# IMPROVED METHODS FOR THE PREPARATION OF THE SPECIFIC POLYSACCHARIDES OF PNEUMOCOCCUS*

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Exposure of the specific polysaccharide of Type I pneumococcus (S I) to alkali is known to cause changes involving removal of an O-acetyl group and alterations in chemical, physical, and immunological properties (1–3). Analogous effects do not occur with S II and S III (3). S V, however, is probably hydrolyzed by alkali because the reducing power which it showed toward alkaline sugar reagents varied from 8 to 30 per cent with increasing alkalinity (4).

In view of the sensitivity of S I and S V to alkali, Dr. O. T. Avery questioned the use of 5 N sodium hydroxide to neutralize acid formed during the growth of Types I and V pneumococci, since high local concentrations of alkali might cause degradation of the polysaccharides even before their removal from the living cells. If this occurred, it seemed to the writers that it might account for the absence of a sharp equivalence zone in the reaction of many preparations of S I with rabbit anti-S I, as well as the poor antigenic properties of S V in human subjects noted on several occasions (5). Preparations of the specific polysaccharides of Types I and V pneumococcus were therefore isolated under as mild conditions as possible from cultures which were not neutralized. Several modifications of the methods used in reference 3 are given, as well as others introduced since the inclusion of this laboratory's methods in reference 6. Preparations of S VII and S XII are also described.

## EXPERIMENTAL

*Cultures.*—Types I and V pneumococci were grown in a partially defined medium (7) containing 1 per cent of glucose, without the intermittent addition of alkali to neutralize the acid formed during growth, a procedure which greatly adds to the yields when permissible.

7.5 liter batches of medium, warmed to 37°C., were inoculated with 75 ml. of an 8 hour

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culture of pneumococcus in the same medium. After incubation for 7 to 8 hours the cultures were chilled in an ice-salt bath. Growth was heavy, autolysis was minimal, and only rarely were Gram-negative forms seen. The final pH was 6.2 to 6.7, except for one instance of Type V in which it was 4.5. In order to kill the pneumococci under conditions which might inhibit autolytic enzymes and prevent their possible action on the capsular polysaccharides, an equal volume of 95 per cent ethanol, chilled to 0°C., was added. This resulted in agglomeration of the organisms. The suspensions were held in the cold for a few hours until settling had taken place. The supernatant was siphoned off and the sediment centrifuged and extracted as described below to obtain the polysaccharides.

Addition of alcohol quickly rendered the organisms Gram-negative but their morphology was otherwise unchanged as long as they were kept in the alcohol. Conversion to the Gram-negative state was more rapid at room temperature than in the cold, also with 1 volume of alcohol than with 2 or 3 volumes. The capsular swelling reaction was unaltered. A similar, less uniform conversion to the Gram-negative state in the presence of ethanol occurs with pneumococci grown in meat infusion–peptone broth.

In the preparation of the specific polysaccharides all operations were carried out in the cold (0–5°C.) and preferably within a period not exceeding 2 to 3 weeks.

*S I, Lot 144.*—The supernatant from the centrifuged organisms gave a negative test for S I and was discarded. The pneumococci were suspended in 80 ml. of acetate buffer[1] and ground in a slowly rotating 1 liter round bottom pyrex flask for 6 hours with stainless steel balls 2 mm. in diameter, with the flask partially immersed in ice water. The suspension was transferred to 50 ml. plastic tubes with a little buffer and centrifuged at 8,000 R.P.M. for 30 minutes.[2] The slightly yellow, viscous supernatant was poured off and the polysaccharide precipitated by the addition of 0.5 volume of cold isopropyl alcohol (fraction A). The residue from the first grinding was again taken up in 80 ml. of acetate buffer and ground for 6 hours as before. The suspension was centrifuged and the supernatant precipitated with isopropyl alcohol (fraction B). The cell residues were discarded since few intact cells remained and earlier runs had shown that almost all S I was removed in two grindings. As both fractions were worked up in the same manner only the isolation of fraction A is described below.

