THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

SECOND PAPER.

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The so called "soluble specific substance" of Pneumococcus, it will be recalled, was discovered in 1917 by Dochez and Avery (1) in filtrates from pneumococcus cultures, and was found to be present not only in the intact bacterial cell but also in the body fluids of the infected host. It yielded precipitates only with immune sera of the homologous type of Pneumococcus and, in addition to its high degree of specificity, possessed a stability to heat, enzymes, and many chemical reagents that augured well for its susceptibility to study by the methods of organic chemistry.

In a recent paper by the present writers (2) a preliminary report of such a study was made. A method was described which permitted the concentration of the specific substance of Type II pneumococcus and its separation from a large portion of the accompanying impurities. The resulting product appeared to consist chiefly of a polysaccharide built up of glucose molecules, gave a specific rotation of $+ 55.7°$, contained 1.2 per cent of nitrogen, yielded 79 per cent of reducing sugars on hydrolysis, and still reacted with immune serum when present in as low a concentration as 1:3,000,000. At that time the question was left open as to whether the specific substance itself consisted of polysaccharide or merely accompanied a polysaccharide impurity.

Not only has further evidence on this point now been obtained, but by refinements in the method of purification it has also been possible to reduce the percentage of nitrogen tenfold, and, in a comparative study of the specific substances of Type II and Type III pneumococci, to establish definite chemical differences between them.

EXPERIMENTAL.

Type II Pneumococcus.

1. Method of Purification.

The following modification of the original method of purification of the specific substance is now being used.

8 day cultures of Pneumococcus Type II in meat infusion phosphate broth are concentrated in 21 liter lots, precipitated with alcohol, and put through the initial three layer separation as described in the previous paper. The gummy middle layer is taken up in about 200 cc. of hot water and the resulting mixture from successive lots is kept on ice until 300 to 325 liters of culture have been worked up. The liquid portion of the combined concentrates is decanted from any salts that have separated and these are dissolved in hot water. The solutions are mixed and run through a Sharples supercentrifuge, washing through thoroughly with water. In a representative run, the volume of the resulting somewhat turbid solution was 4,200 cc. and complete precipitation of the active material required 5.5 liters of alcohol. The mixture is allowed to stand overnight, the clear supernatant liquid is siphoned off, and the precipitate again centrifuged at high speed. The top and bottom layers are discarded and the middle layer is taken up in hot water. The turbid solution is centrifuged and the precipitate washed twice with water and then discarded. 20 gm. of sodium acetate are then dissolved in the solution and washings, since if an insufficient concentration of electrolyte is present an unduly large amount of alcohol must be used to precipitate the active material. The aqueous solution, which should now measure about 1.5 liters, requires 2.5 to 3 liters of alcohol for precipitation. The upper layer is again drawn off and the precipitate treated as before, any bottom layer formed after centrifugation being discarded. The active material is dissolved in water, centrifuged, and the solution, at a volume of 1 liter, treated with 1.5 gm. of sodium acetate and precipitated with 1.5 liters of alcohol. The precipitate is treated as before, after which the aqueous solution of the specific substance, at a volume of about 500 cc., is acidified with acetic acid until no further permanent precipitate forms. The mixture is then repeatedly centrifuged until most of the precipitate separates as a solid cake, from which the almost clear supernatant liquid can be poured off. The precipitate is washed with water and discarded and the solution and washings, at a volume of 750 cc., are treated with 10 gm. of sodium acetate and precipitated with 1 liter of alcohol. The precipitate is centrifuged off, redissolved in hot water containing a little acetic acid and sodium acetate, centrifuged if necessary, and, at a volume of 600 cc., precipitated with 1,100 cc. of alcohol. After centrifugation the active substance is dissolved in water, the solution is treated with 50 cc. of 10 per cent aqueous sodium hydroxide, centrifuged if necessary, and made up to 500 cc.

