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# The Specific Polysaccharide of Type XVIIIA Pneumococcus*

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The capsular polysaccharide of the pneumococcal type XVIIIA consists of D-galactose, D-glucose, rhamnose, *N*-acetylglucosamine, glycerol, and phosphate in the approximate ratios 2:3.5:1:1:1:1. Its behavior on oxidation with periodate and some of its serological reactivities resemble those of the *O*-deacetylated polysaccharide of pneumococcal type XVIII. The residues of galactose and rhamnose are probably linked 1,3-, and the *N*-acetylglucosamine is also stable to periodate.

The capsular polysaccharides of pneumococcus are the antigenic determinants of type specificity in this heterogeneous group of microorganisms. The type-specific antibody found in animals as a result of infection, or injection of the killed cells or the polysaccharides (in man and the mouse) is both curative for and protective against a given pneumococcal type. Hence the study of the chemistry of these capsular substances advances both the practical and theoretical knowledge of the relation between chemical constitution and immunological specificity (for a review see Heidelberger, 1960). As the fine structures of relatively few pneumococcal substances are known, this field is being actively developed in several laboratories.

Clarification of the major structural features of the capsular polysaccharide of type XVIII pneumococcus (Estrada-Parra and Heidelberger, 1963) led to an interest in the closely related type XVIIIA. An early preparation of its polysaccharide, S XVIIIA, was available (Brown and Robinson, 1943) and was further purified. The substance not only showed many similarities to the type-specific determinant of type XVIII, but exhibited several major differences as well.

## Experimental

*Fractionation of S XVIIIA.*—Preparation 1 (Brown and Robinson, 1943), 2.2 g, was dissolved in 50 ml of water, chilled, treated briefly in a Waring Blendor with chloroform-butanol (5:1), and centrifuged in the cold. The aqueous supernatant and the washings of the not very large middle layer were combined, diluted to a concentration of 3% NaOAc with neutralized saturated sodium acetate solution, and precipitated with an equal volume of chilled 2-propanol. A small fraction, deposited upon addition of another volume of ethanol, was not further characterized. The principal fraction was taken up in 70 ml of water and precipitated with 125 ml of saturated ammonium sulfate solution at *p*H 5.4. This step had been found to remove the group-specific C-substance from S XVIII (Estrada-Parra, *et al.*, 1962). After thorough dialysis of the precipitate against neutralized 5% aqueous sodium acetate in the cold, the S XVIIIA was precipitated with chilled ethanol, redissolved in water, dialyzed against water, and poured into 500 ml of cold ethanol containing 7 g of sodium acetate neutralized with a few drops of glacial acetic acid. The fibrous product was squeezed out, broken up into redistilled ethanol, treated with redistilled acetone, and filtered off. The yield was 1.45 g. A comparison of the material with the original substance and with S XVIII is given in Table I.

TABLE I
PROPERTIES OF THE SPECIFIC POLYSACCHARIDES OF TYPE XVIII AND TYPE XVIIIA PNEUMOCOCCUS

| Preparation | $[\alpha]_D$ (°C) | N (%) | P (%) | Acetyl (%) | Ash (%) |
|---|---|---|---|---|---|
| S XVIIIA | | | | | |
| Original No. 1[a] | +82 | 1.7 | 2.9 | 6.1 | 10.6 |
| Purified | +97[b] | 1.5 | 2.9 | 0[c] | 1.7[d] |
| S XVIII | | | | | |
| Fraction C[b] | +79 | 0.3 | 3.0 | 4.6[c] | 1.8[d] |

[a] Brown and Robinson, 1943. [b] Ash-free; Estrada-Parra and Heidelberger, 1963. [c] *O*-Acetyl. [d] As $Na^+$.

Immunochemical methods used were the same as described in other papers (Rebers and Heidelberger, 1959, 1961; Rebers *et al.*, 1962). Rabbit antipneumococcal (anti-Pn) type XVIIIA sera were kindly supplied by Miss Jessie L. Hendry of the Division of Laboratories and Research, New York State Dept. of Health, Albany, N.Y. Anti-Pn XVIIIA horse serum was not available. The present initial chemical study was carried out as far as possible along the same lines as that of S XVIII.