Fraction A was centrifuged off[3] and the supernatant discarded since it contained no S I. The precipitate was dissolved in 100 ml. of cold distilled water and the resulting turbid, greenish solution was deproteinized (8) by addition of 50 ml. chloroform and 10 ml. *n*-butanol to 150 ml. of solution and intermittent agitation in a Waring blendor in a cold room at 3°C. In order to prevent excessive warming the material was mixed for 5 minutes and the blendor was allowed to cool for 5 minutes, this cycle being repeated twice during 30 minutes. The resulting emulsion was centrifuged and the aqueous layer containing the polysaccharide was separated and the chloroform layer washed according to the method described in reference 3. The aqueous layer, now 400 ml., was adjusted to a concentration of 3 per cent sodium acetate with saturated sodium acetate solution brought to pH 6 with acetic acid. 210 ml. (0.5 volume) of cold isopropyl alcohol was added to precipitate the S I and the suspension left overnight. The entire procedure was repeated twice until no middle layer of denatured protein formed. The S I was centrifuged off, dissolved in 150 ml. of water, and dialyzed under negative pressure in Visking sausage tubing with daily changes of water as described in reference 6 until negative tests for sodium, phosphate, and (on two lots) acetate (9, 10) were obtained.

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[1] 157.5 gm. $NaC_2H_3O_2 \cdot 3H_2O$ was dissolved in distilled water, made up to 3 l., and adjusted to pH 6.05 with glacial acetic acid. Toluene was added as a preservative and the solution was stored in the cold.

[2] In a Sorvall SS-2 vacuum centrifuge.

[3] In an International Equipment Co. refrigerated centrifuge, model PR-1.

The solution was centrifuged in the cold at 10,000 R.P.M. for 30 minutes in order to remove a small amount of difficultly sedimentable material. The clear, colorless, highly viscous super-

TABLE I

*Specific Polysaccharides of Types I, V, VII, and XII Pneumococcus*

| Preparation No. | Ash as Na | $[\alpha]_D$* | N* | Acetyl* | P*, free and labile | P* total | Relative viscosity of 0.2 per cent solution, $H_2O$, at 20°C. | Relative viscosity of 0.1 per cent solution, 0.9 per cent NaCl, at 20°C. |
|---|---|---|---|---|---|---|---|---|
| | *per cent* | *degrees* | *per cent* | *per cent* | *per cent* | *per cent* | | |
| I 120 (from reference 3) | 3.4 | +278 | 4.6 | 7.1 | | 0.0 | 9.0 | 1.69 |
| 140A | 4.5 | +267 | 5.0 | | | 0.8 | | |
| 142‡ | 0.6§ | +264 | 5.3 | 9.3 | | 0.8 | 8.64 | 1.88 |
| 144A | 3.0 | +256 | 5.2 | 9.3 | 0.09 | 0.8 | 8.15 | 1.67 |
| 144B | 4.0 | +242 | 6.9 | | 0.32 | 1.9 | | |
| V 8C (from reference 4) | 3.2 | −45 | 5.0 | 12.7 | | 0.25 | | 1.06 |
| 501 | 5.3 | −78 | 4.3‖ | | | 0.5 | 1.53 | 1.13 |
| 503A‡ | 0.37§ | −63 | 4.6 | 12.3 | 0.09 | 1.1 | 3.59 | 1.50 |
| 505A | 2.2 | −62 | 5.2 | 15.0 | 0.24 | 1.5 | 5.1 | 1.82 |
| 506A | 2.5 | −78 | 5.0‖ | 15.3 | 0.22 | 0.53 | 4.05 | 1.66 |
| 506B | 1.9 | −53 | 6.0 | 13.1 | 0.16 | 1.9 | 4.80 | 1.68 |
| $506B_1$ | 2.1 | −74 | 5.0 | | | 0.9 | 3.34 | 1.46 |
| 506C | 4.8 | 0 | 9.6 | 8.4 | 0.41 | 4.1 | 3.08 | 1.37 |
| 506D | 2.3 | −61 | 5.7 | 13.1 | 0.24 | 1.4 | 3.34 | 1.50 |
| VII (from reference 4) | 3.4 | +71 | 3.2 | | | 1.2 | | |
| 703A | 0.8 | +82 | 2.7 | | | 0.6 | | |
| XII (from reference 4) | 4.3 | +4 | 4.7 | | | 0.2 | | 1.25 |
| $1201A_1$ | 2.5 | 0 | 4.2 | | | 0.3 | 2.31 | 1.36 |
| $1201A_2$ | 2.2 | −2 | 4.0 | | | 0.3 | 2.28 | 1.33 |

