The Specific Capsular Polysaccharide of Type VII *Pneumococcus**

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ABSTRACT: The specific capsular polysaccharide of type VII *Pneumococcus* (S VII) is composed of D-galactose, D-glucose, L-rhamnose, and the *N*-acetyl derivatives of D-glucosamine and galactosamine (probably D-), roughly in the ratios 4:2:3:2:2. The sugars were isolated in crystalline form. Galactose, glucose, and glucosamine are destroyed by oxidation of S VII with periodate and specific precipitation in type VII antisera is abolished. The cross-reactions of diverse polysaccharides in these antisera indicate that the specificity of the antibody is directed mainly toward terminal nonreducing galactopyranose residues, particularly if β linked, and to a small extent toward 1,6- and 1,4-linked glucose residues. Inhibition studies on the heaviest cross-reaction, that of tamarind seed mucilage, warrant the prediction that β-linked nonreducing terminal galactopyranose residues will be found to occur in S VII.

The pneumococcal capsular polysaccharides, determinants of the immunological type specificity of this large group of microorganisms, offer ideal material for exploration of the relation between chemical constitution and immunological specificity (for reviews, *cf.* Heidelberger, 1956, 1960). Because of the variety of cross-reactions shown by antipneumococcal (anti-Pn) type VII sera, studies of the capsular polysaccharide of type VII *Pneumococcus* (S VII) were initiated. Preliminary hydrolyses by S. A. Barker in this laboratory indicated the presence of galactose, rhamnose, amino sugar, and possibly glucose. In a preliminary report (Tyler and Heidelberger, 1962) we characterized D-galactose, D-glucose, and L-rhamnose, and tentatively identified galactosamine and glucosamine. These and additional experiments are now presented together with quantitative data on the homologous precipitin reaction and the cross-reactions of numerous polysaccharides in anti-Pn VII sera. Certain structural features of S VII are deduced from the data.

Materials and Methods

Lot 215 of S VII, isolated by the phenol procedure (Palmer and Gerlough, 1940; Gerlough, 1944), was provided by E. R. Squibb and Sons, courtesy of Mr. T. D. Gerlough; it was further fractionated. Antipneumococcal horse sera were supplied by the Bureau of Laboratories, New York City Department of Health, courtesy of Miss A. W. Walter. Rabbit anti-Pn VII serum was given by Lederle Laboratories of American Cyanamide Co. and was diluted with an equal volume of 0.85% saline containing 0.02% merthiolate and 0.3% phenol. Tamarind seed polysaccharide (White and Rao, 1953; Savur, 1955, 1956; Khan and Mukherjee, 1959; Kooiman, 1961) was obtained through Dr. F. E. Brauns and Dr. E. V. White. Karaya gum from *Cochlospermum gossypium* (Hirst and Dunstan, 1953; Aspinall *et al.*, 1962) and carob mucilage (Hirst and Jones, 1948; Smith, 1948) were provided by Professor Sir Edmund L. Hirst, guar mucilage (Wise and Appling, 1944; Ahmed and Whistler, 1950; Rafique and Smith, 1950) by the late Professor F. Smith, the gums of *Khaya grandifolia* (Aspinall *et al.*, 1956), *Combretum leonense* (Aspinall and Bhavanandan, 1965), and *Anogeissus latifolia* (gum ghatti) (Aspinall *et al.*, 1965; and earlier papers) by Dr. G. O. Aspinall, and Ketha gum (from *Feronia elephantum*) (Heidelberger *et al.*, 1962) by Dr. S. Mukherjee. Polysaccharides with blood-group agglutinating activity from *Taxus cuspidata* (Springer *et al.*, 1956) and *Sassafras albidum* (Springer *et al.*, 1965) were furnished by Dr. G. F. Springer, dextrans N236 (Kabat and Berg, 1953) and 1355-S4 (Jeanes *et al.*, 1951, 1954), and blood-group substances by Dr. E. A. Kabat. Dr. P. W. Robbins supplied lipopolysaccharides of *Salmonella anatum* (Robbins and Uchida, 1962; Uchida *et al.*, 1963).

Sugars for the inhibition studies were tested chromatographically and recrystallized when necessary. *N*-Acetylglucosamine was freed of glucosamine with IR-120 (H^+) resin; the recovered sugar was recrystallized. Maltose was purified as the octaacetyl derivative which was deacetylated with sodium methoxide and recovered as β-maltose monohydrate, $[\alpha]_D$ +110–126°. 4-*O*-β-D-Galactopyranosyl-*N*-acetyl-D-glucosamine was provided by Dr. E. A. Kabat. Dr. E. Pacsu furnished two galactobioses, A and B (Turton *et al.*, 1955), corresponding to 6-*O*-α-, and, possibly, 6-*O*-β-D-galactopyranosyl-D-galactose, respectively. About 50–60 mg

* From the Institute of Microbiology, Rutgers, the State University, New Brunswick, New Jersey. *Received December 13, 1967.* Supported by Grant G-19968 from the National Science Foundation.