## Results and Discussion

*Hydrolysis of S XVIIIA by Acid.*—A solution of S XVIIIA was heated in 1.5 N $H_2SO_4$ at 100° for 17 hours, passed through Duolite A in the $OH^-$ form, and concentrated *in vacuo* to small volume. A chromatogram in solvent A, 1-butanol-pyridine-$H_2O$-benzene, 5:3:3:1 (Jeanes *et al.*, 1953), showed components corresponding to glucose, galactose, rhamnose, and glycerol when

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TABLE II
COMPOSITION OF S XVIIIA

| Components | μg/ml | μmoles/ml |
|---|---|---|
| Total sugars by phenol | 628 | |
| Rhamnose by Dische | 74 | 0.45 |
| Total hexose by Dische | 416 | 2.3 |
| Ratio of hexose to rhamnose | 5.1 | |
| After separation | | |
| Galactose by phenol | 137 | 0.76 |
| Glucose by phenol | 247 | 1.37 |
| Rhamnose by phenol | 67 | 0.41 |
| Ratios galactose-glucose-rhamnose | 1.9:3.3:1 | |
| Galactose by galactostat | 124 | 0.69 |
| Glucose by glucostat | 235 | 1.31 |
| Rhamnose by Dische | 61, 64 | 0.38 |
| Ratios galactose-glucose-rhamnose | 1.8:3.4:1 | |
| Glycerol by dehydrogenase | 46 | 0.42 |
| Ratio above sugars to glycerol | 5.7 | |

sprayed with silver nitrate–ammonia and only the first three when sprayed with aniline oxalate. A similar hydrolysate, neutralized with $Ba(OH)_2$, concentrated, and chromatographed in ethyl acetate–pyridine–acetic acid–$H_2O$, 5:5:1:3 (Fischer and Nebel, 1955), showed an additional constituent corresponding to glucosamine when sprayed with ninhydrin and no components aligned with glucuronic and galacturonic acids when sprayed with silver nitrate–ammonia.

For isolation and estimation of the neutral sugars and glycerol, S XVIIIA was hydrolyzed in 1 N HCl at 100° for 26 hours and passed through Duolite A, and the effluent was concentrated to small volume. The neutral sugars were separated on paper with solvent A and eluted with water. The solutions and washings, after passage through sintered-glass filters, were concentrated *in vacuo* and the content of each was determined by the phenol method (Dubois *et al.*, 1956). The molar ratios found were: galactose-glucose-rhamnose, 0.8:1.4:0.4, or 2:3.5:1. D-Glucose by glucostat (Saloman and Johnson, 1959) was 95% of the color value and D-galactose by galactostat (Avigad *et al.*, 1962) was 91% of that found colorimetrically, showing that both of these sugars occurred in the D series. Rhamnose, by the method of Dische and Shettles (1948), gave a value 91% as great as that by the phenol method. Glycerol found by glycerol dehydrogenase (Hagen, 1962) in the eluted rhamnose-glycerol spot was in the molar ratio 0.4, so that in S XVIIIA these components appear to be present roughly in the ratios 2:3(or 4):1:1. The actual values found are given in Table II.

For an assumed repeating unit containing 2 residues of D-galactose, 3 D-glucose, 1 *N*-acetylglucosamine, 1 rhamnose, 1 glycerol phosphate, 1 Na, mw 1350, nitrogen and phosphorus would be 1 and 2.3% instead of 1.5 and 2.9%, as actually found. An *O*-acetyl determination (Hestrin, 1949) gave a negative result, but *N*-acetyl (Ludowieg and Dorfman, 1960) was 1.2 equivalents per N.