* Calculated to the ash-free basis.

‡ Not lyophilized, isolated as the Li salt from redistilled ethanol with the use of neutralized LiCl in the final steps instead of NaOAc.

§ Ash as Li.

‖ Contains 25 per cent hexosamine, calculated as glucosamine.

natant was lyophilized (*cf.* reference 6, p. 437). The properties of the product are given in Table I.

*Yields:* Fraction A: 0.50 gm. of Na salt; fraction B: 0.77 gm. of Na salt.

*S V, Lot 506.*—The Type V pneumococci required three grindings to remove most of the capsular polysaccharide (fractions A, B, and C). The dilute alcoholic supernatant culture fluid contained S V, which was precipitated by an additional volume of ethanol (fraction D). Purification of the fractions was carried out as for S I, except that twice as much ethyl or

isopropyl alcohol was necessary for each precipitation. Since S V is almost quantitatively precipitated from aqueous solution by one-half saturation with ammonium sulfate, fraction A was given a final purification in this manner, followed by dialysis against water, 0.9 per cent sodium chloride solution, water, and lyophilization. The effect of two precipitations with $(NH_4)_2SO_4$ on fraction B is seen by comparison of B and $B_1$. (Table I).

*Yields*, as Na salts: Fraction A: 0.05 gm.; fraction B: 0.62 gm.; fraction C: 0.43 gm.; fraction D: 0.09 gm.

*S VII.*—Sixteen liters of autolyzed, phenolized culture which had been neutralized during growth was run through a Sharples centrifuge to remove $Mg_3(PO_4)_2$, treated with celite, filtered on a Buchner funnel, and further clarified by passage through alundum candles. The filtrate was ultrafiltered through 4 per cent parlodion-coated alundum candles (4) with a final washing of neutral 3 per cent sodium acetate solution. Very little S VII passed through. The collodion was scraped off, washed with water, and the main outer solution and washings, at a volume of 1.5 liters, were treated with 150 gm. of sodium acetate and a few drops of acetic acid, and precipitated with 6 liters of ethanol. The precipitate was centrifuged and taken up in water, depositing on recentrifugation a considerable insoluble residue from which additional amounts of S VII could be recovered by washing. The aqueous solution was treated with sodium acetate, precipitated with alcohol, redissolved, deproteinized with chloroform and butanol, and precipitated with alcohol in the presence of sodium acetate. The gummy precipitate was taken up in water and centrifuged. To the supernatant and washings from the insoluble portion at a volume of 50 ml., was added a neutralized saturated solution of 5 gm. of sodium acetate and an initial precipitate obtained in the cold with 100 ml. of chilled methyl alcohol. After 1 hour the solution was decanted from the oily deposit, which contained relatively little S VII, and precipitated with an additional 100 ml. of methyl alcohol. The supernatant was decanted from the heavy, gummy precipitate of S VII, which was repurified by a repetition of the process, followed by solution in chilled water and pouring into redistilled ethanol. The precipitate was washed with redistilled alcohol and redistilled acetone, filtered off, and dried *in vacuo*. Yield: 1.75 gm. The properties are recorded in Table I since they differ somewhat from those described in reference 4.

