The Combining Regions of the Type III Pneumococcus Polysaccharide and Homologous Antibody*

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Received June 3, 1963

The combining regions of the type III pneumococcus polysaccharide (S III) and homologous antibody were studied by the hapten-inhibition method with oligosaccharides prepared by acid-catalyzed and enzymic hydrolyses of the polysaccharide and purified chromatographically. The antibodies in two rabbit sera differed in ease of inhibition by such oligosaccharides. It is postulated that they differ in average sizes of their combining sites. Precipitin and inhibition data are compatible with the concept of anti-S III combining sites varying in size and directed toward antigenic determinants recurring in S III at intervals along a linear chain.

Serologic type-specificity among pneumococci is determined by capsular polysaccharides (reviewed in White, 1938) and it was recognized that these are ideal materials through which to relate the chemical composition of an antigen to its serological specificity (Heidelberger and Avery, 1923, 1924).

The work herein reported extends the observations on antibodies to dextrans (Kabat, 1961) to a system with a carbohydrate antigen of greater complexity. Type III pneumococcal polysaccharide (S III) was chosen for the following reasons:

(1) The structure of S III has been studied by physical (Record and Stacey, 1948; Koenig and Perrings, 1955) and chemical methods (Heidelberger and Avery, 1924; Heidelberger *et al.*, 1925; Heidelberger and Goebel, 1926, 1927; Hotchkiss and Goebel, 1937; Reeves and Goebel, 1941; Adams *et al.*, 1941). The results indicate that S III is a linear polymer of repeating units of cellobiuronic acid [4-*O*-(β-D-glucopyranosyluronic acid)-D-glucose] in β-1,3- glucosidic linkage.

(2) S III, in contrast to dextran, possesses acidic and strongly ionic properties. Moreover, uronic acids have long been known to play a role in the immunological specificity of polysaccharides and artificial conjugates of carbohydrates to proteins (Goebel, 1936; Goebel and Hotchkiss, 1937; Goebel, 1938).

(3) S III is easily obtained in highly purified form, free of nitrogen, a characteristic which dictated its use in the classic studies of the precipitin reaction (Heidelberger and Kendall, 1929, 1933, 1935a, 1935b, 1937) that laid the foundations of quantitative immunochemistry.

(4) Fragments of S III, obtained by acid hydrolysis, with estimated average molecular weights of 550–1800, precipitate with horse, but not with rabbit antibody (Heidelberger and Kendall, 1933), and inhibit the precipitation of the latter with unhydrolyzed polysaccharide. It seemed of interest to reexamine these earlier observations, and to isolate and characterize the oligosaccharides by the more powerful methods that have since become available.

MATERIALS AND METHODS

Rabbit antiserum 134, prepared by immunization with formalinized type III pneumococci (0.1 mg N/ml), was supplied by Dr. Richard E. Rosenfield. Animals were bled on three successive days and the sera were pooled. Rabbit antiserum R-3-129 was a gift of Dr. Lawrence Levine, who found by micro complement-fixation inhibition (Wasserman and Levine, 1961) that its reaction with S III was not inhibited by D-glucuronic acid.

Horse anti-Pn III serum 393,[1] bled 4-16-34, was obtained from Dr. Jessie L. Hendry, Division of Laboratories and Research of the N. Y. State Department of Health. A Felton antibody solution (Felton, 1931) was prepared from horse anti-Pn III H403, bled 6-28-34; it contained 1.62 mg of total N/ml, and 0.95 mg of antibody N/ml, or 59% antibody N.

S III, preparation 36a, from Dr. Rachel Brown, was used to obtain oligosaccharides and for precipitin and inhibition assays; it contained 12.6% ash. S III preparation 108a, E. R. Squibb and Sons, was also used; both behaved similarly in quantitative precipitin determinations with rabbit antisera.

Monosaccharides used as inhibitors were: glucose from Mallinckrodt and sodium glucuronate from Mann Research Laboratories. The latter was prepared from glucuronolactone (Corn Products Refining Co.) by neutralization with NaOH in 50% methanol (Hach and Benjamin, 1954).

Crystalline synthetic cellobiuronic acid was a gift of Dr. Georg Jayme (Jayme and Demmig, 1960). For use as inhibitor, it was titrated with NaOH until neutral to bromothymol blue, and the solution was adjusted with NaCl to a salt concentration of 0.9%. Cellobiose was from Mann Research Laboratories, and laminaribiose was obtained from Dr. S. A. Barker.

S III depolymerase, lot 1759D, was prepared from the Dubos strain of S III bacillus by Dr. Alan W. Bernheimer (1953). β-Glucosidase was obtained from Sigma Chemical Co. (lot G21B-077).

Descending chromatography was carried out with Whatman 1, 3MM, and 17 papers, and with Schleicher and Schuell 589 papers. The Whatman 3MM and 17 papers were used for preparative chromatography after prewashing with 1 N acetic acid and water. Solvents employed were butanol–acetic acid–water, 50:15:35 and propanol-water, 7:2. Spots were detected with alkaline silver nitrate (Partridge, 1948; Trevelyan *et al.*, 1950), and fixed with 10% aqueous sodium thiosulfate.

Separation of compounds by column chromatography was based on the method of Weissmann *et al.* (1954). Dowex 1 × 10, 200–400 mesh, was cycled three or more

* Aided by grants from the National Science Foundation (G-18727) and the general Research Support Grant of the National Institutes of Health, U. S. Public Health Service. Part II of a dissertation submitted by Rose G. Mage 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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[1] Abbreviations used in this work: Pn, pneumococcal.

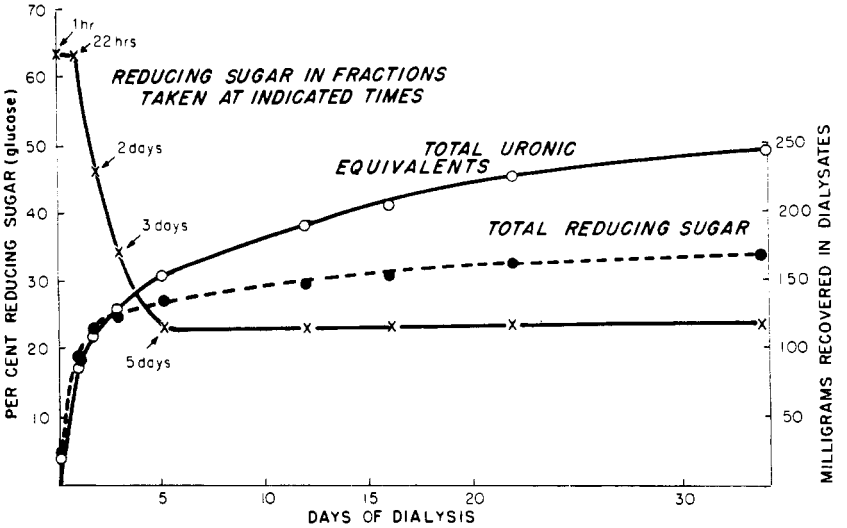

FIG. 1.—Results of dialysis of S III enzyme digest. Total quantities of uronic acid and reducing sugar accumulated by successive dialyses of enzyme-digested S III, and reducing sugar (as glucose equivalents) in dialysis fractions, taken at indicated times, as per cent of sugar weight. The sugar weight was estimated by multiplying the uronic equivalents found in each dialysate by 1.74.

times with 2 N NaOH and 2 N HCl, and once with 2 N NaOH and 2 N formic acid. It was then stored as the chloride and converted just before use to the formate with M sodium formate. A two-step gradient, with two mixing vessels each containing 500 ml, was employed. Uronic acids were determined by a modification (Khym and Doherty, 1952; Weissmann *et al.*, 1954) of the orcinol method (Brown, 1946), and by the carbazole method (Dische, 1947). Some uronic acid analyses were carried out by a more sensitive carbazole reaction (Bitter and Ewins, 1961). Reducing sugars were determined by a modified alkaline ferricyanide method (Park and Johnson, 1949; Schiffman *et al.*, 1958). Whenever phosphate was present, ferric Duponol reagent containing 6 ml of concentrated sulfuric acid per liter was used. Glucose was determined by the primary cysteine method (Dische *et al.*, 1949). Reduction of all volumes to one-third in the orcinol reaction and to one-fifth in determinations of uronic acid and glucose permitted a decrease in the size of sample used.

