THE PRECIPITATION OF BACTERIAL POLYSACCHARIDES WITH CALCIUM PHOSPHATE

PNEUMOCoccus

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Many of the recent immunological studies of the pneumococcus have been concerned with the specific precipitating agent (SSS) described by Dochez and Avery in 1917, and since studied in detail by Heidelberger and Avery (1923, 1924), Heidelberger, Goebel and Avery (1925) and Heidelberger, Sia and Kendall (1930). Essentially their method of preparation consists of evaporation of the pneumococcus broth, separation of active material by repeated fractional precipitation with alcohol from neutral and acid solutions, and further purification varying with each of the three fixed types. Schiemann and Casper (1927–8), and later Saito and Ulrich (1929) prepared specific precipitating material from pneumococcus cells only, of Types I and II, by alcoholic precipitation from a sodium taurocholate solution of the organisms which had first been heated with acetic acid to remove protein. In 1930 Goebel reported modifications of Heidelberger's method, which made the technic adaptable to storing crude material made from large volumes and purifying as desired. Wadsworth and Brown (1931, 1933) prepared a "cellular carbohydrate" by the method of Schiemann and Casper except that solution was obtained by alternate freezing and thawing of the organisms. In like manner, Tillet, Goebel and

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Avery (1930), prepared the species-specific substance from both rough and smooth strains by using large quantities of alcohol for precipitation. In 1933, Dudley and Smith suggested that, for preparation from broth, the protein be first removed from the hot broth by precipitation with acetic acid, and the large volume then concentrated by evaporation. Avery and Goebel (1933) reported "acetyl derivatives" of Type I SSS prepared from both broth and cells, the former prepared by a modification of the SSS technic of Heidelberger and Avery (i.e., avoiding the use of alkali), and the latter prepared by heating autolysed cultures with acetic acid to remove protein—a method similar to Zinsser's technic (Zinsser and Parker, 1923) for preparing "residue antigens"—followed by precipitation of the carbohydrate with alcohol from acid solution.

In the above procedures in which broth was the source of the SSS, the initial concentration of the large volume was effected by means of evaporation on a steam bath. To avoid this procedure, both because of the time required and because of the possibility of some alteration in the activity of the product due to prolonged heating, a method of precipitation of the active material from the unconcentrated culture broth was sought. During a preliminary study in which various calcium salts were used as precipitants, Dr. C. H. Fiske suggested that, inasmuch as the phosphate of calcium is a selective adsorbent for complex carbohydrates, this salt might be used to advantage (Felton, 1932). Calcium phosphate was used for enzyme separation as early as 1860 by Brücke (cited by Effront, 1917). Its use for precipitation of active immunological material was advocated for the purification of diphtheria toxin first by Roux and Yersin in 1889, and later continued by Abt in 1928 and Ohyama in 1931.

Preliminary work with pneumococcus filtrate in this laboratory demonstrated that in appropriate concentration and at optimum hydrogen ion concentration, either calcium or strontium phosphate when formed in situ will carry down most of the specific substance. The present study therefore reports the development of a practical method of preparing SSS by the use of calcium or strontium phosphate as the initial precipitant.
MEDIA

The media used were a haddock infusion broth, and a horse-meat infusion broth. In both cases, the media were prepared by Hartley's (1922) modification of Douglas' technic. Although fish broth gives a somewhat higher yield in weight and in precipitin titer of SSS, both media so prepared proved satisfactory. In general SSS production is greater the longer the period of incubation of the culture up to eight days.

