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Chemical Constitution of the Specific Polysaccharide of Type XVIII Pneumococcus[1a,1b]

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The preparation and properties of the specific polysaccharide of Type XVIII pneumococcus (S XVIII) are described and physical, chemical and immunochemical criteria of purity are given. Acid hydrolysis showed D-glucose and L-rhamnose to be the only sugars present, and these were isolated as β-glucose pentaacetate and L-rhamnose hydrate. S XVIII also contained 3 to 4% of organically bound phosphorus in secondary linkage. Quantitative paper chromatography and colorimetric estimation of rhamnose indicated the ratio glucose:rhamnose:phosphorus to be about 5:1:1. α- and β-amylases had no effect on S XVIII while an acid phosphatase was active only after treatment of S XVIII with alkali.

I. Introduction

Although the production by pneumococci of immunologically active type-specific capsular polysaccharides which give precipitates with antibodies has long been known[2] the difficulty in obtaining amounts sufficient for chemical study has limited the exploration of these substances. The only pneumococcal specific polysaccharide of which the structure is practically completely understood is that of the Type III pneumococcus, a polycellobiuronic acid with 1,3-links, probably β-, between cellobiuronic acid units.[3] As for the polysaccharides of other pneumococcal types, galacturonic acid has been identified as a constituent of that of Type I, (SI),[4,5] rhamnose, glucose and glucuronic acid in SII,[2,6] cellobiuronic acid and glucose in S VIII[7] and galactose and N-acetylglucosamine in S XIV.[8]

Only one previous isolation of the specific polysaccharide of Type XVIII pneumococcus has been recorded.[9] Since nothing was known of the constituent sugars it was decided to attempt their isolation.

II. Experimental

Preparation of S XVIII, No. 1801.—Two volumes of cold ethanol was added to 5 l. of a 12-hr., alkali-neutralized culture of virulent Type XVIII pneumococci in Adams–Roe medium.[10,11] After 12 hr. the supernatant was siphoned off and the pneumococci were centrifuged in the cold and processed as follows by modifications of recorded methods.[12,13] The organisms were suspended in 130 ml. of 3% sodium acetate solution at *p*H 6 and shaken 2 hr. with small glass beads, 0.3 mm. in diameter.[14] After centrifugation the supernatant was treated with 1 vol. of isopropyl alcohol to precipitate the crude polysaccharide which was worked up as in earlier studies.[12] The product contained 6.6% N, some of which was precipitated by $CuSO_4$. The supernatant was passed through a cation-exchange resin (Dowex 50 in the H^+ form) to remove Cu^{++}, centrifuged at 33,000 *g* to eliminate turbidity, dialyzed and lyophilized; yield 1801 A, 0.21 g.

To the culture supernatant, which gave a precipitate when tested with rabbit antipneumococcus Type XVIII serum, one more volume of ethanol was added. After 3 days the precipitate was collected and processed as before, but Cu^{++} was removed from the aqueous solution with a chelating agent, Versene, the *p*H being kept at 6 by addition of acetic acid. One and a half volumes of isopropyl alcohol gave a precipitate, which, after passage through a cation-exchange resin, dialysis and centrifugation at 145,000 *g* still contained 1.3% N. Fractionation with 1 and 3 vol. of warm, saturated sodium sulfate[13] yielded precipitates 1801 B, 0.04 g., and 1801 C, 0.38 g.

Preparation 1800 was obtained through the courtesy of E. R. Squibb and Sons, New Brunswick, New Jersey, and was repurified as above.

Chemical and immunochemical properties of the preparations are given in Tables I and II. The amount of antibody N precipitated by the S XVIII preparations (Table II) was determined[15] by addition of known amounts of S XVIII in physiological saline to rabbit antiserum; after 48 hr. at 0° the precipitates were separated by centrifugation and washed free from serum contaminants with physiological saline. Total N was estimated by a modification of the micro-Kjeldahl method.[16]

Purity of S XVIII Preparations.—Three criteria of purity were used: (1) attainment of the lowest possible nitrogen content. Lots 1800 and 1801 C, containing 0.3 and 0.4% N, respectively, were used in this study. (2) Electrophoretic homogeneity: Preparation 1800 showed only one slowly moving peak in the Perkin–Elmer apparatus at *p*H 5.35 and at *p*H 4.70. (3) A method. described in greater detail in a subsequent section, which makes use of the complete precipitation of most pure antigens by excess homologous antibody.

Hydrolysis of Lot 1800 and Determination of Constituents.—0.15-ml. portions of a solution of lot 1800 containing 2.62 mg. per ml. in 0.5 *N* H_2SO_4 were pipetted into small tubes which were sealed and placed in a boiling water bath. Samples were removed in duplicate at intervals and reducing sugar estimated by the method of Schales and Schales.[17] The data are given in Table III. Stronger acid, even after neutralization, gave lower recoveries of reducing sugar.

