THE CAPSULAR POLYSACCHARIDE OF PNEUMOCOCCUS TYPE IX*

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The immunologically specific capsular polysaccharide (S IX) of Type IX pneumococcus was shown by chromatographic methods to contain glucose, glucosamine, glucuronic acid, and ribose, the last probably because of contaminating nucleic acid. Absorption in the infrared around 850^{-1} cm indicated α-linkages (1). Since S IX is the antigenic determinant of pneumococcal Type IX specificity and Type IX antipneumococcal (anti-Pn) sera cross-react with a wide variety of natural and synthetic polyglucoses and other gums (2, 3), further knowledge of the chemistry of S IX and the basis for its cross-reactivity seemed desirable. Data obtained thus far are presented herewith.

Materials and Methods

S IX was supplied by E. R. Squibb and Sons, New York, kindness of Mr. T. D. Gerlough, and was further purified. Isolichenin was a gift from Dr. D. G. Manners. Anti-Pn sera were derived from the sources listed in references 2 and 3.

Quantitative precipitin reactions were set up at 0° C and kept in a bath at 0° C. Analyses in homologous systems were usually completed after 48 hr while cross-reactions and tests with degraded S IX were allowed to stand up to 2 wk or more, depending upon the rapidity of precipitation. A cold box was used for draining the tubes during the analyses (4). Nitrogen was estimated by Markham's method (5).

Whatman No. 1 filter papers served for chromatographic identification of sugars and the 3 mm grade for their isolation. Solvent systems were: (*a*) pyridine-ethyl acetate–acetic acid–water, 5:5:1:3; (*b*) ethyl acetate–acetic acid–water, 3:1:1; (*c*) *n*-butanol-ethanol–water, 4:1:5; and (*d*) *n*-butanol-pyridine–water–benzene, 5:3:3:1 (upper layer).

Spray reagents were: (*a*) aqueous saturated aniline oxalate, (*b*) alkaline silver nitrate, and (*c*) 0.1% ninhydrin in *n*-butanol. Hexoses were estimated with L-cysteine (6), uronic acid by carbazole (7), and hexosamines by the modified Elson-Morgan reaction (8). Consumption of oxidant during oxidation by periodate was estimated spectrophotometrically (9). All evaporations were carried out at 35–40° C *in vacuo*.

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EXPERIMENTAL

Purification of S IX.—15 g of Squibb lot 143 were taken up in 750 ml of 5% sodium acetate solution adjusted to pH 6.05 with acetic acid. All operations were carried out in the cold. The turbid solution was centrifuged 1 hr at 40,000 rpm and the clear supernatant mixed in a Waring Blendor with $CHCl_3$: C_4H_9OH, 8:1, to remove protein. After 7 such Sevag treatments (10) the interfacial layer was negligible. The concentration of sodium acetate in the aqueous layer was adjusted to 3% and the polysaccharide was separated into two fractions, A and B, by addition of chilled 2-propanol. These were triturated with alcohol and acetone, and dried. Washings from the preceding operations were combined and Fraction B′ was precipitated with 2-propanol. Fraction A was dissolved in 400 ml of 3% sodium acetate solution at pH 6.05 and reprecipitated with an equal volume of ethanol. Alternate precipitation with

TABLE I

Properties of S IX and of Periodate-Oxidized S IX

Constituent	Lot 143, Fr. A_1	Lot 141, Fr. A	IO_4-oxidized S IX, Fr. A_1
Ash, as Li, %	0.8	0.8	
Nitrogen, %	3.0	3.1	
Phosphorus, %	<0.1		
Anhydroglucose,* %	28	29	22
Anhydro-*N*-acetylglucosamine,* %	25	24	24
Anhydroglucuronic acid,* %	23	17	5

* Ash-free

ethanol and 2-propanol was repeated and the polysaccharide was washed with ethanol and acetone, and dried. Yields: Fraction A, 5 g; B, 0.5 g; B′, 1 g.

Of the three fractions, A was free from C-substance as tested for on Ouchterlony plates with heterologous anti-Pn serum high in anti-C. Ash, as Na, 3.2%; no *O*-acetyl (11); *N*-acetyl, 9.5% (12); no inorganic P; total P, 0.5% (13). The aqueous solution showed high absorption at 260 mμ due to nucleic acid.