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of allolactose (6-*O*-β-D-galactopyranosyl-D-glucose) and 6-*O*-β-D-galactopyranosyl-D-galactose was procured by the action of β-galactosidase (Nutritional Biochemicals Corp.) on lactose (Pazur, 1962); the disaccharides were fractionated on paper by prolonged descending multiple development. This afforded a homogeneous sample of allolactose ($R_{lactose}$ 0.78 (butanol–pyridine–H_2O–C_6H_6, 5:3:3:1, upper phase), $[\alpha]_D$ +26° (*c* 1.56, H_2O)) but the galactobiose ($R_{lactose}$ 0.60, $[\alpha]_D$ +31° (*c* 2.03, H_2O) (lit. value $[\alpha]_D$ +34°)), contained traces of allolactose. The constituent sugars of both were verified. Allolactose octaacetate was synthesized in 60–70% yield by Koenigs–Knorr condensation of 2,3,4,6-tetra-*O*-acetyl-α-galactopyranosyl bromide with 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose (*cf.* Reynolds and Evans' (1938) synthesis of β-gentiobiose octaacetate, also Talley (1963)). After deacetylation, allolactose crystallized: mp 179–180°, $[\alpha]_D$ +47–30° (*c* 1.35) (Helferich and Sparmberg (1933) give $[\alpha]_D$ +31°, mp 174–176°). The osazone had mp 189–190° dec (lit. mp 188–189°). Allolactose produced enzymatically was chromatographically identical with the synthetic sample.

Standard methods were used for analysis and hydrolysis of the fractions, and for paper chromatography, with exceptions noted in the text. The purest fractions of S VII were accounted for analytically to the extent of 89–90%. Quantitative microestimations of the amounts of antibody nitrogen precipitated in the homologous and cross-reactions were carried out on duplicate samples (0.20–2.0 ml) of sera as in earlier papers (Rebers and Heidelberger, 1959, 1961; Rebers *et al.*, 1961). In most of the inhibition tests 0.20 ml of antiserum was used, the inhibitor was added in 0.20 ml of saline (0.9% solution of sodium chloride), and the mixture was left at 0° for 1–2 hr before addition of antigen in 0.10 ml of saline. After 10–14 days in a bath at 0° the analyses were completed. The anti-Pn VII rabbit serum was diluted 1:10 and horse serum 937C 1:3 for inhibition tests in the homologous system; in the cross-reactions only the rabbit serum was diluted, and by 1:2. Inhibition tests with dextran required 1.0 ml of serum.

Purification of S VII. Sample 703A (Heidelberger *et al.*, 1950), used in some of the work, contained group-specific pneumococcal C substance. All preparations were examined for this by double diffusion in agar gel on Ouchterlony plates (*cf.* Kabat and Mayer, 1961) *vs.* horse anti-Pn I 1057 or anti-Pn VII 1074, both of high anti-C content (Heidelberger *et al.*, 1962).

Samples 711–714 were also prepared from the Squibb material. Type VII S (6 g) in 100 ml of 1% sodium acetate at pH 6.4 was freed of debris at 40,000 rpm in a Spinco ultracentrifuge. The sediments were washed with 80 ml of sodium acetate solution, and the combined supernatants were repeatedly deproteinized (Sevag, 1934) in the cold with $CHCl_3$–BuOH (8:1). The aqueous solution was adjusted to 5% sodium acetate and pH 6.4 and precipitated by alcohol at 1.6, at 1.8–2.0 (main fraction), and at 3.5 volumes. Although analyses for N and P showed some fractionation, all contained C substance. Nucleic acid in sample 711, from the optical density at 260 mμ, corresponded to only 2% of the P content.

Because of the difficulty of separating S VII from C substance, fractionation was attempted with ammonium sulfate, which had been useful in the purification of S XVIII (Estrada-Parra *et al.*, 1962; Estrada-Parra and Heidelberger, 1963) and S XVIIIA (Heidelberger *et al.*, 1964). Saturated ammonium sulfate (20 ml) containing 5% sodium acetate to give pH 6.7 was gradually added to a solution of 500 mg of sample 713 in 4 ml of water. The precipitated S VII was dissolved in 2 ml of water and twice reprecipitated with 10 ml of the ammonium sulfate solution. The precipitate, P3, was dissolved in water, dialyzed, and lyophilized, yield 290 mg. P3 contained <2.5% of C substance by comparative gel diffusion with known amounts of C substance. C substance (159 mg) recovered from the first supernatant after dialysis and freeze drying contained <5% of S VII. The second and third supernatants contained mixtures of S VII and C substance.