*S XII.*—The initial stages of isolation were similar to those for S VII. Three volumes of ethanol were required for the first two precipitations. After deproteinization and reprecipitation with alcohol a slightly acidified solution of 30 gm. of sodium acetate was added to the soluble material. At a volume of 400 ml. this yielded the S XII with 600 ml. of ethanol. The solid was dissolved in water, made up to 50 ml., and precipitated with 75 ml. of anhydrous propionic acid (*cf.* reference 11). The precipitate was dissolved in chilled sodium acetate solution and the S XII thrown down with an equal volume of reagent grade methyl alcohol and washed as rapidly as possible with ethanol. After an additional precipitation with sodium acetate and ethanol the product was reprecipitated with redistilled ethanol and washed with redistilled alcohol, but became gelatinous. Water was therefore added in the cold until a clear solution resulted and into this was mixed a saturated, slightly acidified solution of 5 gm. of sodium acetate. Part of the S XII separated in the cold overnight. The remainder was precipitated with more ethanol. The fractions, $A_1$ and $A_2$, respectively, were redissolved, reprecipitated with several volumes of redistilled ethanol, washed with a little redistilled acetone, filtered off, and dried.

*Yields:* Fraction $A_1$: 0.29 gm.; $A_2$: 0.44 gm.

Both portions had practically identical chemical, physical, and immunological properties and the combined yield was 68 per cent of the S XII present in the original, centrifuged, autolyzed culture, as determined by means of a calibrated antiserum (12). The analyses showed that 0.18 gm. of S XII was lost by the treatment with celite and filtration through alundum.

*Physical and Chemical Properties of S I, S V, S VII, and S XII (Table I).*—In this table S I 120 is an earlier preparation (reference 3). Comparative data for S V, S VII, and S XII from reference 4 are also given. S I 140A was the principal fraction obtained from a commercial batch (13), for which the culture had been neutralized from time to time with NaOH. The culture for S I 1012 B (Squibb) was neutralized during growth with $NaHCO_3$—$Na_2HPO_4$ buffer (14) and the S I was only slightly damaged. Lots 142 and 144 were from cultures which were not neutralized. S V 503 was obtained from an alkali-treated culture and was isolated according to the method described in reference 3 except that it was precipitated as the copper salt with copper acetate adjusted to pH 5.4 with NaOH and was finally isolated as the lithium salt.

Analyses for nitrogen were made by a modified micro Kjeldahl method. Acetyl was determined by hydrolysis of the polysaccharide with 25 per cent *p*-toluenesulfonic acid for 3 hours in a boiling water bath and distillation of the acetic acid formed in a micro Kjeldahl apparatus. Successive 75 ml. portions were collected, heated to boiling under a condenser with an attached soda-lime trap, and then cooled in an ice bath. The acetic acid was estimated as described in reference 15 by addition of 2 gm. of KI and 2 ml. of a 4 per cent $KIO_3$ solution to each portion, incubation in a stoppered 125 cc. Erlenmeyer flask for 10 minutes at 37°C., and titration of the liberated iodine with 0.0100 N $Na_2S_2O_3$. A blank was run by distillation of an equal amount of *p*-toluenesulfonic acid. No other correction was made for $SO_2$ (15).

Viscosities were measured in both water and 0.9 per cent NaCl solution at 20.00° ± 0.05°C. with an Ostwald-Fenske viscometer.

Phosphorus was determined according to a modification of the method given in reference 16. Since this involved heating in 2 N $H_2SO_4$ for 20 minutes in a boiling water bath, free and labile P were estimated together and constituted about 10 per cent of the total P which was determined essentially according to the method in reference 17.

Reducing sugars as glucose were estimated as described in reference 18 or 19.

The ultraviolet absorption[4] of S 142 showed the shift with increasing acidity reported for ribonucleic acid in reference 20. If the absorption is calculated as that of ribonucleic acid, S 142 contains 3.2 per cent, a value less than one-half that found if the total P is assumed due to nucleic acid. The absorption shows also that only a small proportion of the N of S 142 can be nucleic acid N. S 141 gave no peak in the ultraviolet.