DETERMINATION OF OPTIMUM CONDITIONS FOR PRECIPITATION

The method of determining optimum conditions for the precipitation of SSS depends upon three variables: calcium content; phosphorus content (in the form of phosphate); and hydrogen ion concentration. An experiment showing the results of such a study is given in table 1. The procedure was as follows: After three-day growth of Type I pneumococci (Neufeld) on beef infusion broth, most of the organisms were removed from the broth in a Sharples centrifuge. (The cells were worked up separately.) Specific serum was then found to be precipitable by a 1:256 dilution of the supernatant broth. A series of 10-cc. samples of this broth was set up in three groups as shown in the table. The reagents, all C. P., were used in the following concentrations: 11.6 per cent Na₂HPO₄·12H₂O to give 10 mgm. phosphorus per cubic centimeter; normal sodium hydroxide; and 10 per cent calcium chloride in a saturated solution of calcium hydroxide. After standing at room temperature until flocculation had begun, the precipitates were centrifugalized and dissolved in normal HCl to pH 1 (pink with phenol red). The SSS was then precipitated by the addition of three volumes of alcohol, and to hasten the precipitation, one-fourth to one-third volume of ether. After centrifugalizing, this precipitate was dissolved, neutralized and made up to 10 cc. in 0.85 per cent sodium chloride, the original volume of broth taken. Dilutions were then made for tests with serum, the results of which are shown in the table. Briefly, the protocol shows that the variable of greatest significance is hydrogen ion concentration. For, with a constant amount of calcium chloride-
calcium hydroxide solution, as little as 0.1 mgm. phosphorus per cubic centimeter of broth, in addition to that present in the

original broth, is sufficient to carry down the SSS providing the pH is optimum.

TABLE 1

Determination of optimum conditions for precipitation
Constant calcium, varying pH and phosphorus. Type I

<table>
<thead>
<tr>
<th>11.6 PER CENT Na₃H₂PO₄·12H₂O</th>
<th>NORMAL NaOH</th>
<th>10 PER CENT CaCl₂ IN Ca(OH)₂ (saturated)</th>
<th>pH</th>
<th>Precipitation with immune serum diluted 1:5</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
<td>Dilutions of samples</td>
</tr>
<tr>
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<td></td>
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<td></td>
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<tr>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>2.5</td>
<td>9.6</td>
<td>+</td>
</tr>
<tr>
<td>0.5</td>
<td>0.4</td>
<td>2.5</td>
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</tr>
<tr>
<td>0.5</td>
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<td>9.6</td>
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<td>1.0</td>
<td>2.5</td>
<td>9.6</td>
<td>+</td>
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</tbody>
</table>

* The remainder of the series were at pH above 9.6, i.e., maximum color with thymol blue—exact pH not tested.
Table 2 shows the effect of varying the calcium and the phosphorus content at a constant hydrogen ion concentration (pH 9). The technic of handling the precipitates was similar to that in the above experiment. Inasmuch as calcium phosphate is less soluble in calcium ions than in sodium ions, the hydroxyl ions in this experiment were furnished by calcium hydroxide instead of the sodium hydroxide used in the preceding experiment. Two in-

TABLE 2

_Determination of optimum conditions for precipitation_

Constant pH, varying phosphorus and calcium. Type I. Sixty-six-hour growth on fish medium; all samples made pH 9 with 10 per cent Ca(OH)₂.

<table>
<thead>
<tr>
<th>11.6 PER CENT Na₂HPO₄·12H₂O</th>
<th>10 PER CENT CaCl₂</th>
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<tr>
<td>cc.</td>
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<td>0</td>
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<td>3</td>
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<table>
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<th>Dilutions of samples</th>
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<th>1:8</th>
<th>1:16</th>
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<th>1:1024</th>
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<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

Differences may be made from the results of this experiment shown in the protocol. First, addition of calcium in the form of calcium chloride causes a more complete precipitation of SSS; but, with this medium at least, if sufficient phosphorus is present, 1 per cent of added calcium chloride is as effective as double or triple that amount. Second, this medium prepared from fish by Hartley's method (the same is true for media prepared from
horse meat), contains insufficient phosphorus for maximum precipitation. Only on the addition of phosphorus in concentration of 0.05 to 0.1 mgm. per cubic centimeter of broth, and under otherwise optimum conditions, is SSS obtained in the amount approaching that of the original broth.

From similar experiments it has been found that the optimum hydrogen ion concentration varies slightly with the different types: for Type I, about pH 8.6 to 9, and for Types II and III, pH 9 to 10 (or with thymol blue as indicator, a greenish blue for Type I and a definite blue for Types II and III). Too great an alkalinity however will redissolve some of the SSS from the precipitate.