P3 (107 mg) was further purified by chromatography on DEAE-cellulose (16 × 1.6 cm) in the phosphate form (pH 6.7). Elution with 250 ml of water followed by 250 ml each of 0.01 and 0.02 M phosphate (pH 6.7) and 0.2 M sodium hydroxide gave fractions i–iv. Eluates i–iii were evaporated to small volume *in vacuo*, traces of insoluble material were centrifuged, and the supernatants were dialyzed and freeze dried (26, 35, and 6 mg). Fraction iv was neutralized and similarly treated, yield 29 mg (total recovery 90%.) Fractions i and ii contained S VII and no C substance and were combined (D3) and analyzed (*cf.* Table I). Fraction iii consisted of S VII and traces of C substance; fraction iv contained both. Persistent traces of inorganic phosphorus were removed by treatment of aqueous solutions with Dowex 50 (H^+) followed by Dowex 2 (CO_3'').

Results and Discussion

Infrared Absorption Spectrum. From the spectrum of S VII (D-3) in KBr, the following assignments could be made (Barker *et al.*, 1956): 3.0 (OH), 3.45 (CH), 5.8, 6.05, 6.45, and 7.2 μ typical of the acetamido group (*cf.* also Levine *et al.*, 1953). Confirmation is thus provided that the amino sugars of S VII occur as *N*-acetyl derivatives.

Hydrolysis of S VII and Chromatographic Identification of Its Constituents. In 2 N H_2SO_4 $[\alpha]_D$ of 703A fell to +50° in 6 hr at 98°; darkening made further measurements impossible. However, the hydrolysate (4 N HCl, 3 hr, 100°) of the purest sample (D-3) was almost colorless. Preparation 703A (11 mg) in 2 ml of 2 N HCl was hydrolyzed in a sealed tube at 100° for 6 hr and neutralized with Ag_2CO_3. Galactose, glucose, rhamnose, galactosamine, and glucosamine were detected on chromatograms. Mannosamine and galacturonic and glucuronic acids were absent, as were also glycerol, erythritol, threitol, and ribitol.

A galactosamine:glucosamine ratio of 0.6:1 was indicated after separation on and elution from chroma-

TABLE I: Analyses of S VII.[a]

Constituent or property	703A[b]	711[c]	R-1[d]	R-4[d]	P-3[e]	D-3[f]
N	2.7	3.8	2.7	3.1		2.4
P	0.6	2.5	0.1	0.3	0.2	0.2
$[\alpha]_D$, deg	+82	+69	+66	+91	+92	+80 ± 5
	Analyses on Polysaccharide					
Rhamnose[g]	15.5–19	10	15	14.5–16	16.5	18
Hexoses[h]					36	38
Hexoses[i]					38	37
Gal:Glc (approximately)[j]	2.5:1		2:1	1.5:1		
Hexoses:Rham (approximately)					2:1	2:1
	Analyses on Hydrolysates					
Rhamnose[g]	13.8–15.8		14	14.5		
Galactose[k]	15.8–18.9		21.5	17		
D-Galactose[l]	11.1–14			17.5		
D-Glucose[m]	10–11		9.5	8.5		
N-Acetylhexosamine[n]	31–33[o]		21	25.5[p]	33[q]	33.5[r]
Galactosamine:glucosamine	0.6:1[s,t]			1:1.1[s]		1:1.1[t]
Hexose:rhamnose	1.7:1		2.0:1	1.6:1		
D-Galactose:D-glucose	1.7:1		2.3:1	2:1		

[a] Percentages of neutral sugars calculated as anhydro sugars; released hexosamines as anhydro-*N*-acetylhexosamine. [b] Prepared and partially analyzed by Heidelberger *et al.* (1950). [c] Principal product of fractionation by alcohol. [d] Recovered from specific precipitates. [e] Fractionation by $(NH_4)_2SO_4$ of a sample analogous to 711. [f] P-3 further purified on DEAE-cellulose. [g] Usually according to Dische and Shettles (1948). [h] By primary cysteine reaction and calculated from most probable galactose to glucose ratio, 2:1; *cf.* footnotes *i* and *k*. [i] Phenol–H_2SO_4 (Dubois *et al.*, 1956), corrected for rhamnose determined as in footnote *g*. [j] Determined by primary and secondary cysteine reactions (Dische and Danilchenko, 1967). [k] By primary cysteine reaction, corrected for D-glucose determined by Glucostat. [l] Determined with Galactostat, corrected for galactosamine present. [m] Determined with Glucostat. [n] Usually on 2 N HCl, 100° hydrolysate by method of Rondle and Morgan (1955). [o] Hydrolysis for 2, 4, 6, or 9 hr with 2 N HCl. [p] Hydrolysis for 6 hr with 2 N HCl. [q] 6 N HCl, 1–3 hr at 100°. [r] 4 N HCl, 3 hr at 100°. [s] After chromatographic separation on paper. [t] After chromatography on Dowex 50 (H^+). Glucostat and Galactostat furnished by Worthington Biochemical Corp.