Alcohol is then cautiously added with vigorous stirring until a slight permanent turbidity remains. This is centrifuged off and the clear solution poured from the slight precipitate which generally consists of inactive material. Addition of alcohol is then continued until all of the specific substance has been precipitated, about 700 cc. in all being required. The solid is centrifuged off, dissolved in cold water, centrifuged again, and to the supernatant and washings are added 7 gm. of sodium acetate and 25 cc. of normal sodium hydroxide. The volume is adjusted to 400 cc. and the specific substance precipitated with 600 cc. of alcohol. After centrifugation it is redissolved in water and the solution acidified with acetic acid. At a volume of 180 cc. the solution gives a slight precipitate with about 90 gm. of ammonium sulfate, consisting mainly of impurities precipitable by tannic acid. After centrifugation the supernatant is saturated with ammonium sulfate, precipitating most of the active material as a gum which can be separated by centrifugation. The precipitation with ammonium sulfate is repeated twice, each time from a smaller volume. Small amounts of active material remain in solution and may be recovered by dialysis, concentration to very small volume, and saturation with ammonium sulfate. The main precipitate is dissolved in water and dialyzed in parchment bags against running water until most of the sulfate is removed. The solution is then concentrated to about 100 cc., cooled, diluted with 25 cc. of 1:1 hydrochloric acid, and again subjected to dialysis. The bags are finally transferred to several changes of distilled water until they are entirely free from chloride and sulfate. The solution is then concentrated to dryness on the water bath and the residue taken up in about 100 cc. of hot water. After centrifugation the solution is poured into 1,200 cc. of redistilled acetone, precipitating the specific substance as a white, asbestos-like mass which crumbles readily when dried. The yield is about 4.5 gm. from 300 to 325 liters of culture. From the acetone solution only a very little dark-colored residue is obtained on evaporation.

As stated in the previous paper, variations in the exact volumes given are often necessary with different lots of cultures, but this will occasion little difficulty if all fractionations are controlled by the specific precipitin test.

The properties of the specifically reacting polysaccharide material obtained in this way are summarized in Table I, in which all figures except those in the last two columns are calculated on the ash-free basis. Preparations 21 A and 24 were prepared by the above method,[1] while in No. 21 the alkaline purifications were omitted.

It thus appears that the specific soluble substance of Type II pneumo-

[1] Recent tests have indicated that the calcium oxide found as ash in the various preparations may have been derived at least in part from the parchment bags and experiments are now in progress in which collodion membranes were substituted.

coccus, in its present state of purity, consists of polysaccharide material containing 0.2 per cent or less of nitrogen, yielding about 75 per cent of reducing sugars on hydrolysis, and rotating the plane of polarized light about 55° to the right. It contains about 47 per cent of carbon

TABLE I.

Preparation No.	Specific rotation.	N	Reducing sugars on hydrolysis.*	C	H	Ash.	Precipitation with anti-pneumococcus serum.†
Type II pneumococcus.							
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	
21	+55.2°	0.46	70.5	46.8	6.0	3.7	1:10,000,000
21 A	+55.8°	0.20	67.2			3.2	1:5,000,000
24	+58.2°	0.16	74.8			3.7	1:2,000,000
22 A	+52.2°	0.41	65.8			4.2	1:5,000,000
22 B	+58.5°	0.31	62.6			4.2	1:5,000,000
22 F	+53.9°	0.12	72.5			3.5	1:2,000,000
22 H	+56.3°	0.05	76.1			4.1	1:2,000,000
23	+53.9°	0.39	62.3			6.5	1:5,000,000
24	+58.2°	0.16	74.8			3.7	1:2,000,000
24 A	+50.0°	1.0	74.3			4.0	1:2,000,000
24 C	+50.0°	0.19	75.0			4.0	1:5,000,000
Type III pneumococcus.							
27	−33.0°	0.11	73.0	42.3	5.2	0	1:2,000,000
28	−34.0°	0.05	73.0	42.6	5.6	0	1:3,000,000
31	−34.1°	0.10	74.0			0.2	1:3,000,000

*Calculated as glucose.
†After 2 hours at 37° and overnight at 4°.

and 6 per cent of hydrogen, and is free from sulfur and phosphorus, as determined on 0.2 gm. portions. It contains about 4 per cent of ash, left on ignition as calcium oxide, indicating perhaps that the product isolated is the salt of an acid. It gives no color with iodine and in 0.5 per cent solution is not precipitated by heavy metal salts

except basic lead acetate and, strangely enough, uranyl nitrate. It gives no biuret reaction, nor does it precipitate with phosphotungstic acid or tannic acid.