(1) (a) A preliminary report was presented at the 122nd Meeting of the American Chemical Society in Atlantic City, New Jersey, September 14–19, 1952. This work was sponsored by the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board, and supported in part by the Office of the Surgeon General, Department of the Army, and in part by the Harkness Research Fund of the Presbyterian Hospital. (b) Submitted by Harold Markowitz in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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TABLE I

PROPERTIES OF S XVIII PREPARATIONS

Prepn.	Ash as Na. %	$[\alpha]_D$,[a] degrees	N,[a12] %	Total P,[a12] %
1800[b]	2.0	+86	0.3	3.4
1801 A	3.6	+66	6.6	
1801 B	0.7	+97	0.9	
1801 C	0.6	+89	.4	3.5
R. Brown's[9c]	9.9	+76	.6	3.1

[a] Corrected for ash as Na. [b] Tests for S, halogens and free phosphate[18] were negative. [c] Data not corrected for ash.

TABLE II

PRECIPITATION OF 1.0 ML. RABBIT ANTIPNEUMOCOCCUS TYPE XVIII SERUM[a] BY S XVIII PREPARATIONS

Prepn.	Amount S XVIII used, μg.	Antibody N[b] pptd., mg.
1800	50	0.380
1800	100	.629
1800	150	.782
1801 B	68	.386
1801 B	136	.632[c]
1801 C	78	.471
1801 C	157	.677[c]

[a] Concentrate from Lederle Laboratories, Pearl River, N. Y., kindly supplied by New York City Department of Health laboratories. It was diluted 1 → 10 with saline and preserved with phenol. [b] All supernatant tests showed excess antibody when tested with S XVIII. [c] The lowered reactivity of 1801 may have been due to the neutralization of the culture during growth. NaOH added at this point has a similar effect on S I.[12]

TABLE III

LIBERATION OF REDUCING SUGAR FROM LOT 1800 ON HYDROLYSIS WITH 0.5 N H_2SO_4 AT 100°

Time, hr.	Reducing sugar as glucose, μg.	Reducing sugar as glucose, %	Time, hr.	Reducing sugar as glucose, μg.	Reducing sugar as glucose, %
0	0	0	2	257	65
0.5	136	35	4	286	73
1	193	49	6	311	79
1.5	229	58	12	312	79

Before it was known that weaker acid would suffice, a portion of no. 1800 was hydrolyzed with 2 N H_2SO_4. After removal of sulfate with $BaCO_3$ the solution gave positive anthrone[19] and Molisch tests and negative results with the naphthoresorcinol, Dische diphenylamine desoxypentose,[20] Seliwanoff and Tauber benzidine tests.[21] By descending paper chromatography[22] of the hydrolysate with 1-butanol, ethanol, water, 4:1:5, and use of aniline phosphate spray[23] spots with R_f of 0.15 and 0.35 were obtained. These corresponded to D-glucose and L-rhamnose, as was confirmed when these sugars were spotted alongside the hydrolysate. The same sugars were indicated with collidine, water and with 1-butanol, acetic acid, water as solvents. No evidence was found of the presence of other sugars.

On hydrolysis preparation 1801 C also yielded chromatograms with spots corresponding to glucose and rhamnose.

Quantitative Chromatograms.—Ten mg. of no. 1800 was sealed in a test-tube with 2 ml. of 0.5 N H_2SO_4 and heated at 100° for 6 hr. The solution was placed on a column of IR4B anion-exchange resin in the OH^- form and eluted with water until the Molisch test on 0.1-ml. portions of the effluent was negative. The eluate was lyophilized, dried to constant weight over P_2O_5 *in vacuo* and dissolved in 0.50 ml. of water. Two hundred and thirty-two μg. was spotted on Whatman #1 paper,[22] also 156 μg. of D-glucose and 187 μg. of L-rhamnose, with additional spots of the last two to serve as guide strips[22] for the location of reducing sugars after chromatography. The solvent was 1-butanol, ethanol, water 4:1:5. After 20 hr. at room temperature and location of the sugars by means of the guide strips, sections were cut and extracted with about 3 ml. of water. The results of estimations for reducing sugars in these eluates are given in Table IV. Spot I, with the mobility of a disaccharide, yielded only D-glucose on hydrolysis. Spot II corresponded to D-glucose and III to L-rhamnose.