In studying the structures of the isolated oligosaccharides, reduction with sodium borohydride was used to modify the reducing end. The reaction was carried out at 0–4° for 48 hours, with 100–500 μg of oligosaccharide in 100–500 μl of water and an equal volume of sodium borohydride at a concentration of 10 mg/ml. To analyze for the remaining glucose and glucuronic acid, reduced oligosaccharides were acidified with HCl, evaporated to dryness, repeatedly taken up in small volumes of methanol, and dried *in vacuo* to remove boric acid as methyl borate. Finally the reduced sugars were dissolved in a suitable volume of water. For colorimetric analyses, especially for glucose, it was important to include standards added to identical volumes of a water blank run through the borohydride reduction procedure, since color development is strongly dependent on chloride ion concentration. Controls of glucose, sodium glucuronate, and, in some experiments, cellobiuronic acid were carried through reduction procedures, sugar analyses, and formaldehyde determinations following oxidation with periodate.

Reduced oligosaccharides were prepared for use as inhibitors by neutralization with Dowex 50 (H^+), and were quantitatively recovered by repeated washing of the Dowex. Boric acid was removed as described above and the final products were dissolved in 0.9% sodium chloride.

After the reduced compounds were oxidized with sodium metaperiodate for one-half hour at room temperature (Schiffman *et al.*, 1962a), formaldehyde was determined by the chromotropic acid method of Smith and Montgomery (1956). Erythritol was used as a standard, and reduced glucose, sodium glucuronate, and cellobiuronic acid gave within 10% of theoretical formaldehyde. Oligosaccharides were treated with β-glucosidase to establish whether or not they contained a beta-linked glucose at the nonreducing end; the conditions used were 48 hours at 37° in 0.01 M sodium phosphate buffer at *p*H 5.6. Under these conditions, paper chromatographic separation on Schleicher and Schuell 589 paper in propanol-water (7:2) showed that cellobiose and laminaribiose were split to glucose, but cellobiuronic acid was unchanged.

Precipitin curves for the various anti-Pn III sera with S III were obtained by the standard procedures (Kabat, 1961). Precipitated antibody nitrogen was measured by the Markham micro-Kjeldahl nitrogen and Folin-Ciocalteu tyrosine determinations. Inhibition assays were set up as described by Kabat and Schiffman (1962). Nitrogen analyses were carried out by the adaptation of the ninhydrin procedure of Rosevear and Smith (1961) described by Schiffman *et al.* (1962b), with the use of not more than 5–8μg of antibody N per point.

Enzymic Hydrolysis.—S III, 868 mg (759 mg, ash-free, preparation 36a, as a 3.2 mg/ml solution), was treated with depolymerase at 37° in 0.05 M potassium phosphate buffer containing 0.006 M NaCl, *p*H 5.86, at a substrate-enzyme ratio of 20:1, with toluene as preservative. Enzymatic action was followed by assay of reducing sugar. After 189 hours at 37°, when values of reducing sugar had reached 25%, the digest was dialyzed against nine successive changes of distilled water, each about 2 liters, during a total of 34 days, at 0°. Successive dialysates were kept separately in an attempt to fractionate oligosaccharides by size and to keep the later dialysates salt-free. All dialysates were concentrated and chromatographed on Whatman 3MM paper to obtain oligosaccharides. Corresponding areas on paper chromatograms were eluted, pooled, and rechromatographed until the components obtained gave only one major spot on paper. Fractions containing

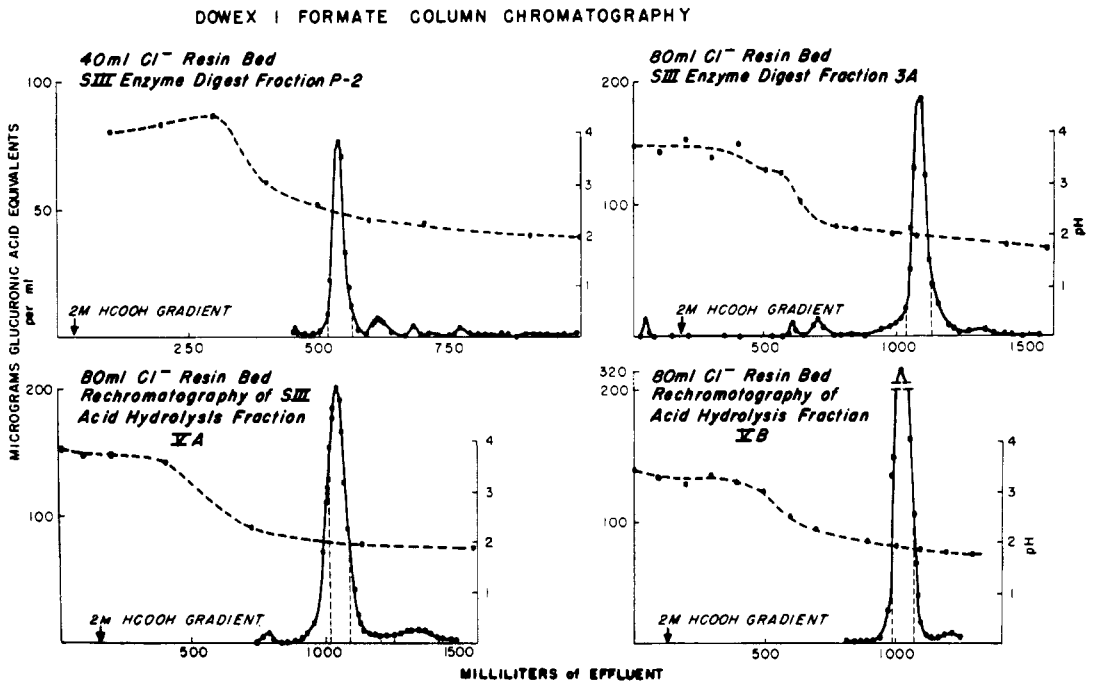

FIG. 2.—Results of repurification of P-2, 3A, VA, and VB on Dowex-1 formate columns.

salts were more difficult to resolve into component sugars. Such salt areas were cut out and chromatographed repeatedly. Some separations of salt-containing fractions were made on Whatman 17 paper. Two of the components were purified further on Dowex 1 columns.

Acid Hydrolysis.—To 1.4 g. of the Na salt of S III (1.225 g, ash-free), preparation 36a, in 40 ml of water, an equal volume of 1.97 N HCl was added. The mixture was held at 90–94° for one-half hour. Insoluble polysaccharide separated, but the soluble sugars in the supernatant were recovered by lyophilization and the precipitate was repeatedly treated with acid for further recovery of sugars. The second hydrolysis was carried out for 1 hour in 80 ml and hydrolyses three through seven were each run for one-half hour in 40 ml total volume. The lyophilized sugars were chromatographed preparatively on Whatman 17 paper. Three areas were cut from the papers: the first corresponding in R_F to cellobiuronic acid, the second containing slower migrating oligosaccharides estimated to be ten monosaccharide units or less in size, and the third containing all more slowly migrating material. This last fraction was added to the insoluble portion after the seventh hydrolysis and heated at 90–94° first in 20 ml of water for 1 hour, then in 1 N HCl for one-half hour. Ninth, tenth, and eleventh treatments were carried out similarly. The remaining insoluble polysaccharide was dissolved by cautious neutralization with NaOH; the solution was readjusted to 0.5 N with HCl, and hydrolyzed at 90–94° for 1 hour, most remaining in solution. Sodium ion was removed with Dowex 50 and the effluent was lyophilized. A thirteenth hydrolysis of the chromatographically slowly moving fraction from hydrolysates 8–12 was carried out in 1 N HCl at 90–94° for one-half hour and this material was rechromatographed on Whatman 17 paper. The oligosaccharide fraction of intermediate size (less than 10 monosaccharide units) was chromatographed on Dowex 1 formate (160 ml of resin bed measured in Cl^- form). Five-ml fractions were collected. A two-step gradient, feeding 1 M formic acid into two mixing vessels each containing 500 ml of water, was established after 215 ml of water had emerged from the column. After 1270 ml of effluent had been collected, the 1 M formic acid was replaced by 2 M formic acid and 2340 ml was collected.