PROCEDURE OF PRECIPITATION

First step—calcium precipitation

Because in general the media used in this study did not contain sufficient phosphorus to allow for complete precipitation of SSS, a preliminary test similar to the ones described above was necessary to determine the amount of disodium phosphate to be added. For our media this was usually 10 to 20 cc. of the 11.6 per cent disodium phosphate solution per liter of broth, i.e., from 0.1 to 0.2 mgm. phosphorus per cubic centimeter of broth. Furthermore, because of the results shown in table 2, a suspension of calcium hydroxide of 10 per cent concentration has been adopted to furnish the hydroxyl ions simultaneously with the calcium, without increasing the content of sodium ions. The decision as to the use of calcium chloride to increase the calcium ion content and thus decrease the solubility of calcium phosphate depends upon the medium in which the organisms are grown. Since the calcium phosphate precipitation carries down other polysaccharides such as glycogen, a high content of these inert polysaccharides in the broth may mean too great an increase of inactive material which can be separated from the SSS precipitate only with great difficulty. The lower yield obtained by the use of less calcium may be advantageous because of the ease of separation and the degree of purity of the final product.

Thus the procedure for large volumes (100 to 120 liters) has
been developed as follows: addition of phosphate in appropriate amount, followed by calcium chloride if found to be necessary, and by sufficient 10 per cent calcium hydroxide suspension to produce the desired hydrogen ion concentration.

To test whether the SSS precipitation was complete, when this technic was applied to large volumes, a 1-liter sample of the calcium supernatant broth was dialyzed to remove inorganic salts and then evaporated before a fan to the original volume of broth represented by the 1 liter of supernatant fluid taken. Serum tests have shown precipitation in about one-eighth or one-sixteenth of the dilution in which the original material precipitated, and this, in all cases tested, was a non-type-specific precipitation. One example, for instance, shows the original broth with a specific precipitate in dilution 1:128 and the dialyzed material, a non-specific precipitation in dilution 1:8. Thus, it is possible that this calcium precipitation method may leave in solution one-sixteenth to one-eighth of the specific substance along with non-specific material.

Second step—calcium removal

The possibilities for removal of calcium from this precipitate are: (1) to precipitate calcium and leave SSS in solution; (2) to precipitate SSS and leave calcium in solution; and (3) to dissolve both, and then separate by a differential precipitating agent. The first of these possibilities was tested in three ways: (a) with oxalic acid in the presence of either sodium or ammonium oxalate, (b) with ammonium sulfate and sulfuric acid, and (c) with sodium hydroxide. The second possibility, in the case of Type I, was tested several times with glacial acetic acid. Third, an acid, the calcium salt of which is soluble in alcohol, i.e., hydrochloric, was used for solution and alcohol for differential precipitation of SSS (leaving calcium chloride in solution). The first and second of these methods have thus far given irregular results; both serum precipitin tests and yield varied over too wide a range to consider them practical methods. Nevertheless it is possible that under optimum conditions, either would give results of practical importance.
The third procedure has, however, given consistently better results both as to yield and as to purity of the final product. Consequently this has been the method of choice. The hydrogen ion concentration necessary is pH 2 or below. In order to avoid an undue increase in the total volume, 4 N HCl has been used for this adjustment. At 4°C. more concentrated acid, although apparently without effect on the polysaccharides of Types I and III, may hydrolyze that of Type II material.

Of the possible reagents to precipitate SSS from this acid solution, ethyl alcohol has been found to be the most satisfactory. A study of the volume relationships of this and other reagents has been carried out on acid solutions of purified SSS. It was found that in the absence of neutral salts alcohol precipitates Type I SSS completely from an acid solution when two volumes are added; Type II requires three volumes; and Type III only one volume. Later work has indicated, however, that, if salts are present, more than one volume may precipitate non-specific material along with specific. These findings are a confirmation of previous work: for with five volumes of alcohol Tillett Goebel and Avery (1930) obtained non-specific material from a method by which Wadsworth and Brown (1931, 1933), using the small quantities of alcohol first advised by Heidelberger and Avery, obtained a specific precipitating substance.