tograms and confirmed when a 2 N HCl, 6-hr hydrolysate of 5 mg of 703A, free of excess HCl, was chromatographed with 0.33 N HCl on a Dowex 50 (H^+) column (1.0 × 37 cm) previously calibrated with glucosamine and galactosamine (Gardell, 1953). Neutral sugars were eluted first with water. The ratio of the eluate volumes (R_V) ($GalNH_2$:$GlcNH_2$) at the peak positions for the amino sugars was 1.17 (calcd from Gardell's peaks, 1.20), identical with the value for the mixture of authentic galactosamine and glucosamine. With the same eluent and a column of Zeocarb-225 (H^+) Crumpton (1959) also found R_V ($GalNH_2$:$GlcNH_2$) 1.20, as well as R_V ($TalNH_2$:$GlcNH_2$) 1.60. This would appear to exclude the presence of talosamine in S VII.

Traces of ninhydrin-positive components corresponding to alanine, lysine, glycine, and/or glutamic acid, probably derived from C substance or other unrecognized impurity, were detected in 4 and 6 N HCl hydrolysates. Maximal release of amino sugars, tested with P-3, occurred with 4 N HCl in 3–6 hr, with 6 N HCl in 1–3 hr.

Recovery of S VII from Its Precipitate with Anti-Pn VII and Hydrolysis with Acid. Partially purified antibody was prepared from a pool of 290 ml of C-absorbed anti-Pn VII horse sera by precipitation with 10–15 volumes of 0.005 M KH_2PO_4, adjusted to pH 6.0 (Felton, 1931, and earlier papers). The insoluble proteins containing the antibodies were centrifuged, dissolved in 120 ml of 0.9% salt solution, and brought to pH 7–7.5 with minimal Na_2HPO_4. After addition of 30 mg of S VII 703A in 10 ml of saline the mixture was left at 0° for several days, centrifuged, and the precipitate was washed three times with saline and once with ice-cold water. Trituration with 2–3 ml of 5% CCl_3COOH gave a gel, while 14 ml of acid resulted in denaturation and precipitation of the antibody, which was washed five times with small volumes of 5% CCl_3COOH. The combined supernatants were dialyzed in the cold against distilled water, and the solution was evaporated to dryness *in vacuo*. S VII recovered (R-1) was 15.5 mg.

In other experiments washed specific precipitates were dissolved in 0.15 N NaOH solution (15–20 ml) at 0° and poured into twice the volume of 10% CCl_3COOH. S VII was recovered, much as before. No degradation was detected on chromatography of a sample of 703A similarly treated with alkali.

S VII R-1 was hydrolyzed in 2 N HCl at 100° for 6

TABLE II: Antibody Nitrogen Precipitated from Type VII Antipneumococcal Rabbit and Horse Sera at 0° by S VII and Other Pneumococcal Polysaccharides.[f]

Polysaccharide	Quantity Used (μg)	Rabbit C[a] (μg)	H 895 C[a] (μg)	H 937 C[a] (μg)	H 1007 C[a] (μg)	H 1074 C[a] (μg)
S VII[b]	100				360	451
	200				352	522
	300		570			
	400		494	880		479
	500			840		
	1000	4930				
S XIV	50				40[d]	
			7[e]	35[e]		24[e]
	100				46[d]	
S XVIII			7[e]	0[e]		4[e]
S XIX	50				16[e]	
			1[e]	70[e]		10[e]
	100				15[e]	

[a] Absorbed with pneumococcal C substance; H, horse. [b] Periodate-oxidized S VII failed to precipitate the horse anti-Pn VII sera. The rabbit serum was not tested. [c] From Heidelberger and Tyler (1964). The values given are for maximal precipitation. [d] Supernatants plus S XIX at the 50-μg level gave 8 μg of N. [e] Supernatants plus S XIV at the 50-μg level gave 34 μg of N; plus dextran N236, 16 μg of N; intact serum gave 40 and 17 μg, respectively. From *d* and *e* the remaining supernatants which had reacted with both S XIV and S XIX were combined and precipitated with tamarind fraction A and *K. grandifolia*, yielding 114 and 18 μg of N, respectively; intact serum gave 156 and 28 μg of N. [f] All results calculated to 1.0 ml of antiserum. Analyses on antiserum 1007 in this and the next table carried out by W. P. Grosvenor.

hr and the hydrolysate was neutralized with $AgCO_3$. The filtrate was concentrated and examined by chromatography. The sugars detected (galactose, glucose, rhamnose, galactosamine, and glucosamine) were identical with those from 703A from which R-1 was derived. Two amino acids were also present and obviously arose from the small amounts of denatured protein which contaminated all samples of S VII recovered from specific precipitates. The amino sugars were eluted from chromatograms and degraded by ninhydrin (Stoffyn and Jeanloz, 1954). Chromatography of the products showed that arabinose and lyxose were produced from the glucosamine and galactosamine fractions, respectively.