RESULTS

Enzymic Hydrolysis.—Figure 1 shows the decrease in reducing equivalents, as per cent of sugar weight, in successive dialysates at the times indicated, through the fifth day. After the fifth day, the per cent reducing sugar became relatively constant. Also shown are the total quantities of uronic acid equivalents and reducing sugar accumulated in the successive dialysates. The lower reducing power of the sugars in the successive dialysates and the slower rate of dialysis suggested that the later fractions were relatively richer in oligosaccharides of longer chain length. After the nine dialyses, the dialyzable sugars from 868 mg of starting material (759 mg on an ash-free basis) totaled 242 mg of uronic equivalents or about 420 mg of sugar (55%). The nondialyzable fraction contained 113 mg of uronic equivalents or about 197 mg of sugar (26%). After exhaustive dialysis, lyophilization, and six deproteinizing treatments with chloroform-butanol (Sevag, 1934; Heidelberger *et al.*, 1936), the final alcohol-precipitated sodium salt weighed 195 mg and contained 93 mg of uronic equivalents. Eighty one per cent of the total sugar was recovered.

Paper chromatographic separation of the dialyzable sugars from the enzymic hydrolysate on Whatman 3MM paper with butanol–acetic acid–water, (50:15:35) for 24–48 hours, gave a series of spots designated in order of decreasing R_F as P-2, 3A, 4A, and 4B; the unresolved slowly moving material between 4B and the origin was called P-5. P-5 was shown to contain five components slower than 4B as well as material that did not move from the origin, even after prolonged paper chromatography in which the solvent was allowed to drip off the end of the paper for periods as long as one week. A spot which moved more rapidly than P–2 with an $R_{glucose}$ similar to cellobiuronic acid, was also seen. It represented less than 5 mg of material and was not studied further. Salts moved in this area of the paper,

TABLE I

ANALYTICAL PROPERTIES AND PROBABLE STRUCTURES OF OLIGOSACCHARIDES FROM ACID AND ENZYMIC HYDROLYSES OF S III

Oligosaccharide														
Code Name	Purified on Paper	Purified on Dowex	Type of Hydrolysis	$R_{glucose}$[a]	Glucose Liberated by β-Glucosidase	Total Uronic Acid: Before $NaBH_4$ (μmoles)[g]	Total Uronic Acid: After $NaBH_4$ (μmoles)[g]	Glucose: Before $NaBH_4$ (μmoles)[g]	Glucose: After $NaBH_4$ (μmoles)[g]	HCHO/μM Oligosaccharide after $NaBH_4$ and IO_4^- (μmoles/μmole oligosaccharide)	Uronic Acid (Orcinol) after IO_4^-: Before $NaBH_4$ (μmoles)	Uronic Acid (Orcinol) after IO_4^-: After $NaBH_4$ (μmoles)	Reducing Sugar (%)	Proposed Structure[f]
P-2	Yes	Yes	Enzyme	0.65	Yes	Orcinol 0.9 Dische 1.1	1.1	1.9	0.93	2.4				Glc-(1 → 3)-GlcUA-(1 → 4)-Glc
3A	Yes	Yes	Enzyme	0.46	Yes	Orcinol 3.7 Dische 3.7	1.9 2.1	3.7	3.3	1.0	2.6–3	2.0	38–41	Glc-(1 → 3)-GlcUA-(1 → 4)-Glc-(1 → 3)-GlcUA
4A	Yes		Enzyme	0.34	Yes	0.56 2.6	0.52 2.2	0.87 4.2	0.54 3.4	1.4		0.43	24–30	Glc-(1 → 3)-GlcUA-(1 → 4)-Glc-(1 → 3)-GlcUA-(1 → 4)-Glc[b]
4B	Yes		Enzyme	0.24	Yes	1.2 4.5	0.9 3.6	1.5 6.2	1.1 6.4	1.1		0.8	24–28	Glc-(1 → 3)-GlcUA-(1 → 4)-Glc-(1 → 3)-GlcUA-(1 → 4)-Glc-(1 → 3)-GlcUA[c]
P-5			Enzyme	[d]									12	Average degree of polymerization 7–14 by HCHO
VA		Yes	Acid	0.42	No	0.9	0.8	1.0	0.4	1.8	0.2-0.3		20	GlcUA-(1 → 4)-Glc-(1 → ?)-GlcUA-(1 → 4)-Glc[e]
VB		Yes	Acid	0.38	No	1.0	0.9	1.2	0.44	2.4	0.5		31	GlcUA-(1 → 4)-Glc-(1 → 3)-GlcUA-(1 → 4)-Glc

[a] $R_{glucose}$ in butanol–acetic acid–water 50:15:35, Whatman 3MM. [b] Contaminated with 4B. [c] Contaminated with 4A and possibly 7-unit sugar. [d] One-week drip in BuOH. HAc-H_2O gave five spots slower than 4B and material at the origin. [e] One of numerous possible structures compatible with the data. [f] Glc = glucose, GlcUA = glucuronic acid. [g] Micromoles of uronic acid and glucose actually found in solutions for analysis.

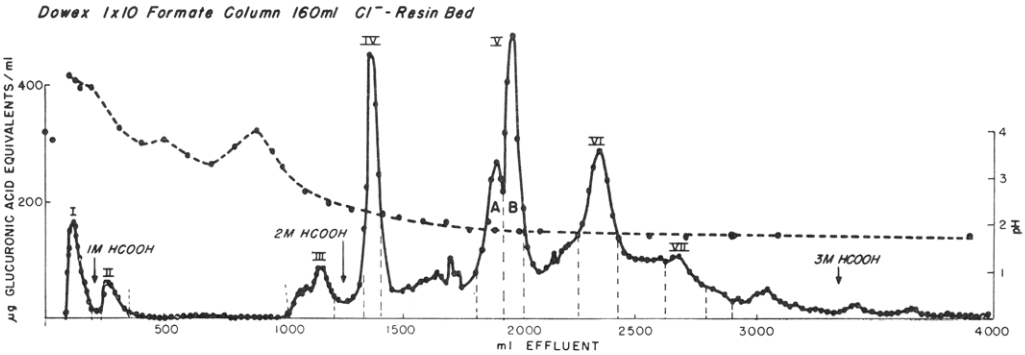

FIG. 3.—Results of chromatography of oligosaccharides from acid hydrolysis of S III.

and may have reduced the yield of components with this mobility. Some material faster moving than the disaccharides was present but was not purified or characterized.

Repurification of P–2 and 3A by column chromatography on Dowex-1 formate (Figure 2, upper two graphs), gave oligosaccharides, structural studies on which are described in a later section and are summarized in Table I. Compounds 4A and 4B were not purified further and were probably cross-contaminated. However, interpretation of their analytical properties becomes simpler if these oligosaccharides are considered to be members of a homologous series and if the cross contamination is borne in mind. The tetrasaccharides VA and VB obtained by acid hydrolysis and described below have clearly been shown to differ from 3A, giving further support for its proposed structure. Yields were P-5, 121 mg; 4B, 41 mg; 4A, 16 mg; 3A, 26 mg; and P-2, 5 mg.

Acid Hydrolysis.—The three fractions obtained by paper chromatography from the thirteen successive hydrolyses of 1.4 g (1.225 g, ash-free) of S III were as follows:

	Weight (mg)	Per cent of recovered sugar
From disaccharide area	258	23.4
From intermediate area	745	67.4
From most slowly moving area	102	9.2
Total in the three fractions	1105	90%, over-all yield

The chromatogram of the intermediate area on Dowex-1 formate is shown in Figure 3. Peak I, eluted with water, contained a mixture of sugars which did not bind to the resin, probably because of formation of lactones and anhydrides. Peak II was chromatographically identical with glucuronolactone. Peak III gave three major spots on paper chromatography in butanol–acetic acid–water, but the components were not obtained in high enough yield for further study. Peak IV was chromatographically identical to cellobiuronic acid. The double peak V was separated into fractions VA and VB and these were rechromatographed separately on Dowex-1 formate columns. Their paper and column chromatographic properties resembled those of fraction 3A obtained by enzymic action. In Figure 2, the results of repurification of these three oligosaccharides on separate Dowex columns are shown together for comparison. The materials that were pooled for further use were those from the regions of the peaks shown between dotted lines. For 3A, VA, and VB, respectively, these were eluted when 855–945, 860–945, and 880–960 ml had emerged from the columns after the start of the formic acid gradients. The peaks marked VI and VII in Figure 3 were recovered and used for quantitative precipitin studies without further purification. In Figure 3, peaks I, II, III, IV, VA, VB, VI, and VII, pooled as indicated by the dotted lines, contained 399 mg or 54% of the 745-mg charge.