2 ½ g of fraction A in 250 ml of water were precipitated with 1% aqueous cetyl pyridinium bromide. The complex formed with S IX was separated from that with nucleic acid by solution in 350 ml of 0.15 M aqueous sodium sulfate (14, modified). The dissolved complex was dissociated by addition of 3% w/v LiCl and S IX was precipitated with ethanol containing 3% LiCl, dissolved in water, and dialyzed against it. After reprecipitation and drying, the yield (Fraction A_1) was 80%, $[\alpha]_D^{23}$ + 121° in H_2O (c, 0.9) calculated to ash-free basis.

In a second preparation of S IX from 30 g of Squibb lot 141, the fraction

corresponding to A showed little absorption at 260 mμ and also contained no C substance. Properties of these two preparations are listed in Table I.

S IXA$_1$, 200 mg, was hydrolyzed with N H_2SO_4, at 100° C for 11 hr. After neutralization with $BaCO_3$ and concentration, the hydrolysate was separated by paper chromatography into its component sugars, glucose, glucosamine, glucuronic acid, and presumably a disaccharide. Glucose was crystallized twice from aqueous ethanol, mp 144–146° C, mixed mp with D-glucose, 145–146° C; literature (lit.) for D-glucose, 146° C; $[\alpha]_D^{23}$ + 50.9° in H_2O (*c*, 1); lit., 52.5°. Part of the fraction was treated with *p*-nitroaniline and acetic acid in methanol at 60° C for 2 hr. The yellow crystals of glucose *p*-nitroanilide obtained on cooling were recrystallized twice from methanol, mp 173–175° C, mixed mp with authentic D-glucose-*p*-nitroanilide, 174–175° C; lit., 175° C; $[\alpha]_D^{23}$ − 209° in pyridine (*c*, 0.3); lit, −212°. The glucose is therefore at least largely the D-isomer.

The fraction corresponding to glucosamine was eluted with water, passed through Dowex 1-X4 (OH^-) resin, evaporated to a syrup, taken up with ethanol, and crystallized twice from ethanol, mp 108–110° C (decomp.); lit., 110° C (decomp.); $[\alpha]_D^{23}$ + 44.5° in H_2O (*c*, 0.9); lit. for D-glucosamine, + 47.5°.

The eluted barium salt of glucuronic acid was passed through IR 120 (H^+), evaporated to a syrup *in vacuo*, and taken up with ethanol. Evaporated *in vacuo* almost to dryness and kept in a 0° C bath for 2 days, the solution deposited crystalline glucuronic acid, mp 154–155° C, mixed mp with authentic D-glucuronic acid, 160–161° C; lit. 154° C, 165° C; $[\alpha]_D^{23}$ + 37.7° in H_2O (c, 0.6); lit. + 36°.

The slow-moving component in the total hydrolysate of S IX appeared to be a disaccharide. On hydrolysis and chromatography it gave spots corresponding to glucose and glucuronic acid. Reduction of 5 mg of "disaccharide" with $NaBH_4$, followed by hydrolysis, yields glucuronic acid as the reducing sugar, showing that a portion, at least, of the glucuronic acid in S IX is linked glycosidically to glucose.

Graded Hydrolysis of S IX.—4 g of S IX, lot 141, Fr. A, were dissolved in 400 ml of 0.5 N H_2SO_4 and heated in a boiling water bath for 3 hr. The solution was neutralized with $BaCO_3$, centrifuged, passed through Dowex 50 (H^+), and Dowex 1-X4 HCO_3^-, and concentrated to small volume. Chromatographic examination disclosed at least four relatively slowly moving spots besides those corresponding to glucose and *N*-acetylglucosamine. The mixture was separated on thick filter papers and the zones containing glucose and *N*-acetylglucosamine were eluted with water. Glucose was characterized as the D-isomer as reported above. The other fraction was crystallized from methanol-ethanol-ether, mp and mixed mp with authentic *N*-acetyl-D-glucosamine, 189–190° C (decomp.); lit. 189–190° C, 205° C (decomp.); $[\alpha]_D$ + 41° in H_2O (c, 0.6); lit. + 41.8°.