It will be recalled that in the first paper glucosazone was isolated from the products of hydrolysis of the specific substance. Since this could have been derived from glucose, fructose, or mannose, a clue was sought in the determination of the optical rotation during the course of the hydrolysis. In one experiment in which the initial specific rotation was + 62° in one-half normal hydrochloric acid, the identical value was obtained after 1½ hours boiling. Since the equilibrium value of the specific rotation of glucose is +52.8°, while the values for mannose and fructose are much lower, it would appear that the polysaccharide contained in the specific soluble substance of Type II pneumococcus is built up of glucose units.

2. Attempts to Separate the Specific Substance from the Carbohydrate.

Efforts at dissociation of the specific substance from the polysaccharide portion of the material were directed along the following lines: (*a*) hydrolysis of the carbohydrate; (*b*) precipitation of the specific substance with immune serum; (*c*) precipitation with uranyl nitrate; (*d*) precipitation with basic lead acetate; and (*e*) precipitation with safranine.

(a) Relation of Specificity to Acid Hydrolysis.

The followng experiment shows, in a rough, qualitative way, how the specific reaction diminishes only as the polysaccharide is hydrolyzed by strong acid, and also illustrates the remarkable stability of the specific substance to strong acid in the cold.

1:1 Hydrochloric Acid Used at Room Temperature.

Original concentration of Preparation 21, 1:1,000.

Test No.	0	1	2	3	4	5
Time	0	2 hrs.	19 hrs.	2 days.	3 days.	6 days.
Immune serum	+++	+++	+++	++±	+	−
Cu reduction	−	−	−	+	++	+++

(*b*) *Precipitation of the Specific Substance with Immune Serum.*

0.25 gm. of Preparation 24 was dissolved in 250 cc. of 0.9 per cent salt solution and treated with Type II immune horse serum until no further precipitate formed. 900 cc. of serum were required. After several hours at 37° the mixture was centrifuged and the gelatinous precipitate ground up in a mortar as well as possible with saline, diluted to 200 cc., and centrifuged. Washing with saline was repeated, both washings failing to react with immune serum. The precipitate was ground up with water and the suspension diluted to about 600 cc., acidified slightly with acetic acid, and boiled for 10 minutes. The resulting coagulum retained all of the specific substance, however, so that some other scheme of dissociation became necessary. After considerable experimentation it was found that the specific substance could be leached out by repeated extraction on the water bath with approximately normal ammonium hydroxide. The opalescent solutions so obtained, which contained much protein material, were neutralized with acetic acid, combined, concentrated on the water bath, centrifuged from precipitated protein derivatives, and dialyzed. The resulting solution was concentrated to 25 cc., treated with 2 gm. of sodium acetate, and precipitated with alcohol, the process being repeated twice from slightly smaller volumes. The precipitated specific substance was then dissolved in water, centrifuged, and evaporated dry on the water bath. The residue was taken up in water, centrifuged, and the process repeated until only a trace of insoluble material remained after drying. The residue was then dissolved in 4 to 5 cc. of water and poured into 60 cc. of redistilled acetone. The specific substance precipitated as a white, fibrous mass, and was washed with acetone and dried. The yield was 0.1 gm.

A comparison of the recovered specific substance (No. 24 A) with the original (No. 24) is given in Table I. It will be seen that precipitation of purified soluble specific substance with the homologous immune serum, followed by recovery of the specific substance from the immune precipitate, leads to a product essentially the same in optical activity, reactivity with immune serum, and percentage of reducing sugars on hydrolysis; in other words, a polysaccharide derivative greatly resembling the starting material. That the higher percentage of nitrogen in the recovered product is due to contaminating protein degradation products originating in the relatively enormous amounts of protein from which a small amount of specific material had to be separated, is indicated by the fact that the recovered specific substance, in contradistinction to all other preparations, gave a weak biuret test and a slight haze with tannic acid.