Table I lists analytical properties and probable structures of the oligosaccharides isolated by the acidic and enzymic hydrolyses. The values given represent uronic acid and glucose actually found in solutions for analysis before and after reduction with sodium borohydride. In assignment of structures, earlier evidence was tentatively accepted that S III consisted largely of a linear chain of cellobiuronic acid 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucose units, linked β-1,3-(Reeves and Goebel, 1941; Adams *et al.* 1941). Oxidation by periodate has given some confirmatory evidence for 1,3-linkages, but no data have been obtained to show that the isolated oligosaccharides are completely β-linked.

Compound P-2 is considered to be the trisaccharide *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyluronic acid-(1→4)-D-glucose. It contained twice as much glucose as uronic acid and reduction of the reducing end with sodium borohydride halved the glucose value. Oxidation of this reduced trisaccharide with periodate gave 2.4 moles of formaldehyde per mole of reduced P-2. Treatment with β-glucosidase as shown in Figure 4 (l) and (m) gave two new spots, one with the

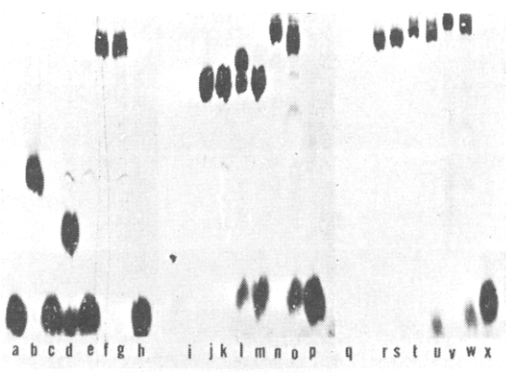

FIG. 4.—Effect of β-glucosidase on oligosaccharides isolated from S III. Descending chromatography on S & S 589 paper in propanol-water, 7:2. Zero-time samples (T = 0) were taken as soon as enzyme had been added at 0°; 48-hour samples (T = 48) after incubation at 37°. With cellobiose, laminaribiose, and P-2, visible splitting had occurred in T = 0 samples by the time they had dried on the paper. (a) D-glucose; (b) cellobiose, T = 0; (c) cellobiose, T = 48; (d) laminaribiose, T = 0; (e) laminaribiose, T = 48; (f) VA, T = 0; (g) VA, T = 48; (h) D-glucose; (i) enzyme alone, T = 48 (the spot is an artifact); (j) cellobiuronic acid, T = 0; (k) cellobiuronic acid, T = 48; (l) P-2, T = 0; (m) P-2, T = 48; (n) 3A, T = 0; (o) 3A, T = 48; (p) D-glucose; (q) enzyme alone, T = 0; (r) VB, T = 0; (s) VB, T = 48; (t) 4A, T = 0; (u) 4A, T = 48; (v) 4B, T = 0; (w) 4B, T = 48; (x) D-glucose.

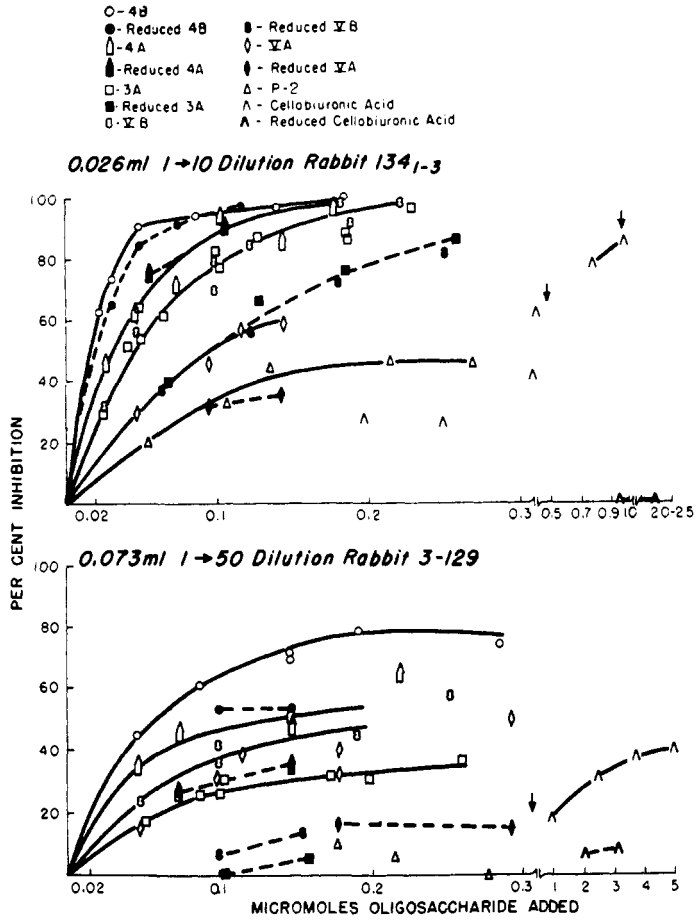

FIG. 5.—Inhibition by oligosaccharides of S III anti-Pn III precipitation.

R_F of glucose, and the other with that of cellobiuronic acid.

Compound 3A contained equimolar proportions of glucose and glucuronic acid, and approximately 50% of the uronic acid was destroyed by reduction with sodium borohydride. It is considered to be the tetrasaccharide *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyluronic acid-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-D-glucuronic acid. Oxidation of the reduced compound with periodate gave 1 mole of formaldehyde per mole from carbon 1 of the reduced uronic acid, and the internal uronic unit remained intact as would occur if it were substituted on carbon three (Table I). β-Glucosidase split glucose from 3A, Figure 4, (n) and (o), producing a second new spot which moved faster than 3A, but more slowly than P-2. From the chromatogram it is difficult to be sure whether complete splitting of 3A occurred after 48 hours. Oxidation of 3A itself with periodate decreased uronic acid from 3.7 to approximately 2.6, or 3.0 moles. The theoretical decrease to 2.0 moles was obtained by borohydride reduction. The basis for this is not clear. Appropriate 3-substituted reducing uronic acids to serve as controls were not available.

For 4A and 4B, which were purified only by repeated paper chromatography, the analytical data suggest some cross contamination, since each compound showed partial destruction of both glucose and glucuronic acid after reduction with sodium borohydride. With higher oligosaccharides, the experimental error of the colorimetric determinations makes structural interpretations more difficult. Compound 4A contained 3 moles of glucose to 2 of glucuronic acid, e.g., 4.2/2.6 or 0.87/0.51 in Table I. Reduction with sodium borohydride in two independent sets of assays destroyed one-fifth and two-fifths of the glucose and about one-twelfth and one-sixth of the uronic acid. Only 1.4 moles of formaldehyde were produced per mole of reduced compound after oxidation with periodate, based on the formula in Table I.