Isolation and Characterization of the Neutral Sugars of S VII. Preparation 703A (500 mg) was hydrolyzed with 2 N H_2SO_4 for 5 hr on a boiling-water bath, cooled, and neutralized with $Ba(OH)_2$ solution. $BaSO_4$ was centrifuged, washed twice with water, and the combined supernatants were evaporated to small volume *in vacuo*. Inorganic material precipitated by ethanol was removed. The solution was evaporated to dryness, the residue was dried over P_2O_5 *in vacuo*, and a portion (30 mg) was removed for analyses. The remainder (408 mg) was dissolved in 4 ml of water and freed of amino sugars on Dowex 50 (H^+) (200–400 mesh). The eluate and washings were combined, evaporated *in vacuo*, and dried over P_2O_5 to give a syrup (245 mg) which contained mainly galactose, glucose, and rhamnose on chromatography. The monosaccharides were separated on Whatman No. 3MM paper by multiple development and eluted with water.

Portion i (60 mg), a syrup, was homogeneous on chromatograms and on ionophoresis (Foster, 1952) in borate buffer (pH 10) and identical with authentic galactose. Crystallization from aqueous ethanol gave two fractions, of which the first had $[\alpha]_D$ +62.5–79.7° (*c* 0.69), mp 146–147°, solidified and remelted at 166–168°. The second had $[\alpha]_D$ +122.4–75.3° (*c* 0.43), mp and mmp 166–168° with authentic α-D-galactose (β-D-galactose has $[\alpha]_D$ +53–80° and α-D-galactose $[\alpha]_D$ +151–80°). Both fractions were combined to prepare the methylphenylhydrazone (Hirst *et al.*, 1947): mp 185–186°; mmp 187–189° with the authentic derivative, mp 188–189°.

Chromatographic fraction ii (34 mg) was glucose with a trace of galactose. The syrup ($[\alpha]_D$ +53° (*c* 0.53)) yielded crystals from aqueous ethanol of mp 146–147°, mmp 147–148° with authentic α-D-glucose. The *p*-nitroanilide failed to crystallize.

Chromatographic fraction iii (58 mg), with $[\alpha]_D$ +16° (*c* 1.00), was chromatographically and ionophoretically identical with rhamnose but contained a trace of material which stained as a hexose and moved slightly faster than rhamnose on chromatograms. It was crystallized as the hydrate: $[\alpha]_D$ +11–14° (*c* 0.36), mp 87–92°; mmp 86–91° with an authentic sample, mp 86–90°. The benzoylhydrazone (Hirst *et al.*, 1947) had mp and mmp 188–189° with an authentic specimen of L-rhamnose benzoylhydrazone. The neutral sugars

TABLE III: Maximal Antibody Nitrogen[a] in Micrograms Precipitated from Type VII Antipneumococcal Sera at 0° by Polysaccharides Containing D-Galactose, also by Dextrans.[cc]

Polysaccharide	Antipneumococcal VII Sera				
	Rabbit C[b]	H 895C[b]	H 937C[b]	H 1007C[b]	H 1074C[b]
S VII	4930	570	880	360	522
Tamarind A[c]	653[d]	193[e,f]	349[g]	156	193[h]
Tamarind B[c]	553[i]	170	284		177
Carob		55	143		69
Guar		4	4[j]		26[k]
Karaya[l]			116[m]		69[h]
Ketha[n]	32	79[o]	88[p]		86[q]
K. grandifolia (treated with alkali)		47	67[r]	28[s]	
T. cuspidata		82[t]	221[u]		
Gum ghatti			37[v]		
Combretum leonense			58[w]		
Sassafras albidum			58[x]		
Blood group					
A (human cyst)			5		6
B (human cyst)					21
A + O (hog mucin)			23		12
Salmonella anatum					
Antigens 3, 10			14		
Antigens 3, 15			36[y]		
Antigens (3), (15), 34			24		
Dextran N326		0	46	17	22[z]
Dextran 1355-S4		7	27[aa]		35[bb]