(c) *Precipitation with Uranyl Nitrate.*

To a solution of 2.2 gm. of a mixture of equal parts of Preparations 22 A and 22 B in 440 cc. of water, 5 per cent uranyl nitrate solution was cautiously added, with vigorous stirring, until present in slight excess. The mixture, from which a heavy, curdy yellow precipitate had settled, was neutralized to litmus with sodium hydroxide and allowed to stand overnight in the ice box. The supernatant liquid, after removal of the excess of uranyl ion with ammonia, reacted only feebly with Type II serum, but was concentrated to small bulk, treated with 1:1 hydrochloric acid in excess, and dialyzed against running water, and finally concentrated to dryness. 0.018 gm. of residue was obtained. The material was partially soluble in water, feebly dextrorotatory, reduced Fehling's solution after hydrolysis, and, in 1:200 solution, reacted strongly with Type II serum. It apparently consisted largely of specific substance that had escaped precipitation, together with accumulated impurities.

The specific soluble substance was liberated from the main uranium precipitate by repeated extraction on the water bath with 200 cc. of normal ammonium hydroxide solution. The centrifuged solutions were neutralized, combined, made slightly alkaline with ammonia, and heated to boiling, after which the small amount of ammonium uranate which separated was centrifuged off. The supernatant liquid was concentrated *in vacuo* to about 800 cc. and heated on the water bath for 1 hour after addition of 10 gm. of sodium acetate, 5 cc. of acetic acid, and a concentrated solution of 3 gm. of sodium dihydrogen phosphate. Small amounts of uranium still in solution were precipitated as uranyl phosphate, which was filtered off after the mixture had been cooled. The filtrate was neutralized to litmus, concentrated to 400 cc., filtered from a slight precipitate, cooled, and made strongly alkaline with 10 per cent aqueous sodium hydroxide. The resulting precipitate was centrifuged off in the cold and the clear supernatant neutralized with hydrochloric acid, concentrated to 150 cc., and dialyzed against running water with occasional additions of 10 cc. of 1:1 hydrochloric acid during the few days of dialysis. After removal of all phosphate and chloride ions the solution was concentrated to 20 cc., centrifuged from a slight precipitate, and poured into 210 cc. of redistilled acetone. The precipitate was washed with acetone and dried. The yield was 1.4 gm. and the color faintly yellow.

A comparison of the properties of the product (No. 22 F) with the original material (Nos. 22 A and B) shows that again a polysaccharide of substantially the same properties and specific activity was recovered.

(*d*) *Precipitation with Basic Lead Acetate.*

1.93 gm. of Preparation 24 were dissolved in 500 cc. of water and precipitated with a slight excess of basic lead acetate solution. The mixture was allowed to stand in the ice box overnight and was then centrifuged. The supernatant, freed from lead with hydrogen sulfide, and dialyzed, left only 0.7 mg. on evaporation, showing that the original material had been quantitatively thrown down.

The main precipitate was freed from lead by precipitating the metal ion as chromate, a preliminary experiment having shown difficulties in the customary removal of lead as sulfide owing to the tendency of the specific material to act as a protective colloid. The basic lead acetate precipitate was accordingly dissolved in dilute acetic acid and the very viscous solution diluted to 500 cc. with water. After addition of 10 gm. of sodium acetate the solution was heated on the water bath and treated with an excess of half normal potassium dichromate solution. After 5 minutes heating it was allowed to cool and was repeatedly centrifuged at high speed until the finely divided lead chromate had settled to the bottom. The yellow supernatant was dialyzed in parchment until it no longer gave a test for chromate ion with barium chloride, although the solution remained faintly yellow. During concentration on the water bath the color changed to a pale green, indicating the presence of traces of chromium ion, and part of the specific substance separated as a scum which had to be redissolved separately in hot water. After an additional dialysis in the presence of strong hydrochloric acid, the solution was further concentrated *in vacuo* to about 65 cc. and poured into 750 cc. of redistilled acetone. 0.82 gm. of solid separated in a somewhat more dense form than in the case of the other preparations.