Third step—purification

For purification of this crude material various means are suggested by the results of other investigators: residual protein may be separated with trichloracetic acid, and SSS obtained by subsequent alcoholic precipitation of the soluble material. Other insoluble constituents may be removed by repeated alcoholic precipitations from alternate acid and neutral solutions. A simple method of purification, which has been the most satisfactory, is dialysis of a slightly acid solution of the material for forty-eight hours, and subsequent precipitation with alcohol from a solution adjusted to the isoelectric point—for Type I pH 4.0 (according to Heidelberger) and for Type II pH 3 (according to our observations). Following such purifications, the final product is usually type-specific, and will precipitate serum when
diluted 1:2,000,000 or 1:5,000,000, or even higher in the case of Type III. Details of various methods for further purification will be reported elsewhere.

OUTLINE OF PROCEDURE

A summary of the procedure as finally developed for practical precipitation of 120-liter lots of supernatant broth of any type (I, II, or III) is as follows:

1. A preliminary test, similar to the two described above, to be carried out on each new batch of medium;

2. To the total volume add proportionate amounts of phosphate and calcium hydroxide (and calcium chloride if necessary), as ascertained above, checking pH with thymol blue.

3. After allowing to settle an hour or more, siphon and discard the supernatant fluid, and filter precipitate over night at room temperature (hardened paper, such as CSS No. 575, is satisfactory).

4. Scrape precipitate into large container and chill; From now on the procedures should be carried out in the cold (4°C.).

5. Add 4 N HC1 to make about pH 2, i.e., pink with phenol red; do not remove acid insoluble at this stage.

6. Add amount of ethyl alcohol appropriate for each type to the acid solution and collect precipitate.

7. Repeat alcoholic precipitations from acid solution until the precipitate is free from calcium. Any acid insoluble material may be removed and worked up separately after the second alcoholic precipitation. Best results are obtained when the two or three successive alcoholic precipitations from acid solution are carried out as rapidly as possible. Indeed these may be done all in one day: for, if insoluble material is not first removed from the acidified solution, the salt content will be sufficient for immediate flocculation when alcohol is added. Then continue purification, as indicated by other workers, by the use of protein precipitants, or by repeated alcoholic precipitations from neutral and acid solutions, or by dialysis. Wash with redistilled acetone and dry in vacuo. To obtain maximum yield, precipitin tests should be made at each step of the procedure.

It should be borne in mind that inasmuch as both broth and
cultures vary appreciably, no standard procedure can be set down even for one type. Because Type II has chemical characteristics similar to glycogen, and both are precipitated with calcium phosphate, some difficulty has been experienced in separating them. The method works best with Type III. As a matter of fact, providing there is sufficient phosphorus present in the form of phosphate, 80 per cent of the Type III SSS is precipitated by simply titrating the broth with calcium hydroxide solution. The simplest method to remove the calcium and simultaneously precipitate this SSS is the use of HCl, as employed by Heidelberger for precipitation of Type III SSS. Because this specific polysaccharide is insoluble in HCl, the procedure up to the stage of purification can be carried out with not too great a loss without the use of alcohol.

A REPRESENTATIVE TYPE I PREPARATION

A representative sample of Type I SSS was prepared as follows: 91 liters of a sixty-hour growth of type I pneumococci in horse meat infusion (glucose to make 0.5 per cent and NaOH to give pH 7.6 being added after twenty-four and forty-eight hours incubation) were precipitated, after a preliminary test, by the addition per liter of broth, of 20 cc. of 11.6 per cent disodium phosphate solution and 20 cc. of 10 per cent suspension of calcium hydroxide. The precipitate was handled as described in the above outline. The weight of the moist precipitate was 2578 grams. For a first solution in acid 700 cc. of 4 N HCl were necessary; for the second, 400 cc. of normal HCl were used. The alcoholic precipitate was then suspended in water, dissolved with a minimum amount of sodium hydroxide, and precipitated with one and one-half volumes of alcohol. Two more acid and neutral solutions were precipitated with alcohol. There was then still some material insoluble in acid, from which SSS was recovered separately. The acid-soluble material was precipitated with alcohol, washed with acetone and dried in vacuo. The yield was 2.5 grams. Serum tests showed precipitation when the material was diluted 1:5,000-000 with Type I serum, but no precipitation with Types II and III sera.
A MODIFIED TYPE II PREPARATION