[a] Values on both sides of the maximum are omitted to save space, but the supernatants from these tubes were usually added to those at the maximum and used for the analyses in the footnotes below. [b] C, absorbed with pneumococcal C substance; H, horse. [c] See Heidelberger (1963). [d] Supernatants plus tamarind B gave no precipitate. [e] Supernatants plus S VII gave 361 μg of N. [f] Supernatants mixed with those of tamarind B, plus *T. cuspidata* gave 6 μg of N. [g] Supernatants plus *T. cuspidata* gave 82 μg of N, plus S VII gave 557 μg of N, plus dextran 1355-S4, dextran N236, gave 15 and 0 μg of N, respectively. [h] Actually run on supernatants from which S II had precipitated 2 μg of N. [i] Supernatants plus tamarind A gave no precipitate. [j] Single determination. [k] Supernatants plus karaya gave 52 μg of N. [l] *Cachlospermum gossypium*, also called kutira. [m] Supernatants plus ketha plus *K. grandifolia* gave 20 and 7 μg of N, respectively. [n] *F. elephantum*. [o] Supernatants plus tamarind A gave 97 μg of N. [p] Supernatants plus karaya gave 50 μg of N. [q] Supernatants plustamarind B gave 104 μg of N. [r] Supernatants plus ketha plus karaya gave 38 and 44 μg of N, respectively. [s] Supernatants plus S XIV gave 18 μg of N. [t] Supernatants plus tamarind A gave 126 μg of N. [u] Supernatants plus tamarind A gave 195 μg of N. [v] Supernatants plus ketha gave 55 μg of N; the doubly absorbed supernatants plus tamarind A, 193 μg of N. Supernatants plus karaya gave 85 μg of N; the doubly absorbed supernatants plus tamarind gave 168 μg of N. [w] Supernatants plus *S. albidum* gave 22 μg of N. [x] Supernatants plus ketha gave 55 μg of N; plus gum ghatti gave 19 μg of N; *K. grandifolia* gave 23 μg of N. [y] Supernatants plus gum ghatti gave 25 μg of N. [z] Supernatants plus dextran 1355-S4 gave 10 μg of N. [aa] Supernatants plus gum ghatti, dextran N236 gave 16 and 6 μg of N, respectively. [bb] Supernatants plus dextran N236 gave no precipitate, nor did one form after further addition of the supernatants of footnote *z*. [cc] Calculated to 1.0 ml of antiserum.

were isolated from the hydrolysate of S VII 703A in the approximate ratio galactose:glucose:rhamnose of 1:0.6:1.

Isolation and Characterization of the Amino Sugars of S VII. Fraction P-3, containing <2.5% C substance, was used, as negligible amounts of amino sugars would be derived from the known contaminant. The fraction (150 mg) was hydrolyzed with 15 ml of 2 N HCl at 100° for 3 hr and neutralized by passage through 30 ml of Dowex 2 (CO_3''). The eluate and washings were concentrated *in vacuo*, and transferred to a column of 10 ml of Dowex 50 (H^+) to absorb amino sugars Neutral sugars (65 mg) were recovered from the eluate; their hexose:rhamnose ratio was somewhat less than 2:1.

Amino sugar hydrochlorides (53 mg) were eluted from the Dowex resin with 20 ml of 1 N HCl and fractionated with 0.33 N HCl as eluent, on a column (2.6 × 36 cm) of Dowex 50 (H^+) (200–400 mesh) calibrated for glucosamine and galactosamine (R_V $GalNH_2$:$GluNH_2$, 1.17) (calcd from Gardell, 1953). The ratio of recovered galactosamine to glucosamine, by assay

TABLE IV: Inhibition of the S VII Anti-Pn VII System by Mono- and Disaccharides.[a,e]

Inhibitor		Rabbit C		Horse 937C	
Substance	Amt Added (μmoles)	Antibody N Pptd (μg)	Inhibn (%)	Antibody N Pptd (μg)	Inhibn (%)
None		82–84[b]		46–49[b]	
D-Galactose	193[c]			28	43
	113	59	28	32	35
D-Glucose	112	69	16	43	10
L-Rhamnose	210[c]			31	37
	112	69	16	32	33
D-Galactose + L-rhamnose	193 + 210[c]			20	59
N-Ac-D-galactosamine	73	78	5	43	12
N-Ac-D-glucosamine	86	70	15	39	21
α-Methyl galactoside	22	77	8	44	4
β-Methyl galactoside	22	69	18	38	17
Gentiobiose	23	91[d]	5	45	8
Lactose	23	70	17	40	13
Allolactose	20	85[d]	11	35	29

[a] The final volume of reaction mixture was 0.50 ml, unless otherwise noted. Maltose, cellobiose, and melibiose at the 23-μmole level gave no inhibition in the horse serum and 12% or less in the rabbit serum. [b] Range of values in different experiments. [c] Final volume, 0.525 ml. [d] In these tests the reaction without inhibitor gave 96 μg of N. [e] Sera absorbed with PnC, reactions at 0°.

of aliquots, was 0.9:1. Appropriate fractions were pooled, concentrated, and dried over P_2O_5 *in vacuo*. Fraction i (16 mg), crystalline, was chromatographically identical with glucosamine. After recrystallization, it had $[\alpha]_D$ +70° at equilibrium (*c* 0.60), decomposing at *ca.* 180°, and was thus identical with D-glucosamine hydrochloride. On degradation with ninhydrin (Stoffyn and Jeanloz, 1954) arabinose was produced. The product of N acetylation (Roseman and Ludowieg, 1954; Crumpton, 1959) was identical by chromatography with *N*-acetylglucosamine and had $[\alpha]_D$ +49° (*c* 0.37), mp 207–208°; mmp 208–209° with authentic *N*-acetyl-D-glucosamine, mp 209–210°.