The product (No. 24 C), as will be seen from Table I, was again essentially the same type of polysaccharide derivative as the original material and the preceding preparations.

(*e*) *Precipitation with Safranine.*

In recent work by Marston (3) safranine is used as a precipitating agent for trypsin and pepsin, resulting in a considerable purification of the enzymes. While safranine is far from a specific precipitant for enzymes, throwing down, as it does, such widely diversified types of substances as serum protein and gum arabic, it nevertheless seemed of interest to study its effect on the soluble specific substance, since as an azine dye, it represented a totally different type of precipitant from the immune serum or metallic salts previously used. A preliminary experiment showed that in suitable concentration the specific substance was quantitatively precipitated by safranine.

0.3 gm. of Preparation 22 F (Table I) was dissolved in 10 cc. of water and treated with a 1 per cent aqueous solution of Grübler's safranine until no further precipitate formed. The dark red, gummy precipitate was washed with 1 per cent safranine solution, dissolved in one-half normal hydrochloric acid, and shaken repeatedly with butyl alcohol, with occasional additions of water to make up for the amount taken up by the solvent. In order to remove all but traces of the dye it was necessary to make the solution alkaline and shake with butyl alcohol, and finally to repeat the process on the acid side. The aqueous solution was then neutralized to litmus, concentrated to dryness on the water bath, taken up in water, centrifuged from traces of insoluble material, and dialyzed after addition of 1:1 hydrochloric acid. The chloride-free solution was concentrated to dryness and the residue taken up in 5 cc. of water. The solution was centrifuged and poured into 60 cc. of acetone, precipitating 80 mg. of faintly pink, fluffy material.

As will be seen from No. 22 H, Table I, precipitation by safranine failed to alter the character of the specific polysaccharide material.

(f) *Preparation from Intact Pneumococci and Purification by Adsorption.*

It was thought that if a dissociation of polysaccharide and specific substance were possible, especially if the carbohydrate were derived from the broth culture medium, it might take place more readily if both the source of the crude specific substance and the method of purification were altered. Accordingly the intact bacterial cell was chosen as the source of the specific substance, and this was removed from solution by adsorption on aluminium hydroxide, a method used with success by Willstätter (4) in the purification of invertase.

120 liters of an 18 hour broth culture of Type II pneumococci were treated in ten lots as follows:

The pneumococci were centrifuged off at high speed, taken up in about 1 liter of 0.85 per cent NaCl solution, and treated with the minimum amount of bile necessary to effect complete solution of the bacterial cells. This solution was acidified with dilute acetic acid to precipitate the bacterial protein, and after removal of this by centrifugation, was neutralized to litmus and autoclaved. Each lot of filtrate, about 1.5 liters in volume, was shaken overnight with an especially bulky form of aluminium hydroxide, about 40 gm. being necessary to remove the specific substance from solution in each case. The hydroxide was allowed to settle, and, after the supernatant liquid had been siphoned off, was washed once with 1.5 liters of water. This was also siphoned

off, and the precipitate extracted overnight in the shaking machine with 1 liter portions of 2 per cent disodium phosphate solution. Three extractions were usually sufficient to remove the specific substance from the aluminium hydroxide. The phosphate extracts were neutralized to litmus with acetic acid, concentrated to small bulk on the water bath, and combined as the different lots were worked up. The total concentrate was then warmed to dissolve salts which had crystallized and any aluminium hydroxide present was filtered off. The filtrate was then precipitated with alcohol and the precipitated specific substance purified by repeated fractionation as in the original method. The resulting opalescent aqueous solution was finally dialyzed until free from salts and passed through a Berkefeld W filter, and concentrated to dryness on the water bath. The residue was taken up in water, centrifuged, and the solution concentrated to 10 to 15 cc. and poured into 160 cc. of acetone. 0.32 gm. of a voluminous, white, fluffy precipitate was obtained.

