The Specific Polysaccharide of Type XVIII Pneumococcus. II.*

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In addition to D-glucose, L-rhamnose, and phosphate, previously shown to be present in the specific polysaccharide of type XVIII pneumococcus (S XVIII), galactose and glycerol are now identified chromatographically. Treatment with alkali yields glycerophosphate and undialyzable polymer containing glucose, galactose, and rhamnose. This polymer precipitates only 15% of the antibodies in Type XVIII antipneumococcal horse serum reactive with intact S XVIII. It also shows increased cross-reactivity with a Type VI antipneumococcal serum which contains antibodies cross-reactive with S II and other polysaccharides with multiple residues of (1 → 3)-linked L-rhamnose. Oxidation of alkali-degraded S XVIII with periodate leaves rhamnose and galactose in resistant linkages and destroys glucose, with production of erythritol and glycerol. The chemical properties and immunologic cross-reactivities of S XVIII and its product of alkaline degradation indicate that a part of the glucose is probably linked (1 → 4)- and (1 → 6)- and that the rhamnose and galactose are bound (1 → 3)-.

The immunologically specific capsular polysaccharide of Type XVIII pneumococcus, S XVIII, was originally shown to contain D-glucose, L-rhamnose, and doubly bound phosphate (Markowitz and Heidelberger, 1954). Since then, ribitol phosphate has been found as a constituent of the specific polysaccharide, S VI, of type VI pneumococcus (Rebers and Heidelberger, 1959, 1961), so that it appeared desirable to study S XVIII in greater detail. This has now resulted in the discovery of two additional components and in the acquisition of data bearing on the structure of the substance.

EXPERIMENTAL

Materials and Methods.—Earlier preparations of S XVIII were used in part, as well as E. R. Squibb and Sons' lot 1104. This preparation was further purified, the most effective method being the following: after precipitation in the cold with glacial acetic acid in the presence of calcium acetate the precipitate was washed with cold ethanol and dissolved in cold water. After addition of sodium acetate to *p*H 4.5 and precipitation by 1.2 volumes of saturated ammonium sulfate solution, a further small amount of the salt was added to attain maximal precipitation. After thorough dialysis of the precipitate against water the content of nitrogen was 0.6%, phosphorus, 3.1%, $[\alpha]_D^{23}$ +96°.

Quantitative microestimations of antibody nitrogen in the homologous reaction and cross-reactions were carried out by the methods given in the papers cited in the introduction.

Hydrolysis of S XVIII with Acid.—Whether hydrolyzed with 0.1 N, 0.5 N, or 2 N H_2SO_4, S XVIII gave, in addition to the glucose and rhamnose spots previously identified on chromatograms, a well-defined galactose spot, developed either with $AgNO_3$-NH_3 or with aniline hydrogen phthalate. 1-Butanol–pyridine–H_2O, 6:4:3, was the best solvent system for resolution of the glucose and galactose spots. The two sugars were also separated by electrophoresis on paper in 0.05 M borate buffer at *p*H 9.9 at 500 v and 7 mamps. Glycerol was identified chromatographically in the 0.5 N H_2SO_4 hydrolysate with the solvent system 1-butanol-ethanol-H_2O-NH_3 40:10:49:1, upper phase.

Recovery of S XVIII from Its Precipitate with Anti-Pn XVIII and Hydrolysis with Acid.—Thirteen mg of air-dried preparation 1800 (Markowitz and Heidelberger, 1954) was dissolved in 5 ml of 0.9% saline and added to a mixture of 45 ml of anti-Pn XVIII 495C and 45 ml of saline. After several days in the cold, the precipitate was washed three times with cold saline, dissolved in H_2O at 0° with the aid of a few drops of N NaOH, made up to 35 ml with H_2O, and treated rapidly with 35 ml of a chilled 10% aqueous solution of Cl_3CCOOH. The mixture was centrifuged at 0° and the supernatant neutralized with NaOH to *p*H 5.8. The residue of denatured antibody was washed twice with small volumes of 5% aqueous Cl_3CCOOH and the washings were neutralized and added to the main solution. After thorough dialysis in the cold against H_2O the solution was concentrated, lyophilized, and dried to constant weight. The 11 mg of S XVIII thus

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recovered had $[\alpha]D^{23}$ + 73° and was hydrolyzed in 0.5 N H_2SO_4 at 100° for 6 hours, cooled, and passed through Duolite A in the OH^- form; the solution was then concentrated to very small volume. A portion, chromatographed with 1-butanol–pyridine–water–benzene, 5:3:3:1, gave spots corresponding, as before, to galactose, glucose, and rhamnose. Rhamnose and glycerol do not separate in this solvent. Another portion, chromatographed with 1-butanol–ethanol–water–ammonia, 40:10:49:1 (upper phase), gave an unresolved spot with $AgNO_3$-NH_3 for glucose and galactose, one for rhamnose, and another corresponding to glycerol, the last absent on a similar chromatogram developed with aniline oxalate.