Fraction ii (20 mg) also crystallized but had $[\alpha]_D$ +54° (*c* 0.93). Owing to its rather low specific rotation we cannot be certain that it is entirely in the D form. On chromatography in ethyl acetate–pyridine–acetic acid–water (5:5:1:3) (Fischer and Nebel, 1955), the product showed galactosamine as the main component, with traces of two slower components. After purification on paper a portion was degraded with ninhydrin. Lyxose was produced and identified on chromatograms in two different solvents. Since lyxose would also be formed from talosamine, the remainder of the sample was N acetylated and chromatographed in butanol–pyridine–water (6:4:3). It had R_{NAcglc} 0.92, as did *N*-acetylgalactosamine (*cf.* also Crumpton, 1959; Heyworth *et al.*, 1961) whereas these workers showed *N*-acetyltalosamine to have $R_{NAcglc} > 1.1$.

Oxidation of S VII-703A by Periodate, Reduction of the Product with Borohydride, and Acid Hydrolysis of the Reduced Material. Periodate consumed and formic acid liberated were determined according to Schiffman *et al.* (1962), but with 200-μl aliquots instead of 20-μl aliquots. Preparation 703A (9 mg) was oxidized with 10 ml of 0.01 M $NaIO_4$. Oxidation was complete in 44 hr at an uptake of 0.77 mmole of $NaIO_4$, with liberation of 0.11 mmole of HCOOH/180 mg of S VII.

After oxidation of 19.8 mg of 703A in 22 ml of 0.01 M periodate the mixture was dialyzed and formaldehyde, measured by chromotropic acid (Lambert and Neish, 1950), was determined on samples of the dialysate; it corresponded to 0.07 mmole/180 mg of S VII. Mannitol was used as the standard. The oxidized S VII in the dialysis bag was reduced with 30 mg of $NaBH_4$ in 3 ml of water and again after 4 hr at room temperature. The mixture was left at 3° overnight, dialyzed, and lyophilized. The product (11 mg) was hydrolyzed with 1 N H_2SO_4 at 100° for 6 hr, neutralized with $Ba(OH)_2$, and chromatographed. Galactosamine, rhamnose, erythritol, or threitol, and glycerol were detected. Limited oxidation of the most highly purified preparation (D3) at room temperature in the dark for 4 hr (Roberts *et al.*, 1963) gave the same products. Arabinose was absent, excluding the presence of nonreducing end groups of galactofuranose in S VII.

Immunochemistry of S VII and Anti-Pn VII Sera. In Table II are shown quantitative data on the precipitation of five anti-Pn VII sera by S VII and on the cross-reactions of the polysaccharides of pneumococcal types XIV, XVIII, and XIX (*cf.* also Heidelberger and Tyler, 1964) in these antisera.

Table III shows the maximal amounts of antibody nitrogen precipitated from anti-Pn VII sera by dextran

TABLE V: Inhibition of the Cross-Reaction between Tamarind A and Type VII Antipneumococcal Sera by Mono- and Disaccharides.[a]

Inhibitor		Rabbit C		Horse 895C		Horse 937C		Horse 1074C	
Substance	Amt Added (μ-moles)	Antibody N Pptd (μg)	Inhibn (%)	Antibody N Pptd (μg)	Inhibn (%)	Antibody N Pptd (μg)	Inhibn (%)	Antibody N Pptd (μg)	Inhibn (%)
None		52–59[b]		29–32[b]		50–56[b]		31–34[b]	
D-Galactose	113	0	100	1	97	4	93	1	97
	26	12	77	6	81	12	79	7	79
	6	26	50			28	49		
D-Glucose	112	23	59	17	41	30	46	19	39
	22	44	15	28	13	46	16	27	13
L-Rhamnose	112	18	68	12	59	25	55	14	55
	22	39	25	22	31	40	27	22	29
N-Ac-D-galactosamine	77	43	17	24	25	43	23	22	35
N-Ac-D-glucosamine	86	49	13	29	0	50	11	28	10
α-Methyl galactoside	22	42	19	15	53	32	42	11	68
β-Methyl galactoside	22	7	87	8	75	15	73	6	82
Maltose	23	47	20	27	16	46	13	25	19
Cellobiose	24	46	22	30	6	46	13	27	13
Gentiobiose	23	35	36			38	24		
Melibiose	23	38	36	22	31	37	30	15	52
Lactose	23	6	90	6	81	22	59	8	77
	6	31	47	16	48	36	35	14	53
Allolactose	20	6	89	4	87	13	74	5	85
	5	23	62	12	61	23	58	9	73
Galactobiose A	4					41	18		
Galactobiose B	11					11	78		
6-*O*-β-D-Galactopyranosyl-D-galactose	23	0	100			10	80		
	4	18	70			22	60		
4-*O*-β-D-galactopyranosyl-*N*-acetyl-D-glucosamine	9	27	51			35	30		