In 4B the glucose value was slightly higher than the uronic acid value on a molar basis. This extra glucose may be due to contamination with some penta-(4A) and possibly some heptasaccharide. In two independent sets of assays, one-fifth and one-fourth of the uronic and 0 and one-fourth of the glucose equivalents were destroyed on reduction with sodium borohydride. One and one-tenth moles of formaldehyde were released on oxidation of the reduced compound with periodate. Compounds 4A and 4B, like 3A, appear to have periodate-insensitive internal uronic acid residues from which one might infer that they are 3-linked. Both 4A and 4B showed two new spots after treatment with β-glucosidase (Figure 4 t, u, v, w). In each instance, one spot corresponds to glucose and the other to a new compound moving faster than the original oligosaccharide. From the formula in Table I, 4A would yield tetrasaccharide VB (see below) by removal of the nonreducing glucose residue. In Figure 4, VB and this new product from 4A have about the same R_F, but the conditions used would not achieve resolution of similar tetrasaccharides. Glucose is the unit at the nonreducing end of the four oligosaccharides obtained by enzymic hydrolysis, i.e., P-2, 3A, 4A, and 4B. VA and VB, obtained by acid hydrolysis, were chromatographically similar to 3A (Figure 2). However, neither VA nor VB was split by β-glucosidase (Figure 4). The analytical data suggest that they are both tetrasaccharides with glucose at the reducing end. Both gave

TABLE II
MICROMOLES OF INHIBITOR NEEDED FOR 50% AND 25% INHIBITION

	Unreduced Inhibitor				Reduced Inhibitor
	R-134		R-3-129		R-134
	50%	25%	50%	25%	50%
Inhibitor					
Cellobiuronic acid	0.4	0.25	5 (40%)	1.9	
P-2	0.14 (44%)	0.072			
3A	0.044	0.02	0.26 (35%)	0.088	0.096
VA	0.096	0.04	0.29 (50%)	0.076	0.15 (35%)
VB	0.044	0.02	0.22	0.054	0.096
4A	0.032	0.013	0.12	0.034	Probably 0.032
4B	0.018	0.007	0.058	0.024	Probably 0.018

equimolar quantities of glucose and glucuronic acid. Reduction with sodium borohydride did not affect the uronic acid but decreased the glucose by one-half. About 2 moles of formaldehyde were produced from the reduced glucose on oxidation with periodate. VA had a lower reducing equivalent (about 20% by weight) than did VB (31%). Both values were lower than the 38–41% found for 3A. Oxidation with periodate resulted in destruction of 50% of the uronic equivalents of VB as measured by the orcinol reaction, compatible with VB being *O*-β-D-glucopyranosyluronic acid-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyluronic acid-(1→4)-D-glucose. Seventy to eighty per cent of the uronic acid of VA was destroyed by periodate. Thus VA and VB, although similar, were separated by column and paper chromatography, and differed in reducing power and behavior toward periodate. A definite structure for VA cannot be formulated.

Inhibition of S III Anti-Pn III Precipitation by Oligosaccharides.—This is shown with two rabbit anti-Pn III sera in Figure 5. Per cent inhibition has been plotted against micromoles of inhibitor added. With all oligosaccharides tested, the antibodies in serum R-3-129 were more difficult to inhibit than those in serum R-134. Four-tenths μmole of cellobiuronic acid gave 50% inhibition with serum R-134 while 5 μmoles of this disaccharide gave only 40% inhibition in serum R-3-129. Table II shows the quantities in μmoles of each inhibitor required for 25 and 50% inhibition with these two sera. Molar inhibiting power increases with chain length. For serum R-3-129, 4B was the best inhibitor, followed in decreasing order by 4A, VB, VA, 3A, and cellobiuronic acid. With the limited amounts of P-2 available it was found less potent than 3A, but could not be compared with cellobiuronic acid at levels at which the latter gave significant inhibition. Sodium glucuronate and glucose were inactive over the range tested.

With serum R-134, 4B was a better inhibitor than 4A. Tetrasaccharides 3A and VB gave identical inhibition curves; both were poorer than 4A and better than tetrasaccharide VA. VA was in turn better than trisaccharide P-2, and P-2 was better than cellobiuronic acid. With this serum, inhibition by sodium glucuronate was demonstrable; 22 and 11.6 μmoles gave 39 and 28% inhibition, respectively. Twenty-two and 11 μmoles of glucose, and 14.5 and 11 μmoles of cellobiose gave no significant inhibition. With R-134, on reduction of the compounds with sodium borohydride, 4B and 4A showed little or no decrease in inhibiting power, but 3A and VB decreased to the level of compound VA. Reduced VA gave inhibition similar to that of unreduced P-2. The inhibiting power of cellobiuronic acid was completely destroyed by reduction with sodium borohydride in the concentration range at which more than 80% inhibition was found before reduction. In contrast to their behavior in serum R-134, when tested with serum R-3-129, reduced 4B and 4A were significantly poorer as inhibitors than were the unreduced compounds. Compounds 3A, VB, VA, and cellobiuronic acid also lost inhibiting potency after reduction with sodium borohydride, but because of the limited quantities of oligosaccharides available it was not possible to carry out tests at the higher concentrations.

Extensive inhibition studies were not possible with horse anti-Pn III, serum 393, because of the limited quantities of oligosaccharides available. Three-tenths and 0.4 μmole of 4B, the best inhibitor, gave 40% inhibition of precipitation, with 5.4 μg antibody N per determination.

Precipitin Studies with S III and Its Degradation Products.—None of the oligosaccharides listed in Table I precipitated rabbit serum 134, but P-5, the mixture of paper-chromatographically slowly moving dialyzable sugars from depolymerase treatment of S III, did precipitate. The precipitin curves obtained with R-134 and S III, the nondialyzable fraction of depolymerase-treated S III (nondialyzable), and the dialyzable P-5 fraction are shown in Figure 6a. The P-5 and nondialyzable fractions precipitated 15 and 25%, respectively, of the antibody nitrogen precipitable by S III.

With horse anti-Pn III, P-5 precipitated 55% of the 950 μg of anti S III N in the Felton globulin solution (Figure 6b), and at least 90% of the total anti-S III N in H 393 (Figure 6c). With this latter serum the nondialyzable fraction and S III itself gave almost identical precipitin curves as did HCl B, a fraction prepared by acid hydrolysis and supplied by Dr. M. Heidelberger, which contained 12.5% reducing sugar and showed no movement on paper chromatography. Peaks VI and VII from the Dowex column fractionation (Figure 3) precipitated 18 and 36%, respectively, of the anti-S III from H 393. Slight precipitation was also evident with 4B and 4A, but no precipitate was demonstrable with the other oligosaccharides listed in Table I. The precipitin curves shown in Figure 6d were obtained with a volume of undiluted H 393 containing 99 μg of antibody N. Compound 4B precipitated 8% and 4A 3% of the anti-S III.

DISCUSSION

In this study, oligosaccharides have been prepared from S III by enzymic and acid-catalyzed hydrolyses. Structures have been proposed for a trisaccharide and tetrasaccharide, P-2 and 3A, which were isolated after depolymerization of S III with an enzyme from a soil bacillus originally described by Dubos and Avery (1931). Two other products of this enzymic depolymerization, 4A and 4B, were partially purified. Although they were still somewhat contaminated with each other, it was possible to propose a structure for

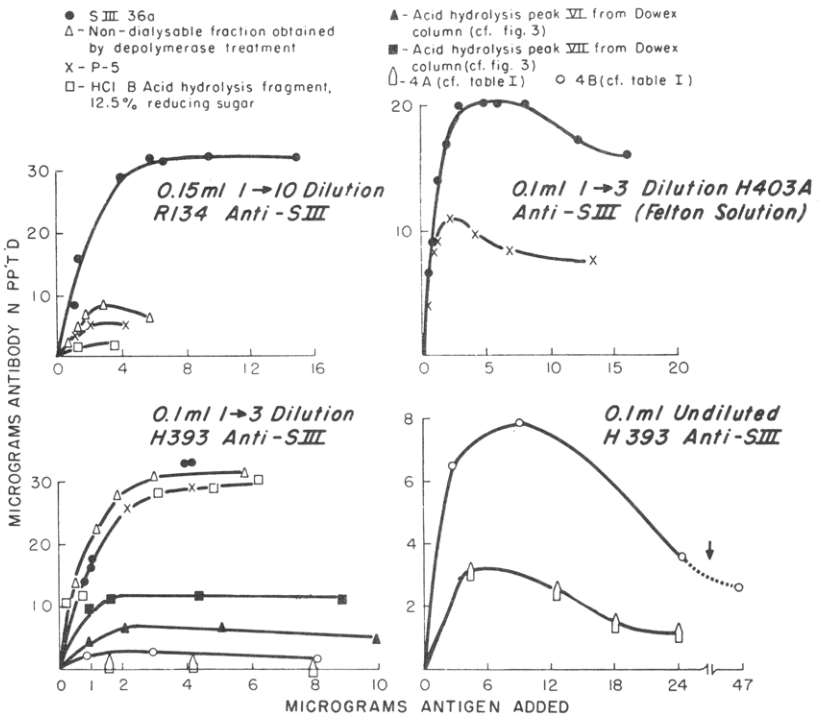

FIG. 6.—Precipitin curves obtained with horse and rabbit anti-Pn III sera, S III, and some degradation products; a, upper left; b, upper right; c, lower left; d, lower right.

each. Enzymic hydrolysis gave oligosaccharides with terminal nonreducing glucose, while acid hydrolysis gave products with terminal nonreducing glucuronic acid. Torriani and Pappenheimer (1960, 1962), who studied the specificity of an S III depolymerase from an independently isolated soil bacillus (Sickles and Shaw, 1934), report that their S III depolymerase acts on internal 1,4-β linkages. The oligosaccharides obtained in greatest yield, 3A and 4B, would result from such enzymatic cleavage. P-2 and 4A, obtained in lesser yield, might have been formed by splitting of glucuronic acid from the reducing ends of the major products. Torriani and Pappenheimer obtained a trisaccharide identical with P-2. They at first (1960) thought that another spot was a tetrasaccharide with terminal nonreducing glucuronic acid, but subsequently (1962) considered the structure as uncertain.