Degradation of S XVIII with Alkali.—S XVIII differed from S VI (Rebers and Heidelberger, 1959, 1961) in two respects in its behavior with alkali: first, higher concentrations of NaOH were required for degradation, and, second, the sugars remained undialyzable (alk. S XVIII), whereas S VI could be wholly split to dialyzable units. Treatment of S XVIII with 0.1 N NaOH at room temperature (about 25–28°) under N_2 split off only 20% of the P in 8 days and 40% (at somewhat lower temperatures) in 3 weeks. A portion of the 20% degraded material was allowed to stand under N_2 at 37° for 17 days in N NaOH. By this time 90% of the original P content was dialyzable through a regenerated viscose bag which had been thoroughly soaked in changes of H_2O to remove all glycerol and nondialyzable ultraviolet-absorbing material. Of the dialyzable P, 5.5% was inorganic PO_4, the remainder glycerophosphate, as shown by a chromatogram run in methanol–formic acid–water 80:15:5, and developed according to Hanes and Isherwood (1949). A spot corresponding to glycerol was also found by chromatography in 1-butanol–ethanol–H_2O–NH_3. An immunochemical comparison of S XVIII and alk. S XVIII is given in Table I.

Oxidation of S XVIII with Periodate.—S 1800, P = 3.4%, 5.4 mg, and 1.0 ml of 0.1 M $NaIO_4$ were allowed to react in a volume of 5.0 ml at 22°. After 2 days the consumption of periodate was 3.6 moles per mole P; after 4 days, 4.0 moles. Another sample, 1104P, 4.4 mg, in 3 ml, was allowed to stand 20 days at approximately 4° with 3 ml of 0.1 M $NaIO_4$. After precipitation with a slight excess of $BaCl_2$ the solution was dialyzed against H_2O in the cold. The outside liquid was Molisch-negative. The final volume of the bag contents was 11.5 ml. Determination of sugars by the phenol method (Dubois *et al.*, 1956) showed 111 μg per ml; calculated for (rham-gal), with formula weight S XVIII Na as 971, 121 μg per ml. Calculation of the formula weight was based on assumed ratios of galactose, glucose, rhamnose, glycerol, and phosphate of 1:3:1:1:1. After reduction with $NaBH_4$ and hydrolysis with N H_2SO_4 for 6 hours at 100° (Smith and Montgomery, 1959), a chromatogram in 1-butanol–pyridine–water–benzene for 48 hours showed spots for galactose, glucose (weak), rhamnose, and erythritol. Duplicate 1.0-ml portions of S 1800 containing 500 μg each were oxidized with $NaIO_4$ in 0.5 N H_2SO_4 for 5 minutes at room temperature (Lambert and Neish, 1950). After addition of Na_3AsO_3, CH_2O was determined to be 1.26 moles per mole P. This rose to 2.24 moles after hydrolysis of S 1800 in 0.1 N H_2SO_4 for 72 hours and conversion of 56% of the P into inorganic PO_4.

Oxidation of Alkali-Degraded S XVIII with Periodate.—The preparation (see above) which had been freed of 90% of its P was oxidized in much the same way as was S XVIII. Galactose and rhamnose accounted for roughly 50% of the product, and were thus retained with little or no loss. The material gave no precipitate in anti-Pn XVIII sera. A chromatogram of the oxidized, reduced, and hydrolyzed product in 1-butanol–pyridine–water also gave a strong spot corresponding to erythritol, while one in 1-butanol–ethanol–water–ammonia gave an additional spot with the R_F of glycerol.