[a] Conditions as in Table IV; final volume 0.5 ml. [b] Range of values in different experiments.

and a number of galactose-containing polysaccharides originating in other microorganisms, plants, and animals. The strongest cross-reactions were given by the two fractions of tamarind seed polysaccharide. Fraction A, the first precipitated by isopropyl alcohol (Heidelberger, 1963) reacted more strongly than did the second fraction (B) but both precipitated the same portion of the antibodies (footnotes *d*, *e*, *g*, and *i*). Earlier studies and those of Kooiman (1961) showed that the tamarind polysaccharide contained xylose, carried nonreducing β-galactopyranose end groups, and that roughly two of every three residues of glucose were linked β-1,4,6 and one β-1,4. The cross-reactivity in anti-Pn VII is undoubtedly due to the multiple residues of β-D-galactopyranose, with possible reinforcement by D-glucose, since the supernatants showed reduced precipitation with dextrans (footnote *g*). Footnotes *k*, *m*, and *n-x* show mainly that all of these gums precipitate the same fraction of antibody or portions of it, undoubtedly because of appropriate residues of D-galactose, as indicated above. Such residues would not necessarily have to be β linked: the galactomannans carob and guar have α-D-galactose end groups. As for the cross-reactions of the dextrans, these appear to be due largely to multiple residues of 1,6-linked D-glucose. This is almost the only linkage in dextran N236 and occurs, with other linkages, in the other dextran. Precipitation of a serum with one of the dextrans greatly reduced or abolished reaction with the other (footnotes *z*, *aa*, and *bb*).

The inhibition tests recorded in Table IV show D-galactose to be the strongest monosaccharide inhibitor of homologous S VII anti-Pn VII precipitation, followed closely by L-rhamnose. High concentrations were required for significant inhibition. At much lower levels β-methyl D-galactoside was a better inhibitor than the α anomer, while allolactose, 6-*O*-β-D-galactopyranosyl-D-glucose, inhibited twice as well as lactose, the 4-*O* isomer, in the horse serum, but less strongly in the rabbit serum.

Tests on the inhibition of the cross-reaction of tama-

rind A in anti-Pn VII sera are given in Table V. These show that D-galactose and its β-methyl glycoside react most strongly and about equally, while α-methyl galactoside is less potent. Less active were the two *N*-acetylamino sugars. The residues of *N*-acetylgalactosamine were resistant to periodate and possibly serve as branch points in S VII and would then be relatively inaccessible as determinants of immunological specificity. Of the disaccharides tested, allolactose was the best inhibitor, followed closely by lactose and 6-*O*-β-D-galactopyranosyl-D-galactose and galactobiose B which is probably identical with it. Again the α anomers melibiose and galactobiose A were less active. 4-*O*-β-D-Galactopyranosyl-*N*-acetyl-D-glucosamine was also a good inhibitor of the cross-reaction.

In Table VI it is shown that D-glucose, not surprisingly, is a better inhibitor of the cross-reaction between dextran and anti-Pn VII than is D-galactose. In this system allolactose has about the same activity as galactose, and lactose is a poorer inhibitor. As has been shown on previous occasions, a sugar such as galactose which is not present in the cross-reacting antigen, may none the less function as an inhibitor of the cross-precipitation if it is a part of the determinant which gave rise to the antibodies.

TABLE VI: Inhibition of the Reaction between Anti-Pn VII Serum 1074C and Dextran 1355-S4 by Mono- and Disaccharides.[a]

Inhibitor	μmoles	N Pptd (μg)	Inhibn (%)
None		26	
D-Galactose	193	11	58
	97	15	42
	48	19	27
None		27	
D-Glucose	112	9	67
	56	8	70
Lactose	57	22	15
Allolactose	50	17	35

[a] 1.0 ml of serum; total volume 1.65 ml.

The inhibition tests thus confirm the evidence adduced by the analyses for cross-precipitation; namely, that at least a portion of the D-galactose in S VII occurs as β-linked nonreducing end groups. Although a second residue of D-galactose may be linked to this, the next sugar is more likely to be D-glucose, attached at 6 or 4, since such galactoglucoses are inhibitors of both the homologous and cross-precipitations. Linkage of the glucose at 2 is not excluded. The corresponding galactoglucose was not available. Since the glucose in S VII is not stable to oxidation by periodate it cannot be linked 1,3. As only *N*-acetylgalactosamine survives the oxidation, it is probable that this sugar provides the numerous branch points which appear to be a structural feature of S VII.

Another possibility is that there may be occasional end groups of L-rhamnose in addition to those of galactose, or that rhamnose is the next sugar to galactose. This was not tested, since galactorhamnoses were not available.

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