Models of structures proposed for two alternative beta-linked tetrasaccharides, 3A and VB, are shown in Figure 7. The dimensions of 3A, Glc-(1→3)-GlcUA-(1→4)-Glc-(1→3)-GlcUA, are 22.2 × 10.6 × 4.2 A, and those of VB, GlcUA-(1→4)-Glc-(1→3)-GlcUA-(1→4)-Glc are 22.8 × 10.6 × 4.2 A. The structure of VA, another tetrasaccharide from acid hydrolysis, is not fully known. Both VA and VB contain equimolar quantities of glucose and glucuronic acid, with glucose at the reducing end. The stability to acid of the glucurono-glucose bond as compared with the glucosyl-glucuronic acid bond, together with the insusceptibility of VA and VB to β-glucosidase, led to the inference that both contained beta-linked glucuronic acid at the nonreducing end. Of the numerous structures possible for VA, perhaps a dicellobiuronic acid linked other than β-1,3 would be most consistent with the structure accepted for S III. VA might be an acid-reversion product, rather than a true structural component with a hitherto unrecognized linkage; but since VA and VB were isolated in comparable yields, the latter alternative must be seriously considered. Since the structure of S III as a linear polymer of cello-

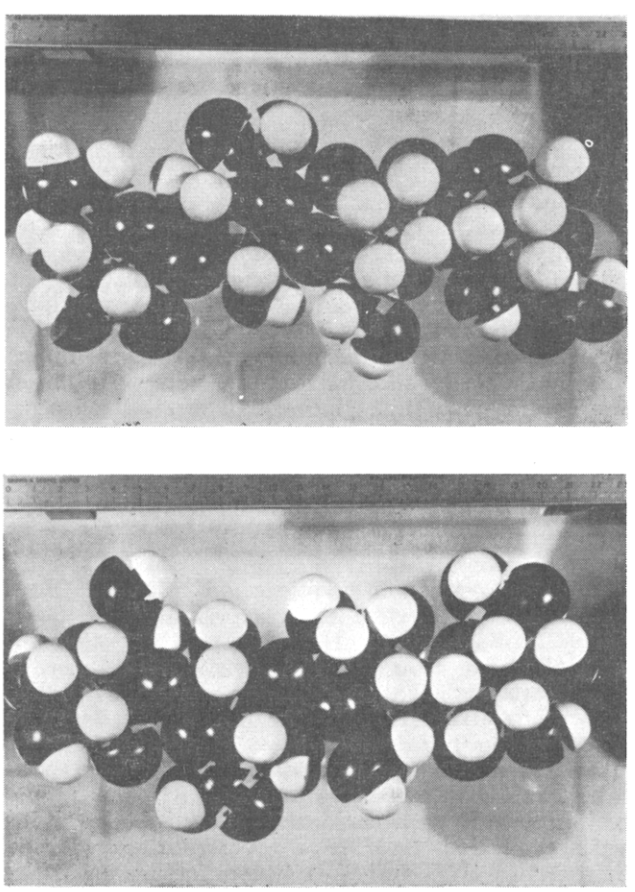

FIG. 7.—Models of structures proposed for two alternative β-linked tetrasaccharides. Top: 3A. Glc-(1 → 3)-GlcUA-(1 → 4)-Glc-(1 → 3)-GlcUA. Bottom: VB. GlcUA-(1 → 4)-Glc-(1 → 3)-GlcUA-(1 → 4)-Glc.

biuronic acid linked by β-1,3 glucosidic bonds was completed in 1941, there has been recognition of the heterogeneity of polysaccharides from a single source (Smith and Montgomery, 1959) and the great structural variation that may occur among products of different strains of closely related microorganisms (McCarty and Lancefield, 1955; Jeanes *et al.*, 1954; McCarty, 1958). A systematic reinvestigation of S III by modern methods (Smith and Montgomery, 1959; Stacey and Barker, 1960) might disclose features not now known, such as other linkages, branch points, and possibly other sugars.

All the oligosaccharides isolated inhibited the precipitation of rabbit anti-Pn III by S III, with some differences shown by individual mono- and oligosaccharides in the two sera studied. With both sera, inhibiting power increased with chain length of oligosaccharide, as in the dextran-antidextran system. With R-134, the increment in inhibiting power decreased in going from glucuronic acid to cellobiuronic acid, to trisaccharide P–2, to tetrasaccharides 3A and VB, to pentasaccharide 4A (Figure 5). When the isomaltose series of oligosaccharides was assayed in the dextran-antidextran reaction with both human (Kabat, 1961) and rabbit (Mage and Kabat, 1963) antisera, the increment of inhibiting power decreased progressively and an upper limit was approached with isomaltohexaose or -heptaose with human antibody, and isomaltopentaose or -hexaose with rabbit antibody. It was therefore inferred that few if any of the antidextran-combining sites were directed toward chains of more than seven glucose units.

The behavior of 4B with R-134 in the S III system is exceptional in that the increment in going from 4A to 4B is greater than or equal to that from 3A to 4A (Figure 5). Such behavior might result if 4B were sufficiently large to exhibit some bivalence in reacting with antibodies with combining sites directed against small chain lengths. In dextrans multiple terminal nonreducing end groups are present and the terminal sugar is viewed as contributing most to hapten-antibody binding. Since S III is a highly linear molecule, its antigenic determinants may occur at intervals along the repeating sequence and some antibodies may have their strongest specificity for one structural feature along the chain (e.g., glucuronic acid), whereas others are most strongly directed toward another feature of the determinant (e.g., glucose). The small oligosaccharides would undoubtedly be monovalent, but with longer oligosaccharides bivalent inhibitors would be expected and such bivalence might first be manifested toward antibodies with small combining regions. The approach to an upper limit in inhibiting potency thus becomes confounded by a superimposed increase in inhibiting power due to bivalence of inhibitors. Both the inhibition behavior and precipitation with horse anti-S III suggest that 4B is bivalent. An oligosaccharide having the average composition of a trimer of the repeating unit (galactoglucorhamnopyranosyl ribitolphosphate) of another linear polymer, S VI, also precipitates with horse antibody (Rebers *et al.*, 1961). Thus its increased effectiveness in inhibiting the S VI-rabbit anti-S VI system as compared with the monomeric repeating unit could be due to its bi- or multivalence. Kabat (1962) has discussed the inhibition data for the S VI anti-S VI system in that light. In their inhibition studies with lupus erythematosus sera and denatured DNA, Stollar *et al.* (1962) consider it possible, though unlikely, that pentathymidylic acid is a polyvalent inhibitor.