Although the broth culture medium as a source of the specific substance was eliminated, and the method of purification radically altered, the properties of the specific substance recovered (No. 23, Table I) remained entirely analogous to those of the other preparations discussed in this section, in each of which highly purified material was taken as a starting point and was treated in a different way.

Efforts at splitting the specific substance-polysaccharide complex, if such it be, with the aid of enzymes have been made, but have so far proved unsuccessful, as the polysaccharide is not attacked by the ordinary carbohydrate-cleaving enzymes. Several molds have also been used without success.

Type III Pneumococcus.

Little work was necessary on the specific soluble substance of Type III pneumococcus to show that marked chemical differences existed between it and the corresponding substance of Type II. 8 day broth cultures were used in this case also and the method of concentration and purification was the same up to the last steps, differences occurring only in the tenacity, appearance, and greater quantity of the precipitates of active material.

It then developed that not only is the Type III specific substance precipitated by heavy metal salts such as those of silver, mercury, and copper, which do not precipitate the Type II substance, but that, in conformity with this, the Type III substance is the soluble alkali

or alkaline earth salt of an *insoluble* strong acid which is thrown out of solution in the presence of an excess of strong hydrochloric acid.

Preparations 27 and 28 (Table I) were obtained from material which had been carried through the usual method of fractionation, including the final dialysis. In No. 27 the specific substance was first precipitated by copper chloride and freed from copper by treatment with hydrogen sulfide, while in No. 28 this step, later shown to be unnecessary, was omitted. The free specific acid itself was obtained as follows, the quantities given referring to the material from 300 to 325 liters of broth:

The final, dialyzed solution of the soluble substance, at a volume of about 600 cc., is treated with 100 cc. of 1:1 hydrochloric acid. After a preliminary interval the specific acid begins to separate slowly in fibrous masses which form gelatinous clumps and do not appear to be crystalline under the microscope. Frequent stirring is necessary to prevent the formation of a stiff jelly. The mixture is allowed to stand overnight in the ice box and is then filtered on a 7 inch Buchner funnel, pressed out with a flat top glass stopper, and washed twice with normal hydrochloric acid. From the filtrate in the case of Preparation 28 another polysaccharide was isolated after dialysis. It had a specific rotation of +185°, gave 100 per cent of reducing sugars on hydrolysis, was readily fermented by ptyalin, contained 0.05 per cent of nitrogen, and gave a purplish red color with iodine. It was therefore indistinguishable from erythrodextrin or glycogen, and was probably derived from the broth culture medium.

After washing on the funnel with normal hydrochloric acid the specific acid is washed repeatedly with 50 per cent alcohol, the suction being disconnected and the substance allowed to soak up the alcohol at each washing. When the washings no longer contain chlorine ion, the specific acid is washed several times with redistilled acetone and dried *in vacuo*, being warmed from time to time to accelerate the progress. The yield varies from 6.5 to 9 gm., and the substance is ash-free.

Since the properties of the specific acid were so characteristic and even permitted removal of an accompanying carbohydrate, it was thought that the long scheme of purification might be shortened with its aid. Accordingly, Preparation 31 was worked up in this way:

After the third reprecipitation with alcohol and subsequent removal of additional impurities by precipitation with acetic acid the centrifuged solution, at a volume of 800 cc., was treated with about 200 cc. of 1:1 hydrochloric acid, occasionally stirred, and allowed to stand overnight in the ice box. The precipitate was filtered off, washed with a little normal hydrochloric acid, suspended in water, and dissolved with the aid of a slight excess of sodium hydroxide. The resulting somewhat turbid solution was acidified faintly with hydrochloric acid,

diluted to about 800 cc., and run through an 8 inch Berkefeld N candle, washing through with much water. The resulting clear solution was concentrated to about 600 cc. and reprecipitated as in the case of Preparation 28. The yield of slightly yellowish material was 9.6 gm. The precipitation of the Type III substance by hydrochloric acid is not entirely quantitative, but the loss is small in proportion to the degree of purification attained.