In another experiment, the fastest of the four spots alluded to above, with $R_{glucose}$ 0.77 in solvent *b*, gave glucose and glucosamine on hydrolysis.

The column containing the acid fractions was washed with water until this was sugar-free, and the acids were eluted with 0.5 N NH_4OH. This solution contained glucuronic acid and at least three other sugars, one of which had the same chromatographic mobility as that of the previously obtained "disaccharide" and gave the same results on hydrolysis and on reduction followed by hydrolysis.

In an attempt at still milder degradation of the polysaccharide, 10 mg portions were heated with 0.01 N and 0.1 N H_2SO_4 in a boiling water bath. After each hour 1 ml portions were removed, cooled, and neutralized with $BaCO_3$. The filtrates were made up to 10.0 ml and 90 mg NaCl added to each. Results of qualitative precipitation tests with anti-Pn IX rabbit serum were:

Time of heating, *hr*.....	1	2	3	4
0.01 N acid.....	4+	4+	4+	3+
0.1 N acid.....	2+	+	±	−

In another run, each hydrolysate was treated with Dowex 50 (H^+) and Dowex 1-X4 (OH^-) resins, concentrated to small volume, and examined by paper chromatography.

Time of heating, *hr*.....	1	2	3	4
0.01 N acid...	No spot	No spot	No spot	No spot
0.1 N acid....	No spot	Spot near base line	2 spots, no maltose	Glucose streak from base line, no maltose

Quantitative data on the amounts of antibody precipitated by the 1- and 2-hr 0.1 N acid hydrolysates are given in Table II.

Oxidation of S IX A_1 by Sodium Metaperiodate.—In the dark at 4° C for 3 days, when the reaction ceased, each anhydrohexose unit (mol wt 162) had consumed 0.27 moles of oxidant, with liberation of 1 mole of formic acid per 132 residues. Excess periodate and iodate ions were removed with Ba^{++} and the filtrate was hydrolyzed. The neutral hydrolysate, obtained as before, was found to contain glucose, glucosamine, and a small amount of glucuronic acid. In another run excess periodate was reduced with ethylene glycol and the solution dialyzed against H_2O. The oxidized S IX was reduced with sodium borohydride, and after removal of boric acid with MeOH the IO_4-oxidized, reduced S IX was precipitated with ethanol. The composition of the product

is given in Table I. The periodate-oxidized, reduced S IX was kept with 0.1 N H_2SO_4 at room temperature for 3 days, neutralized with $BaCO_3$, and centrifuged. On chromatographic examination, the only mobile spot corresponded to

TABLE II

Precipitation of Antipneumococcal Sera by S IX and Derivatives

H, horse; R, rabbit

Polysaccharide, amount	Antibody N precipitated at 0°C calculated to 1.0 ml, from anti-Pn		
	IX H963C*	IX R91₂‡	XIV H635C*
mg	*μg*	*μg*	*μg*
Homologous, at max. pptn.	1655	5340§	1010
S IX 0.03			9‖
0.07			11‖
IO_4-Oxd. S IX			
0.2	1070		
0.4	1115		
0.6	1040		
0.75		2290¶	
0.9		2280¶	
S IX 1.6		5000**	
0.1 N H_2SO_4-degraded S IX			
1 hr at 100°C, 1.6		2170**	
2 hr at 100°C, 1.6		840**	

* Absorbed with pneumococcal C substance.

‡ Contained only 4 μg anti-C nitrogen per ml.

§ 1.5 mg S IX used per ml original serum; analyses carried out with 0.10 ml 1 → 3 dilution, total volume, 0.15 ml.

‖ Supernatants gave 12 μg N with dextran N 236 as against 13 μg for unabsorbed serum.

¶ Supernatants gave 2655 μg N with S IX and 93 μg N with isolichenin as against 127 μg for the latter with unabsorbed serum. After 6 months in the cold, IO_4-oxd. S IX still precipitated 1990 μg N.

** Analyses carried out with 0.10 ml 1 → 10 dilution and 16 μg S IX, total volume 0.3 ml.

glucuronic acid. A second oxidation, followed by hydrolysis, gave a mixture containing glucose and glucosamine.