TABLE IV

QUANTITATIVE ESTIMATION AND RECOVERY OF SUGARS IN 232 μG. OF LOT 1800

Material	Amount spotted, μg.	Optical[a] density	Reducing sugar as glucose, μg.		Recovery, %
Glucose	156	0.585	173		110
Rhamnose	187	.539	210		112
I (R_f 0.058)		.738	39[b]		
II (R_f 0.15)		.665	103	188[c]	81[d]
III (R_f 0.36)		.748	29		
Paper blank		.781	1		
Glucose soln.	348	.385			
Rhamnose soln.	173	.582			
Hot ferricyanide blank		.782			

[a] At 420 mμ in the Beckman Model B spectrophotometer with 1.0 cm. Pyrex cuvettes. [b] As disaccharide, 78 μg. [c] Total recovery of I, II, III: 78 + 103 + 29 − 3 = 207. This divided by 1.1 to correct for recoveries of glucose and rhamnose, gives 188 μg. [d] Since S 1800 contains 3.4% P or 7% :PO·OH this would actually account for 87% of the amount taken.

Additional Estimation of Rhamnose.—16% of L-rhamnose hydrate was found in preparation 1800 by the Dische Cy H_3 reaction,[24] as compared with 11% recovered from the chromatogram. 1801 C gave 19.1% by the former method. Confirmation of the presence of methylpentose was given by the 55% decrease of D_{396}-D_{428} in 2 hr. after dilution of the reaction mixture with water.[24]

Isolation of Hydrolytic Products of S XVIII.—To 0.5 g. of no. 1800 in 20 ml. of water 20 ml. of 4 N H_2SO_4 was added. The solution was heated under reflux at 100° for 2 hr. The clear, colorless solution was cooled and sulfate removed by the addition of $BaCO_3$. The filtrate was lyophilized and the residue acetylated with acetic anhydride and fused sodium acetate at 90° for 1 hr. with exclusion of moisture. The acetates were dissolved in hot methanol and the solution chilled and saturated with pentane. The product which separated was recrystallized to constant melting point and dried *in vacuo;* yield 0.24 g., m.p. 128.5–129.5°, not depressed by β-glucose pentaacetate, $[\alpha]_D$ +5.3 (0.4, $CHCl_3$), molecular weight 409 (Rast). The X-ray diffraction patterns of the substance and β-glucose pentaacetate were identical.[25]

The mother liquor was chromatographed on silicic acid[26]–Celite 535[27,28] (3:1) with benzene, *t*-butyl alcohol as the developer in order to remove colored impurities. The zone reacting with alkaline permanganate was extracted with acetone but yielded no crystals. Catalytic deacetylation of a small portion with barium methoxide and hydrolysis with 0.1 N and N HCl, followed by chromatography on

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(25) Thanks are due Prof. R. J. Holmes, Department of Geology, Columbia University, for the comparison.

(26) Silicic acid ($SiO_2 \cdot xH_2O$), 100 mesh, Analytical Reagent from Mallinckrodt Chemical Works, New York.

(27) A product of the Johns-Manville Co., New York.

(28) J. F. Haskins and M. J. Hogsed, *J. Org. Chem.*, **15**, 1275 (1950).

paper showed glucose and rhamnose to be present. The main portion of the acetate sirup was finally chromatographed on a column of 84 g. of Silene EF[29]–Celite 535, 5:1 with 500 ml. of benzene, *t*-butyl alcohol, 2,000:1, as developer. Zones were observed 0.5 and 0.75 of the way down the column and were sectioned and eluted as previously described. The upper fraction, A, yielded 0.15 g. of solid and the other, fraction B, 0.06 g. Fraction A, crystallized from ethanol–pentane to constant melting point, 88.5–89.5° showed $[\alpha]_D$ +49° (1.1, $CHCl_3$), mol. wt. (Rast) 463, and gave a positive Molisch test and negative benzidine and orcinol tests. Hydrolysis with *N* HCl for 2 hr. at 100° and paper chromatography after removal of chloride showed that the only carbohydrate present was glucose. No crystalline material could be obtained from the mother liquors.

Fraction B was taken up in 2 ml. of absolute methanol and 0.05 ml. of 2 *N* $Ba(OMe)_2$ added. After 18 hr. at 0° Ba^{++} was removed with CO_2 and the filtrate concentrated to dryness, taken up in 0.5 ml. of acetone, treated with one drop of water and allowed to stand at room temperature. After two days about 5 mg. of crystals was separated and air-dried. These melted at 90–92° and gave no depression of m.p. with authentic α-L-rhamnose hydrate. No further crystals could be obtained from the mother liquors.

Alternative Hydrolysis of S XVIII and Isolation of Constituents.—Three-tenths gram of no. 1800 was hydrolyzed with 0.5 *N* H_2SO_4 for 6 hr. at 100° and chromatographed on cellulose. The sugars identified were the same as in the preceding hydrolysis.