TABLE I

ANTIBODY NITROGEN PRECIPITATED FROM ANTI-PNEUMOCOCCAL TYPE XVIII HORSE SERA BY S XVIII AND DERIVATIVES, 0°

All data calculated to 1.0 ml antiserum

Substance and	Amount (μg)	Anti-Pn XVIII 495C[a] (μg)	Anti-Pn XVIII 632C[a] (μg)
S XVIII	100		308
	600	2200	
Alk. S XVIII			
8 days, 0.1 N NaOH[b]	220	466	
8 days, 0.1 N NaOH[b]	320	488	
21 days, 0.1 N NaOH[c]	100	331	
21 days, 0.1 N NaOH[c]	200	349	
21 days, 0.1 N NaOH[c]	300	303	
17 days, N NaOH, 37°[d]	100	285	3
17 days, N NaOH, 37°[d]	200	263	2
S XVIII IO_4[e]	55		134
	111		143
	260	1580	
	540	1750	
	1100	1515	

[a] Absorbed with pneumococcal C-polysaccharide. Pn = pneumococcal. [b] 20% P dialyzed out. [c] 39% P dialyzed out. [d] 90% P dialyzed out. [e] Periodate-oxidized S XVIII.

DISCUSSION

Although it would be premature to draw up a detailed structural formula for the immunologically specific capsular polysaccharide of type XVIII pneumococcus, S XVIII, it is evident that the substance consists of a main chain of several residues of D-glucose, with smaller amounts of L-rhamnose and galactose. If these constitute a repeating unit, roughly one residue of α-glycero-

TABLE II

CROSS-PRECIPITATION OF S XVIII AND ALK. S XVIII IN ANTIPNEUMOCOCCAL HORSE SERA

Micrograms of antibody N precipitated, 0°, calcd. to 1.0 ml

Substance	Amt. Added (μg)	Anti-VI 681C	Anti-VI 771C	Anti-VII 895C	Anti-VIII 1008	Anti-XIV 635C
Homologous polysacch., max.		724	760	576	1288	1010
S XVIII	25			7	14	
	50	8	1			
	75			5	37	
	150	10	2		40[a]	
39% alk. S XVIII	50		15		21	
	100	6	18		27	
	150		14		26	
	300	7				
90% alk. S XVIII	25			3		
	50	6			17	5
	60		18, 22[b]			
	75			2		
	150	7	20, 22		22	1

[a] Reaction inhibited 96% by 3 μmoles lactose or cellobiose, 28% by 28 μmoles of maltose. Serum 1008, which had precipitated 127 μg N with barley or oat glucans gave 25 μg N with 150 μg S XVIII. [b] Reaction inhibited 91% by 55 μmoles L-rhamnose, 68% by 5.5 μmoles, 27% by 0.55 μmoles.

phosphate is attached through the phosphate to each unit. The side-chains are removable by alkali, leaving the main chain of sugars in undialyzable form. It is possible that alkali induces secondary changes, as removal of one-fifth of the phosphate reduces precipitation in anti-Pn XVIII by more than three-quarters. Removal of most of the remaining phosphate has a relatively small effect. Oxidation of S XVIII with periodate apparently leaves the galactose, rhamnose, and a small portion of the glucose intact. The accompanying changes in the glucose and glycerol residues only slightly reduce the amount of antibody precipitable from horse serum 495. Oxidation of the alkali-degraded material results in the modification of all of the glucose, while the galactose and rhamnose remain unchanged. These two sugars therefore appear to be present in (1 → 3)-linked form. If all of the sugars occur as pyranoses, as seems probable, the presence of formaldehyde among the products of oxidation of intact S XVIII would indicate that the side-chains consist of α-glycerophosphate and not the β-form. A portion, at least, of the glucose appears to be linked (1 → 4)-, as shown by the formation of erythritol after oxidation, reduction, and hydrolysis. This would account for the cross-reactivities of S XVIII and its alkaline degradation product in antipneumococcal type VIII horse serum (Table II). The specific polysaccharide of type VIII pneumococcus contains one of its two glucose residues in α-(1 → 4)-linkage, the other as β-(1 → 4)- (Jones and Perry, 1957).

The cross-reactivity of S XVIII in antisera to pneumococcal type VI, also shown in Table II, is slight before alkaline degradation, but becomes ten times as great in serum 771 after removal of the glycerophosphate. As this antiserum contains the highest proportion of antibody reactive with S II and other polysaccharides containing multiple residues of (1 → 3)-linked L-rhamnose (Heidelberger and Rebers, 1961), it would appear that the glycerophosphate residues in S XVIII are either attached to the otherwise (1 → 3)-linked rhamnose or to one of the sugars adjacent to this. Little can be said regarding the other cross-reactivities at present.

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