Preliminary experiments showed that most of the carboxylic groups of S IX could be converted to ethylene glycol esters with ethylene oxide and reduced to $-CH_2OH$ by $NaBH_4$ (15). The product precipitated less antibody from anti-Pn IX than did the original polysaccharide.

DISCUSSION

The data presented indicate that S IX is composed of D-glucuronic acid, *N*-acetyl-D-glucosamine, and glucose, the major portion of which, at least, is the D-isomer. Some of the glucose and glucuronic acid residues are joined

TABLE III

*Inhibition of Precipitation of Isolichenin in Antipneumococcal Type IX Sera**

Total volume, 0.50 ml. H, horse; R, rabbit

Inhibitor	μmoles added	Antibody N precipitated from		Inhibition
		H963C	$R91_2$	
		μg	μg	%
None			35	
D-Glucose	75		3	91
	25		17	51
Cellobiose	30		20	43
None		44		
D-Glucose	100	30		32
Na D-glucuronate	29	46		0
N-Ac-D-glucosamine	45	36		18
None			39	
Methyl α-D-glucoside	31		26	33
Methyl β-D-glucoside	31		26	33
None			31	
N-Ac-D-glucosamine	45		14	55
Cellobiose	24		19	39
Maltose	23		5	84
Maltose	5		15	52
Maltose	2.5		20	36
None		32		
Cellobiose	55	29		9
Maltose	15	21		34
Maltose	5	24		25

In the S IX anti-S IX reaction, under comparable conditions, N-acetyl-D-glucosamine, 45 μmoles, gave 14% inhibition; the other sugars scarcely inhibited.

* In these and other analyses, the assistance of Mr. W. P. Grosvenor is gratefully acknowledged.

glycosidically as an aldobiuronic acid with glucose at the reducing end. On oxidation of S IX by periodate a portion of the glucose and much of the glucuronic acid are degraded, whereas the *N*-acetylglucosamine is completely resistant. From the small amount of formic acid liberated during the oxidation

it also appears that the macromolecule is not very highly branched. The disappearance of glucuronic acid after a second oxidation indicates that this part of the acidic sugars was originally linked at the 3-position and that one or the other of its neighboring sugars was vulnerable to the oxidant. Adjacent residues of *N*-acetylglucosamine and glucose occur. The three constituents do not add to 100%. Only oligosaccharides were found in addition.

Earlier studies (16, 17, 3) showed strong cross-reactions of synthetic polyglucoses, glycogen, dextrans, and isolichenin in anti-Pn IX sera, and the reactions were attributed to multiples of suitably linked glucose units, particularly those linked α-1,4- and α-1,3-. Dextrans containing 1 → 2, 1 → 3, and 1 → 4 linkages showed stronger cross-reactions than those exclusively 1 → 6, and precipitation was inhibited by 1 → 2-, 1 → 3-, and 1 → 4-linked glucose-containing di- and trisaccharides (16). Attempts to detect such among the hydrolytic products of S IX were unsuccessful, probably because the glucose→ glucose linkages are those most readily split, since this sugar is the first liberated on mild hydrolysis of S IX. Linkage of two D-glucose residues 1 → 4, as in maltose, is indicated by the strong inhibition of the cross-reaction of isolichenin in anti-Pn IX by maltose (Table III). The possibility that some of the other glucose residues in S IX are linked 1 → 3, as also occurs in isolichenin (18, 19), is to be considered because of the magnitude of the cross-reaction, which involves up to 38% of the antibody in horse serum IX 623 C. The inhibitory effect shown by *N*-acetyl-D-glucosamine (Table III) is an indication that this substance, also, is a determinant in the overall specificity of pneumococcal Type IX.

As yet the experimental data do not suffice for the setting up of a structural formula for S IX, but the work is being continued.

SUMMARY

The capsular polysaccharide of Type IX pneumococcus contains D-glucose, *N*-acetyl-D-glucosamine, and D-glucuronic acid. Complete hydrolysis is difficult. All of the *N*-acetylglucosamine is resistant to oxidation by periodate, but the other two sugars are degraded in part. Chemical and quantitative serological data are consistent with the linkage of two D-glucose residues 1 → 4, as in maltose; others may be linked 1 → 3. Part, at least, of the glucuronic acid and *N*-acetylglucosamine is linked to glucose.

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