate buffer (2.5 ml, pH 5.4) and mixed with the enzyme (*ca.* 100 units) dissolved in the same acetate buffer (2.5 ml). A layer of toluene was added and the solutions were incubated for 48 h at 35°. A further addition of the same amount of enzyme was then made and the solutions were incubated for another 2 days. The solutions were then deproteinized by precipitation with trichloroacetic acid and examined by paper chromatography for any sugars released (see Table III). For quantitative precipitin reactions, the deproteinized enzymic hydrolysates were dialysed and freeze-dried to yield the degraded polysaccharide. Quantitative precipitin reactions were performed with 0.1 ml of Type VII antipneumococcal horse serum by the usual methods[22]; the values given in Table III were those obtained at equivalence.

The following enzymes were used in these experiments: α-amylase, β-amylase, dextranase, and cellulase from Worthington Biochemical Corp., Freehold, N.J., U. S. A.; β-galactosidase from Sigma Chemical Co., St. Louis, Mo., U. S. A. and chitinase from Mann Research Laboratories, New York, N. Y., U. S. A.

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