Additional Evidence for the Occurrence of Rhamnose and P as Essential Components of S XVIII Preparations.—As previously noted, the method is based upon the complete precipitation of an antigen by excess antibody: with thyroglobulin all of the iodine was precipitated (80–101%)[30a] with blood group A substance all of the fucose (79–129%),[30b] with SII, all of the rhamnose (87–112%).[6] In the case of S XVIII rhamnose and P are determined in the precipitate with excess antibody. Corrections for the amounts present in the antibody were made by means of a control precipitation with S III which does not contain rhamnose or P, and anti-pneumococcus Type III rabbit serum. This is justifiable since antibodies to different antigens of the same class and even the corresponding normal globulin appear to have the same composition.[31] The precipitate formed with lot 1800 and Type XVIII antipneumococcus rabbit serum contained 109% of the rhamnose and 75% of the P added as the polysaccharide (Table V). Similarly, with 1801 C, 75% of the rhamnose was precipitated; the quantities available were too small for an estimation of the P precipitated. It is evident that most of the rhamnose and P in S XVIII come down in the specific precipitate formed in the region of antibody excess.

TABLE V

DETERMINATION OF RHAMNOSE AND PHOSPHORUS IN SPECIFIC PRECIPITATES (METHYLPENTOSE AS RHAMNOSE HYDRATE)

	S III	S XVIII No. 1800	S XVIII No. 1801 C
Polysaccharide added, μg.	83.1	50	39.2
Antibody N pptd., μg.	183	380	236
(1) Methylpentose in specific ppt., μg.	5.5	19.3	12.2
(2) Methylpentose in ppt. due to antigen, μg.	0.3	8.7[a]	5.6[a]
(3) Methylpentose in ppt. due to antibody, μg.	5.2[b]	10.6[c]	6.6[c]
(4) Ratio methylpentose:antibody N	0.028		
% methylpentose of S XVIII pptd.		109	75
Phosphorus			
Polysaccharide added, μg.		600	
P in S XVIII added (Table I), μg.		20.4	
P in specific ppt., μg.		15.2[d]	
% P of S XVIII pptd.		75	

[a] (1)—(3). [b] (1)—(2). [c] Calcd. from (4). [d] A control test with S III—anti S III showed negligible amounts of P.

Tests for Cross-reactivity of S II and S XVIII.—Since both polysaccharides contain rhamnose and glucose it seemed possible that S II might precipitate Type XVIII antipneumococcus serum and that S XVIII might react with the antibodies in Type II serum. However, tests with the corresponding horse sera with amounts of the polysaccharides ranging from 5 to 200 μg. were uniformly negative, as were also tests with the usually less cross-reactive rabbit antisera over a smaller range.

Action of Enzymes on S XVIII.—α-Amylase, β-amylase and acid phosphatase from seminal fluid did not act on preparation 1800. Treatment of no. 1800 with alkali almost completely destroyed its serological reactivity but did not release inorganic phosphate. When the alkali treated product was tested with acid phosphatase 20% of the P was released as $PO_4^{=}$.

III. Discussion

The separation of contaminating impurities from S XVIII was found to be very difficult. However, an electrophoretically homogeneous material with a nitrogen content of 0.3% eventually was obtained. Immunological data showing the essentially complete precipitation of rhamnose and phosphorus in S XVIII by excess antiserum support the electrophoretic evidence of homogeneity and lead to the conclusion that the preparation used for structural studies was substantially pure.

The results of chromatography of the hydrolysate on paper and the actual isolation of β-glucose pentaacetate and α-L-rhamnose hydrate establish the presence of the two sugars in S XVIII. Quantitative paper chromatography (Table IV) and the analyses and tests for P indicate that the polysaccharide is made up of glucose, rhamnose and secondary phosphate in approximately the ratios 5:1:1.

Many instances are known in which an antigen chemically related to the homologous one may precipitate the antibody. These are called cross-reactions and some have been studied in detail.[32] Because S XVIII contains glucose and rhamnose one might expect it to cross-react with antiserum to Type II pneumococci, for S II, the type-specific determinant of these pneumococci also contains glucose and rhamnose.[6] However, precipitation does not occur with either rabbit or horse Type II antipneumococcus sera, nor does the reverse reaction of S II with Type XVIII antipneumococcus sera take place. More profound differences in the structures of S XVIII and S II are therefore indicated than merely the presence of P in the former and of glucuronic acid in the latter.

Although little can be said as to the fine structure of S XVIII there are one or two clues. Perhaps some of the sugar residues are joined by phosphate linkages, much as in the case of the specific polysaccharide of *H. influenzae*, type b,[33] since acid phosphatase reacts only after the polysaccharide is treated with alkali. These phosphate linkages, whether inter-residual or not, seem important for the immunological specificity of S XVIII, for when they are partially opened by alkali, reactivity with antibody is lost.

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(29) From Columbia Chemicals, Grant Building, Pittsburgh 19, Pa.

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