*Reactivity of S Preparations with Type-Specific Rabbit Antisera.*—In Table II are given data on the precipitation of anti-Pn I rabbit sera with various S I preparations. The determinations were made in duplicate at 0° according to the method given in reference 12.

Of the S V preparations, a complete curve was run only with 506B. The other fractions were set up with excess antibody at two points and the values for maximum antibody precipitable and for the quantities of S required for maximum precipitation of antibody were calculated according to the method described in reference 6 (pp. 31, 32) from Equation 6 *a* in reference 21. Representative data are given in Table III.

*Effect of 0.10 N NaOH on S V.*—A solution of S 505A containing 2.0 mg. per ml. in 0.10 N NaOH was kept at about 25°C. At intervals 1.0 ml. of solution was withdrawn, neutralized with 0.10 N HCl, and diluted to 0.20 mg. per ml. with water for determination of pH, reducing sugars as glucose, and reactivity with calibrated rabbit anti-Pn V serum.[5] The undiluted alkaline solution was used for all except the last two measurements of the ultraviolet absorption against a blank of 0.10 N NaOH; owing to increased absorption at 73 hours, dilution was necessary for the final readings.

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[4] A Beckman model D U spectrophotometer was used.

[5] For these last analyses the solution was further diluted to 0.10 mg. per ml. with 1.8 per cent NaCl solution.

TABLE II

*Precipitation of Anti-PN I Rabbit Sera by Various S I Preparations*

| Serum* | Preparation | Antigen used per ml. serum | Antibody N precipitated per ml. serum | Tests on supernatants | |
|---|---|---|---|---|---|
| | | | | + anti-Pn I | + antigen |
| | | *mg.* | *mg.* | | |
| A | 140A | 0.128 | 0.715 | ± | ++++ |
| | | 0.256 | 1.013 | ++ | ++++ |
| | 1012B | 0.32 | 1.40 | − | + |
| | 142 | 0.20 | 1.24 | − | + |
| Pool 957–963 | 1012B | 0.16 | 0.764 | − | +++ |
| | | 0.32 | 0.994 | − | ± |
| | | 0.283‡ | 0.961‡ | | |
| | 142 | 0.094 | 0.621 | − | +++ |
| | | 0.188 | 0.973 | − | − |
| | | 0.235 | 1.067 | +++ | − |
| | | 0.268‡ | 1.085‡ | | |
| | 144A | 0.097 | 0.627 | − | ++ |
| | | 0.194 | 0.972 | − | + |
| | | 0.258‡ | 1.032‡ | | |

* Diluted 1 → 2 with 0.9 per cent NaCl.

‡ Maximum amounts calculated from the equation.

TABLE III

*Reaction of S V Fractions with 1.0 Ml. C-Absorbed Anti-PN V Rabbit Serum Concentrate**

| Fraction | S V | Antibody N precipitated | Calculated maximum S V | Calculated maximum antibody N |
|---|---|---|---|---|
| | *mg.* | *mg.* | *mg.* | *mg.* |
| 501 | 0.128 | 0.716 | 0.257 | 0.96 |
| 503A | 0.20 | 1.102 | 0.329 | 1.30 |
| 505A | " | 1.042 | 0.360 | 1.29 |
| 506A | " | 1.174 | 0.368 | 1.49 |
| 506B | " | 0.990 | 0.464 | 1.42 |
| $506B_1$ | " | 1.117 | 0.316 | 1.29 |
| 506C | " | 0.652 | 0.650 | 1.30 |
| 506D | " | 1.040 | 0.336 | 1.25 |

* Prepared by Lederle Laboratories and obtained through the courtesy of Miss Annabelle Walter, New York City Department of Health Laboratories.