One interesting preparation illustrative of a different method for removing calcium also shows the effect of heat on the resultant material. The calcium precipitate, obtained from 120 liters of Type II supernatant broth, was dissolved to pH 3 with 4 N HCl and excess sodium carbonate added to give maximum color with thymol blue (pH 10). The mixture was then heated at 100°C. in an Arnold sterilizer for fifteen minutes. After cooling, and centrifugalizing the insoluble material (from which SSS was recovered separately), the soluble fraction was neutralized and precipitated with two volumes of alcohol. The precipitate was twice dissolved in alkali and precipitated with one volume alcohol; then precipitated with alcohol from alternate acid and alkaline solutions twice; and finally washed with acetone and dried. The yield from 1630 grams of moist calcium precipitate was 1.4 grams. The material contained less than 0.2 per cent nitrogen, 48.9 per cent hydrolyzable sugar; precipitated only specific serum in a dilution of 1:2,500,000; but failed to produce active immunity in white mice.

DISCUSSION

Routine preparations of SSS for use in the laboratory have been made by this procedure for five years. Approximately a hundred batches each from 100 liters of broth have been so prepared. This material when diluted 1:2,000,000 to 1:5,000,000 precipitated specific immune serum; contained about 4 to 5 per cent nitrogen in Type I preparations, and little or no measurable nitrogen in Type II and Type III preparations; showed a sugar percentage after hydrolysis of 18 to 30 per cent for Type I samples and of 40 to 50 per cent for Type II samples.

The product has from the first shown characteristics different from those of the SSS of Heidelberger and Avery. In the first place, it precipitates more protein from a given immune serum: e.g., 2.8 mgm. precipitable protein were obtained in 1 cc. of serum F146, as compared with the finding of Heidelberger of 1.9 mgm. in the same serum (Felton, 1931). Second, the SSS prepared by the calcium phosphate method has consistently precipitated all
the protective antibody from immune serum. In this respect the results are the same as those obtained by Avery and Goebel with their acetyl polysaccharide. The discrepancy in this respect between Sabin’s (1931) results and ours may be explained by a possible difference in the samples of SSS used. Third, from the earliest use of this method, the preparations have produced active immunity in white mice. However, in a comparative test, a sample of SSS prepared by Dr. Heidelberger by his original method was found by us to produce active immunity in white mice. Indeed, Zozaya and Clark (1933) report active immunity in white mice following injection of samples of SSS some of which were supplied by Dr. Heidelberger and others made according to his technic. Consequently the evidence seems hardly conclusive in support of the explanation of Avery and Goebel (1933) that the original preparations of SSS by the method of Heidelberger and Avery were found to be lacking in antigenic activity due to destruction of acetyl groups during the process of preparation. Confirming the work of Avery and Goebel, observations in this laboratory (Felton, 1934) have shown that Type I SSS heated at 100°C. in alkaline solution is so altered that it no longer causes complete precipitation of the protective antibody in immune serum; on the other hand, such treatment continued for one hour does not completely destroy the antigenic activity of the material. The acetyl groups may play an important part with Type I SSS, but information as to the exact rôle of this chemical group awaits further work.

It should be emphasized that calcium phosphate is not a specific precipitating agent for the polysaccharides of pneumococcus. It is, however, in part specific for complex carbohydrates when used in an alkaline reaction. At neutral reaction the precipitate obtained is largely protein rather than polysaccharide. The same general procedure outlined above has been used for isolation of polysaccharides of microorganisms other than pneumococcus, and it was found that as long as a suitable hydrogen ion concentration was maintained, a great part if not all the polysaccharide specific to the organism studied was precipitated along with other polysaccharides present in the broth.
SUMMARY

A method is outlined for preparation of the soluble specific substance of pneumococci Types I, II, and III from a broth culture by means of an initial calcium phosphate precipitation. Methods for removal of calcium are indicated, and subsequent purification follows procedures already in use.

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