Because of this limitation in interpretation of inhibition data for an essentially linear polymer like S III, no definite estimate of the upper limit in size of the combining regions of S III–specific antibodies has been made. However, differences between two sera have been demonstrated which can best be interpreted on the basis that anti-S III, like antidextran, is made up of a heterogeneous population of antibody molecules with combining sites that vary in size. An antiserum made up mostly of antibodies with larger sites would be less readily inhibited by smaller oligosaccharides than an antiserum containing mostly antibody with smaller sites. When antidextrans from single individuals were fractionated into two populations by specific absorption of antibody on insoluble dextran (Sephadex) and hapten-elution in two steps, it was indeed found that the fractions eluted with isomaltose or isomaltotriose were readily inhibited by the smaller oligosaccharides, whereas the fractions eluted with isomaltohexaose were more readily inhibited only with the larger oligosaccharides (Schlossman and Kabat, 1962). The good inhibition by glucuronic and cellobiuronic acids in serum R-134, in contrast to the negligible and weak inhibition with these substances in serum R-3-129, suggests that the average sizes of the combining regions of the antibodies in the former are smaller than those in the latter. R-3-129 is more difficult to inhibit than R-134 with all the oligosaccharides tested. Reduction of the proposed hexasaccharide 4B and the proposed pentasaccharide 4A with sodium borohydride has slight and no effect, respectively, on their molar inhibiting potency with R-134 (Figure 5), suggesting that with this serum a fifth or sixth unit makes little or no contribution to binding. However in R-3-129 reduced 4A and 4B are significantly poorer inhibitors than the unreduced compounds. The heterogeneous population of antibodies in this serum may contain a significant fraction directed toward an antigenic determinant larger than the six-unit 4B. Whereas 3A and VB give nearly total inhibition in serum R-134, none of the tetrasaccharides produces 100% inhibition with serum R-3-129, and even 4B, the best inhibitor, gives only 73–78% inhibition at the highest concentrations tested. Inability to obtain complete inhibition may indicate antibodies with combining regions insufficiently satisfied by the hapten used in competition with the antigenic determinants on the intact polymer. Antigenic determinants on the polymer may have a kinetic advantage in competition with haptens. The presence of repeating specific groups near each other along a chain, and any stabilization of determinant configuration in the polymer, could contribute to such an advantage. The antibodies of R-3-129 which are inhibitable by the tetrasaccharides may be those with smaller combining sites. R-134 would appear to have only a small proportion of antibodies with sites complementary to more than four or five sugar units, while the anti-S III in serum R-3-129 seems largely composed of antibodies with sites complementary to six or more units. The estimated ranges in sizes for the combining sites of anti-S III is in general agreement with the early precipitin studies of Heidelberger (Heidelberger and Kendall, 1937; Heidelberger, 1938), who found that in large antibody excess the ratio of μgrams of antibody nitrogen precipitated to μgrams of polysaccharide added (N/S) averaged 13.5. Using 150,000 as the molecular weight of rabbit antibody, they calculated that one molecule of antibody was bound for every 1800 weight units of S III. This corresponds to one antibody molecule for every five cellobiuronic acid units (ten sugar units) along the chain. These data also suggest that the antigenic determinant of S III does indeed occur at intervals along the linear chain, since even if there is some branching in S III,

it is unlikely to be extensive enough to provide terminal nonreducing ends to bind such large amounts of antibody.

Since compounds reduced with sodium borohydride have structural features similar to the next smaller oligosaccharide, plus an additional structurally correct glycosidic bond, it is not surprising that reduced hexasaccharide 4B is only slightly better than unreduced pentasaccharide 4A, and reduced 4A is only slightly more active than unreduced tetrasaccharide 3A in serum R-3-129. The attached open-chain sugar does not appear to interfere with the binding of hapten to antibody. From the finding that glucuronic acid and cellobiuronic acid inhibit precipitation of R-134 at concentrations at which glucose and cellobiose do not, it can be inferred that the acidic group is important in hapten binding at least by some of the anti-S III. In R-134, tetrasaccharides VB and 3A, each with two intact uronic acid units, are equally good inhibitors. After reduction with sodium borohydride, when the reducing glucuronic acid of 3A is changed to an open-chain alcohol with its carboxyl group intact, both tetrasaccharides become poorer but remain indistinguishable as inhibitors. Reduced 3A is still much more potent than P-2, the trisaccharide with only one uronic acid group in the molecule. It is possible that enough of the structural integrity of the uronic acid group remains after reduction to satisfy the structural requirements of the predominantly small sized antibody-combining regions in this serum, or that the third glycosidic linkage contributes the additional binding energy.

It is very likely that anti-S III sera possess heterogeneity of specificity in addition to simple variation in sizes of antibody-combining sites (Heidelberger *et al.*, 1942). Heidelberger and Kendall (1935b) concluded from studies of the reactions of horse anti-S III sera with methylated S III that different parts of the S III antigenic determinants may act independently in stimulating the formation of antibodies and in reacting with them. Markowitz and Heidelberger (1954), in later studies of the reactions of anti-S III with chemically modified S III, also found evidence for such heterogeneity. Observed differences in hapten inhibition between sera might reflect varying proportions of antibodies with different specificities, but a detailed interpretation of the data is not possible. In R 3-129, all three tetrasaccharides are relatively poor inhibitors but differ in potency. Tetrasaccharides VA and VB from acid hydrolysis each probably has two β-1,4 linkages, and they are better inhibitors than 3A which has but one such linkage. This serum may have more antibodies specific for the β-1,4 linkage than R-134. If VA contains a linkage not present in VB or 3A, but present in S III, there may be a fraction of antibodies whose combining sites include specificity for this linkage. Even though VA is a better inhibitor than 3A in one of the sera, it could simply be a good cross-reacting substance, not truly present in S III.

Acknowledgment

The authors wish to acknowledge the constant interest and numerous suggestions of Dr. Gerald Schiffman throughout the investigation.

References

Adams, M. H., Reeves, R. E., and Goebel, W. F. (1941), *J. Biol. Chem. 140*, 653.

Bernheimer, A. W. (1953), *J. Exptl. Med. 97*, 591.

Bitter, T., and Ewins, R. (1961), *Biochem. J. 81*, 43P.

Brown, A. H. (1946), *Arch. Biochem. 11*, 269.

Dische, Z. (1947), *J. Biol. Chem. 167*, 189.

Dische, Z., Shettles, L. B., and Osnos, M. (1949), *Arch. Biochem. 22*, 169.

Dubos, R., and Avery, O. T. (1931), *J. Exptl. Med. 54*, 51.

Felton, L. D. (1931), *J. Immunol. 21*, 357.

Goebel, W. F. (1936), *J. Explt. Med. 64*, 29.

Goebel, W. F. (1938), *J. Exptl. Med. 68*, 469.

Goebel, W. F., and Hotchkiss, R. D. (1937), *J. Exptl. Med. 66*, 191.

Hach, W., and Benjamin, D. G. (1954), *J. Am. Chem. Soc. 76*, 917.

Heidelberger, M. (1938), *J. Am. Chem. Soc. 60*, 242.

Heidelberger, M., and Avery, O. T. (1923), *J. Exptl. Med. 38*, 73.

Heidelberger, M., and Avery, O. T. (1924), *J. Exptl. Med. 40*, 301.

Heidelberger, M., and Goebel, W. F. (1926), *J. Biol. Chem. 70*, 613.

Heidelberger, M., and Goebel, W. F. (1927), *J. Biol. Chem. 74*, 613.

Heidelberger, M., Goebel, W. F., and Avery, O. T. (1925), *J. Exptl. Med. 42*, 727.

Heidelberger, M., Kabat, E. A., and Mayer, M. (1942), *J. Exptl. Med. 75*, 35.

Heidelberger, M., and Kendall, F. E. (1929), *J. Exptl. Med. 50*, 809.

Heidelberger, M., and Kendall, F. E. (1933), *J. Exptl. Med. 57*, 373.

Heidelberger, M., and Kendall, F. E. (1935a), *J. Exptl. Med. 61*, 559.

Heidelberger, M., and Kendall, F. E. (1935b), *J. Exptl. Med. 61*, 563.

Heidelberger, M., and Kendall, F. E. (1937), *J. Exptl. Med. 65*, 647.

Heidelberger, M., Kendall, F. E., and Scherp, H. W. (1936), *J. Exptl. Med. 64*, 559.

Hotchkiss, R. D., and Goebel, W. F. (1937), *J. Biol. Chem. 121*, 195.

Jayme, G., and Demmig, W. (1960), *Chem. Ber. 93*, 356.