*Degradation by Alkali.*—An aqueous solution of S XVIIIA was treated with sodium borohydride, left for 2 days at 4°, brought to *p*H 12 with 1 N NaOH, allowed to stand under $N_2$ for 3 days at 28°, run through Dowex 50 ($H^+$ form), concentrated, and dialyzed against water. Under these conditions acetyl and 5.5% of the phosphorus were split from S XVIII, with drastic modification of its immunological specificity. S XVIIIA, which contains no *O*-acetyl, lost 3.6% of its

TABLE III
ANTIBODY NITROGEN PRECIPITATED BY S XVIII, S XVIIIA, AND THEIR DERIVATIVES FROM RABBIT ANTISERA TO PNEUMOCOCCUS XVIIIA[a]

| Substance | Amt. (μg) | Pool, RN 103, RL 936[b] (μg) | F498[c] (μg) |
|---|---|---|---|
| S XVIIIA | 210 | | 1625 |
| | 280 | 1290[d] | |
| | 420 | 1410 | 1845 |
| | 560 | 1370 | |
| 41% Alk S XVIIIA | 210 | 69[e] | 710 |
| | 420 | 38 | |
| | 550 | | 700 |
| S XVIIIA $IO_4$ | 140 | 344[f] | |
| | 280 | 252[f] | |
| S XVIIIA $IO_4BH_4$ | 70 | 511[g] | |
| | 140 | 529[h] | |
| S XVIII | 140 | 252[i] | |
| | 210 | 321 | 646[j] |
| | 400 | | 998[j] |
| | 800 | | 1100 |
| 5% Alk S XVIII | 100 | | 775[k] |
| | 200 | | 985[k] |
| | 400 | | 1050 |
| 90% Alk S XVIII | 150 | 132 | |
| | 300 | 98 | |

[a] All reactions at 0°, calcd to 1.0 ml of original serum. [b] After removal of 8 μg antibody nitrogen to Pn C-polysaccharide. [c] After removal of traces of anti-C. [d] S XVIIIA, reduced with borohydride and kept at *p*H 12 at 28° for 3 days, conditions under which S XVIII was deacetylated with much loss of precipitating power, precipitated the same quantity of antibody as before treatment with alkali. [e] Supernatants from this and other precipitations in the region of excess antigen + S XVIII at the 150 μg level gave 241 μg N. [f] Supernatants + S XVIIIA $IO_4BH_4$ at the 55-μg level gave 8 μg N. [g] One determination only. [h] Combined S XVIIIA $IO_4BH_4$ supernatants + S XVIII at the 150-μg level gave 265 μg N. [i] The preparation of S XVIII used in this determination precipitated only 246 μg N at the 210-μg level. [j] Combined supernatants + 5% alk S XVIII at the 100-μg level gave 200 μg N. [k] Combined supernatants + S XVIII at the 200-μg level gave 97 μg N.

phosphorus and still precipitated rabbit anti-Pn XVIIIA maximally (Table III).

Another sample was reduced as before, dialyzed against water, and treated more drastically in 0.9 N NaOH at 37° under $N_2$ for 26 days. The solution was passed through Dowex 50 ($H^+$) and dialyzed. One per cent of the sugars and 41% of the phosphorus went into the dialysate, not as inorganic $PO_4$ but as glycerol phosphate, as shown on chromatograms of the vacuum-concentrated solution in formic acid–methanol–water, 80:15:5 (Bandurski and Axelrod, 1951), sprayed with periodate-benzidine (Cifonelli and Smith, 1954) or Hanes' reagent (Hanes and Isherwood, 1949). After hydrolysis at 100° in 1 N $H_2SO_4$, glycerol could be demonstrated on a chromatogram in solvent B (Block, 1952). S XVIIIA is accordingly much more resistant to alkali than S XVIII, which loses 90% or more of its glycerol-phosphate side chains under similar conditions.