It will be seen from the remarkable uniformity of the figures in Table I for Preparations 27, 28, and 31 that one is apparently dealing here with a much more definite chemical entity than in the case of the Type II substance, in which, in the absence of so characteristic a property which can be made use of in the process of purification, the variation in the findings is of a much wider range. While the Type II specific substance rotates the plane of polarized light about 55° to the right, the insoluble Type III acid, dissolved by neutralization with sodium hydroxide, gives a rotation of *minus* 34°. Its activity with homologous antipneumococcus serum and the percentage of reducing sugars found on hydrolysis are about the same as in the case of the Type II substance, while the nitrogen content is so low as to be practically negligible. The carbon content is about 4 per cent less and the hydrogen content about 0.5 per cent less. 0.1 gm. of the acid requires about 0.3 cc. of normal sodium hydroxide for neutralization to litmus. The Type III substance, as well as the Type II, gives no color with iodine, no biuret reaction, and no precipitate with tannic acid, but it is precipitated not only by uranyl nitrate and basic lead acetate, but also by many other heavy metal salts which do not affect solutions of the Type II substance. There is thus no question of the marked differences in chemical properties of the two substances, and some insight into the cause of these differences is afforded by the behavior of the Type III substance on hydrolysis.

2.0 gm. of Preparation 28 (corresponding to 1.92 gm. of anhydrous substance) were suspended in water and dissolved by the gradual addition of normal sodium hydroxide, 6.5 cc. giving a solution neutral to litmus when all the acid had dissolved. The solution was made up to 192 cc. and showed a specific rotation of −36.5°. After mixing with 192 cc. of normal hydrochloric acid the rotation was unchanged but on boiling it gradually decreased to 0° and became positive, tending to become constant at about +23° after 5 hours. The hydrolyzed solution was cooled, neutralized to Congo red with 10 per cent sodium hydroxide, concentrated *in vacuo* to 164 cc., and divided into two portions.

By the action of phenylhydrazine on the first portion an osazone was obtained, crystallizing in balls of microscopic needles. After washing with methyl alcohol the yield was 0.062 gm. The osazone softened above 180° and decomposed above 195°, and was levorotatory, but was so insoluble in the usual alcohol-pyridine mixture that a quantitative determination of the rotation was impossible. The remaining half of the hydrolysis mixture was almost neutralized with sodium hydroxide, treated with 1 to 2 gm. of sodium acetate, and to the solution 1.5 gm. of freshly recrystallized *p*-bromophenylhydrazine hydrochloride were added. The solution was then heated on the water bath and filtered from the small amount of reddish tar which separated almost at once. A crystalline osazone soon separated. After 40 minutes heating, the mixture was allowed to cool, and the partially crystalline osazone was filtered off, washed with water, and ground up with glacial acetic acid, finally placed in the water bath for a few moments. The mixture was quickly cooled, diluted with several volumes of ether, and allowed to stand overnight in the ice box. A gelatinous precipitate over the heavy crystalline sediment was poured off and the crystals were filtered off and washed first with glacial acetic acid and finally with ether. The yield was 0.040 gm. When rapidly heated, the *p*-bromophenylosazone derivative softened and darkened at about 180° and melted with decomposition at about 190°. It gave a clear, pale yellow solution in pyridine-alcohol (3:2) with an initial specific rotation of 280° and a final value of $[\alpha]_D$ −230°. According to Neuberg (5) glucuronic acid forms a derivative with *p*-bromophenylhydrazine characterized by an extraordinarily large levorotation, but his product had different properties from the one obtained above. However, a portion of the hydrolysis liquid gave a strong orcinol test for glucuronic acid.[3]

DISCUSSION.