The percentage degradation of S V (Table IV) was calculated by assuming that the precipitate with immune serum was due to unaltered S V. The equation for 505A, antibody N precipitated = 7.2 S–10 $S^2$, was derived from data given in part in Table III. The precipitates were analyzed for N by the Markham modification (22) of the micro Kjeldahl method.

Degradation of S V by alkali was also evident from the changes in ultraviolet absorption. As isolated, S V showed no absorption peaks, the curve gradually decreasing toward a very small value at 320 mμ. At the end of 1.3 hours in 0.10 N NaOH absorption had increased, and after 48 hours a definite peak had developed at 270 mμ, with marked increase in the over-all absorption. After 123 hours there was a sharp peak at 270 mμ, similar to that attributed to furfural derivatives in reference 23 at 277 and 285 mμ when solutions of xylose and glucose were boiled.

TABLE IV

*Effect of 0.1 N Sodium Hydroxide on S V*

| Time | Antibody N precipitated from antiserum by | | Percentage undegraded S V from equation | Reducing sugars as glucose |
|---|---|---|---|---|
| | 0.1 mg. S V | 0.2 mg. S V | | |
| *hrs.* | *mg.* | *mg.* | *per cent* | *per cent* |
| 0 | 0.620 | 1.042 | 100 | 28 |
| 1.3 | 0.502 | | 82 | 26 |
| 48 | | 0.428 | 33 | 26 |
| 73 | | 0.246 | 18 | 24 |
| 123 | | 0.100 | 7 | 24* |

* In another run the reducing value was 12.8 per cent after 6 weeks.

## DISCUSSION

It has become customary, in growing cultures of many bacteria which form acid, to add 5 N sodium hydroxide to restore the neutral reaction of the culture medium and permit enhanced growth, often to previously unheard of densities of cells (7). The studies now reported indicate, however, that certain sensitive substances on the surfaces of the growing bacterial cells, *e.g.* the specific polysaccharides of Types I and V pneumococcus, may be damaged even by the brief exposures to local excess of alkali brought about in this way.

The deleterious effect of the addition of hydroxide to the culture is shown in Table II, both by the smaller amount of antibody precipitated from an anti-Pn I rabbit serum by lot 140A, a purified fraction of a commercial preparation[6] for which the original culture had been neutralized with sodium hydroxide (13), and by the absence of a clear-cut equivalence zone characteristic of preparations, both from these laboratories and from the manufacturer, made from cultures which were neutralized with sodium hydroxide. Even the addition of bicarbonate-disodium phosphate seems to start the process of degradation, as noted in Table II, although this was not the only variable in the method of preparation of the Type I lot 1012 B.

[6] Manufactured by E. R. Squibb & Sons.

As shown in Table IV, the effect of 0.1 N sodium hydroxide on S V is much more drastic than on S I. Exposure for 3 days at room temperature sufficed to reduce the precipitating power of S V toward an anti-Pn V rabbit serum to 18 per cent of the original value, while that of S I was reduced only to 34 per cent.

From the foregoing the conclusion appears inescapable that neutralization of the acidity developed by growing bacterial cultures should be avoided if one wishes to isolate an alkali-sensitive constituent of the cell as nearly as possible in its native state.

Although most of the phosphorus in S I 140 A consisted of free phosphate (13), that in lots 142 and 144 and in the Type V substance was mainly organically bound. Ultraviolet absorption spectra of 142 and 144 showed that not more than 50 per cent of this could be present in nucleic acid. S V contains no nucleic acid, even though most of the preparations were not free from organically bound P.

## SUMMARY

The specific polysaccharides of Types I and V pneumococcus give sharp equivalence zones and show maximal precipitation with homologous rabbit antisera only when carefully prepared from cultures which have not been neutralized with alkali.

The precipitating power of S V toward homologous rabbit antiserum falls off in 0.1 N NaOH even more rapidly than that of S I, dropping to 7 per cent of the original value in 6 days at room temperature. The alkaline solution develops a large absorption peak at 270 mμ.

Directions are given for the preparation of S VII and S XII.

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