*Oxidation with Periodate.*—At 4° in the dark, consumption of $NaIO_4$ by S XVIIIA was constant in 2 weeks at 4 μmoles $IO_4^-$ per μmole P, with 1.2 μmole formaldehyde liberated per μmole P. Following reduction with borohydride and hydrolysis with $H_2SO_4$, chromatograms of the residual material showed major

TABLE IV
ANTIBODY NITROGEN PRECIPITATED BY S XVIII, S XVIIIA, AND THEIR DERIVATIVES FROM ANTISERA TO PNEUMOCOCCUS XVIII[a]

| Substance | Amt. (μg) | Rabbit 43C[b] (μg) | Horse 495C[b] (μg) | Horse 632C[b] (μg) |
|---|---|---|---|---|
| S XVIII | At max | 4590[c] | 2200[c] | 308[c] |
| S XVIIIA | At max | | 357[c,d] | |
| | 30 | | | 25[e] |
| | 100 | | | 21[e] |
| | 550 | 2050[f] | | |
| | 825 | 2010[f] | | |
| Alk S XVIIIA | 55 | 155 | 310 | |
| | 110 | 159 | | |
| | 165 | 135 | 318 | |
| S XVIIIA $IO_4$ | 50 | | 58 | |
| | 150 | | 159 | |
| | 200 | | 86[g] | |

[a] All reactions at 0°, calcd to 1.0 ml of original serum. [b] After removal of anti-C. [c] Estrada-Parra and Heidelberger, 1963. [d] Similar supernatants + 5% alk S XVIII at the 100-μg level gave 91 μg N; + 90% alk S XVIII at the same level gave 39 μg N; unabsorbed 495C serum gave 445 and 285 μg N, respectively (see Estrada-Parra and Heidelberger, 1963). [e] Similar supernatants + 5% alk S XVIII at the 20-μg level gave 3 μg N. [f] Supernatants gave no further precipitation with deacetylated (5% alk) S XVIII. [g] Supernatants opalescent.

components corresponding to galactose, rhamnose, and glucosamine, also others with the mobilities of erythritol, glycerol, and ethylene glycol. It will be recalled that in S XVIII the galactose and rhamnose also survived oxidation with periodate, and erythritol and glycerol were formed from the glucose, most of which disappeared (Estrada-Parra and Heidelberger, 1963). It is probable, therefore, that the neutral sugars in S XVIIIA are linked much as in S XVIII. Further work will be necessary, however, before a structural formula can be proposed.

*Quantitative Serological Data.*—Tables III and IV summarize quantitative data relating the immunological and chemical properties of S XVIII, S XVIIIA, and their derivatives. Precipitin data are given for two anti-Pn XVIIIA rabbit sera in Table III. Removal of nearly one-half the glycerophosphate from S XVIIIA by alkaline degradation (not necessarily the only chemical change) almost abolishes precipitation in one of the sera, while reducing it to 40% in the other. In the former serum, the only one tested, periodate-oxidized S XVIIIA, S XVIIIA-$IO_4$, precipitates only one-fourth of the antibody, while the percentage rises to 38 after reduction of the oxidized material with borohydride. This behavior was not noted with S VI (Rebers *et al.*, 1962) or S XVIII. In both instances reduction of the oxidized polysaccharide further lowered the amount of antibody precipitated. In serum F498, S XVIII and deacetylated (5% alk-) S XVIII show the same extensive cross-precipitation, as might have been anticipated from the lack of an *O*-acetyl group in S XVIIIA.

Data are given for three anti-Pn XVIII sera in Table IV. The rabbit serum showed far more extensive cross-reactivity with S XVIIIA than did the two horse sera, and this might have been expected, also, since precipitation in this serum was the least affected by deacetylation of S XVIII (Estrada-Parra and Heidelberger, 1963, Table III). Moreover, the supernatants from the reaction of serum 43C with S XVIIIA failed to precipitate further with deacetylated S XVIII.

The limited evidence thus far available, including that in the other footnotes of Table IV, indicates, then, that the immunological specificity of pneumococcus type XVIIIA greatly resembles that of the deacetylated determinant of the specificity of pneumococcus type XVIII. This conclusion appears to be valid in spite of the occurrence of *N*-acetylglucosamine and an extra residue of D-galactose in the capsular polysaccharide of type XVIIIA. One would expect, on closer analysis, to find effects and differences referable to these two sugars as well.

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