Jeanes, A., and Haynes, W. C., Wilham, C. A., Rankin, J. C., Melvin, E. H., Austin, M. J., Cluskey, J. E., Fisher, B. E., Tsuchiya, H. M., and Rist, C. E. (1954), *J. Am. Chem. 76*, 5041.

Kabat, E. A. (1960), *J. Immunol. 84*, 82.

Kabat, E. A. (1961), *in* Kabat and Mayer's Experimental Immunochemistry, 2nd ed., Springfield, Ill., Charles C. Thomas.

Kabat, E. A. (1962), *Fed. Proc. 21*, 694.

Kabat, E. A., and Schiffman, G. (1962), *J. Immunol. 88*, 782.

Khym, J. X., and Doherty, D. G. (1952), *J. Am. Chem. Soc. 74*, 3199.

Koenig, V. L., and Perrings, J. D. (1955), *J. Biophys. Biochem. Cytol. 1*, 93.

McCarty, M. (1956), *J. Exptl. Med. 104*, 629.

McCarty, M. (1958), *J. Exptl. Med. 108*, 311.

McCarty, M., and Lancefield, R. (1955), *J. Exptl. Med. 102*, 11.

Mage, R., and Kabat, E. A. (1963), *J. Immonol. 93* (in press), this thesis, Part I.

Markowitz, H., and Heidelberger, M. (1954), *J. Am. Chem. Soc. 76*, 1313.

Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181*, 149.

Partridge, S. M. (1948), *Biochem. J. 42*, 238.

Rebers, P. A., Hurwitz, E., and Heidelberger, M. (1961), *J. Bacteriol. 82*, 920.

Record, B. R., and Stacey, M. (1948), *J. Chem. Soc.*, 1561.

Reeves, R. E., and Goebel, W. F. (1941), *J. Biol. Chem. 139*, 511.

Rosevear, J. W., and Smith, E. L., (1961), *J. Biol. Chem. 236*, 425.

Schiffman, G., Howe, C., and Kabat, E. A. (1958), *J. Am. Chem. Soc. 80*, 6662.

Schiffman, G., Kabat, E. A., and Leskowitz, S. (1962a), *J. Am. Chem. Soc. 84*, 73.

Schiffman, G., Kabat, E. A., and Thompson, W. (1962b), *J. Am. Chem. Soc. 84*, 463.

Schlossman, S. F., and Kabat, E. A. (1962), *J. Exptl. Med. 116*, 535.
Sevag, M. G. (1934), *Biochem. Z. 273*, 419.
Sickles, G. M., and Shaw, M. (1934), *J. Bacteriol. 28*, 415.
Smith, F., and Montgomery, R. (1956), *Methods Biochem. Analy. 3*, 197.
Smith, F., and Montgomery, R. (1959), Chemistry of Plant Gums and Mucilages, New York, Reinhold.
Stacey, M., and Barker, S. A. (1960), Polysaccharides of Microorganisms, London, Oxford University Press.
Stollar, D., Levine, L., Lehrer, H. I., and Van Vunakis, H. (1962), *Proc. Nat. Acad. Sci. U.S. 48*, 874.
Torriani, A., and Pappenheimer, A. M., Jr., (1960), *Bull. Soc. Chim. Biol. 42*, 1619.
Torriani, A., and Pappenheimer, A. M., Jr., (1962), *J. Biol. Chem. 237*, 1.
Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.
Wasserman, E., and Levine, L. (1961), J. Immunol. 87, 290.
Weissmann, B., Meyer, K., Sampson, P., and Linker, A. (1954), *J. Biol. Chem. 208*, 417.
White, B. (1938), The Biology of Pneumococcus, New York, The Commonwealth Fund.

The Specific Polysaccharide of Type XVIII Pneumococcus. III*

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Received July 9, 1963

The galactose in the specific polysaccharide of type XVIII pneumococcus (S XVIII) has now been identified as the D enantiomorph by means of D-galactose oxidase. Glycerol was confirmed by paper electrophoresis and by the action of glycerol dehydrogenase. *O*-Acetyl was found as an additional constituent very important in the immunological specificity of S XVIII. Molar ratios of the components approximate: D-glucose 3, D-galactose 1, L-rhamnose 1, glycerol 1, *O*-acetyl 1, phosphate 1. The sugars appear to be in the pyranose form. Treatment with strong alkali splits off glycerophosphate and *O*-acetyl, yielding a product, Alk S XVIII, which retains the sugars with no change in ratios. Alk S VXIII, oxidized by periodate and reduced by borohydride, yields, in the proportions indicated: D-galactose 1, L-rhamnose 1, erythritol ≤ 1.5, glycerol 1. Mild acid hydrolysis of this product, followed by a second oxidation with periodate and reduction, results in destruction of the galactose and rhamnose with formation of glycerol and propylene glycol. These results, together with the cross reactions described and tests for their inhibition, point toward one or the other structure:

$$-\left[\rightarrow 3)\text{-D-gal-}(1\rightarrow 4)\text{-}\alpha\text{-D-glc-}(1\rightarrow 6)\text{-D-glc-}(1\rightarrow 3)\text{-L-rham-}(1\rightarrow 4)\text{-D-glc-}(1-\right]-$$
$$\left.\begin{array}{l}\text{Ac—}\\ \text{HOCH}_2.\text{CHOH.CH}_2\text{O.PO}_2-\end{array}\right]_x$$

or

$$-\left[\rightarrow 3)\text{-D-gal-}(1\rightarrow 4)\text{-D-glc-}(1\rightarrow 3)\text{-L-rham-}(1\rightarrow 4)\text{-}\alpha\text{-D-glc-}(1\rightarrow 6)\text{-D-glc-}(1-\right]-$$
$$\left.\begin{array}{l}\text{Ac—}\\ \text{HOCH}_2.\text{CHOH.CH}_2\text{O.PO}_2-\end{array}\right]_x$$

Initial studies on the capsular polysaccharide of type XVIII pneumococcus, S XVIII, showed the presence of D-glucose, L-rhamnose, and secondarily bound phosphate (Markowitz and Heidelberger, 1954). Because ribitol phosphate was found in the capsular polysaccharide of type VI pneumococcus, S VI (Rebers and Heidelberger, 1959, 1961), the study of S XVIII was resumed (Estrada-Parra *et al.*, 1962), resulting in the finding of two new components, galactose and glycerol, the latter present as glycerophosphate. The glycerophosphate could be removed by alkali without loss of the sugars (Alk S XVIII). Oxidation of S XVIII and Alk S XVIII by periodate indicated that rhamnose and galactose were probably 1,3-linked and that at least part of the glucose was bound 1,4- and 1,6-. The present paper is a report of further progress which has resulted in the proposal of partial structures for S XVIII.

* Submitted by S. Estrada-Parra in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Institute of Microbiology, Rutgers, The State University, New Brunswick, N. J. Supported in part by a grant (G-19968) from the National Science Foundation.

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EXPERIMENTAL

Earlier preparations of S XVIII were used in part, e.g., 1104P (Estrada-Parra *et al.*, 1962). An additional batch was prepared from a strain of type XVIII pneumococci obtained from Dr. R. Austrian. This was passed through mice and grown in eleven 4-liter flasks, each containing 2 liters of Adams-Roe medium (1945) for 18 hours. S XVIII was obtained both from the cells (fractions A[1] and B) and culture fluid (fractions C and D) as in previous papers (Heidelberger *et al.*, 1950; Estrada-Parra *et al.*, 1962).

Properties of the fractions, as well as of another (1800H) obtained by combination and repurification of earlier preparations are given in Table I. *Aloe vera* polysaccharide was kindly given by Dr. F. Cordoba, S XVIII A by Dr. Rachel Brown, and the polysaccharide of *Klebsiella* E-26 by Dr. J. F. Wilkinson. Immunochemical methods are described in other papers (Rebers and Heidelberger, 1959, 1961; Rebers *et al.*, 1961).

Determination of O-Acetyl.—By the method of Hestrin (1949), with ethyl acetate as standard, duplicate 2.0-mg portions of S XVIII C gave 92 μg, or 4.6%. The molar ratio of *O*-acetyl to phosphate was therefore 0.81:1 (on ash-free basis).

[1] A small, acetic acid–precipitable fraction, apparently mainly protein.