As regards the soluble specific substance of Pneumococcus Type II, it has proved possible to start with purified carbohydrate material, containing 0.2 per cent or less of nitrogen and still capable at a dilution of one part in 2 to 5 million of precipitating immune serum, and with such a product by a number of diverse procedures, to recover in each instance a substance almost identical with the starting material in optical rotation, percentage of nitrogen, percentage of reducing sugars on hydrolysis, and specific activity. Whether the specific substance is precipitated by immune serum, by uranyl nitrate, by basic lead acetate, or by safranine, precipitants of three totally

[3] The writers wish to express their heartiest thanks to Dr. P. A. Levene for his generously given time and assistance in connection with this and other phases of the work, and to Miss I. Weber and Dr. W. A. Jacobs for their help, as well.

unrelated types; whether the intact bacterial cell or the broth culture medium is used as the source of the material; or whether the method of purification is based on simple fractional precipitation, or on adsorption, the product recovered is always essentially the same, and always largely a polysaccharide. Coupled with this are the facts that the specificity of this extraordinarily stable material does not diminish on treatment with strong acid in the cold until reducing sugars begin to appear, and that by the same technique it is possible from Type II and Type III pneumococci to isolate *different* polysaccharide derivatives of equal specific activity. If the polysaccharide encountered were an impurity derived from the broth culture medium, contaminating an unknown specific substance, it might be expected to be the same, or at least very similar, in both instances.

It is thus becoming increasingly difficult to believe that the soluble specific substance is not actually the polysaccharide derivative itself, for there is now accumulated a considerable mass of evidence in favor of this view.

The study of the chemical nature and biologic specificity of this polysaccharide material furnishes a basis for the better understanding of the immunological relationships of the bacterial cell. The serological specificity of the various types of Pneumococcus is intimately related to, if not solely dependent upon, the presence of this specific cell constituent. The synthesis of this polysaccharide material is a cellular function highly developed in those strains of pneumococci which are most capable of multiplying in animal tissues. This substance apparently bears a significant relationship not only to type specificity but to virulence and capsular development. The elaboration of this soluble specific substance during growth in the animal body is so marked that its presence may be detected in the body fluids of experimentally infected rabbits and in the blood and urine during the course of the spontaneous disease in man.

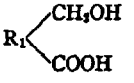
As pointed out in a preceding paper (6), the pneumococcus cell contains among other constituents two substances which are separable and distinct chemically and which possess different properties immunologically. One of these cell constituents is protein in character. Further studies on the biological specificity of pneumococcus protein are now in progress. It need only be mentioned here that

serologically and antigenically this protein fraction seems to be less specific as to type than is the intact bacterial cell. The second, or carbohydrate fraction, the chemical nature of which is dealt with in this communication, is highly and specifically reactive only with antibacterial serum of the same type of Pneumococcus as that from which the substance is derived. Although this bacterial polysaccharide is specifically reactive with antibody produced in response to immunization with the intact organism, it is itself, when dissociated from combination with other cell constituents, but slightly if at all capable of inducing antibody formation. The biologic differences in the specificity of the soluble specific substances of Type II and Type III pneumococcus are reflected in differences in their chemical constitution.

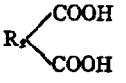
The chemical difference between the soluble specific substances of Pneumococcus Types II and III are so marked as to cause astonishment that analogous fractions of closely related microorganisms can have such widely divergent properties. While both consist of about 75 per cent polysaccharide[3] and 25 per cent of unknown constituents, the Type II substance is dextrorotatory, the Type III substance levorotatory; the former, if the salt of an acid, is the salt of a soluble acid, while the latter is the salt of an extremely strong, difficultly soluble acid, so strong, indeed, that in very dilute aqueous solution it turns Congo red paper blue. The polysaccharide portion of the Type II specific substance appears to be built up of glucose units, while that of the Type III substance seems, on the basis of preliminary evidence, to be composed either of glucuronic acid itself or of some analogous acid. The lower percentages of carbon and hydrogen in the Type III substance are also in harmony with this view, since the replacement of $-CH_2OH$ groups by $-COOH$ (the difference between glucose and glucuronic acid) would have this effect.

The differences between the two specific substances, if they may be assumed to be chemical individuals, may be presented by the following two expressions:

Type II.



Type III.



[3] In the calculation it is assumed that the reducing power of the sugars obtained on hydrolysis is the same as that of glucose.

It is hoped soon to report on the positive identification of the sugar unit of the Type III substance, on the nature of the unknown portion of the specific substance, and on the soluble specific substance of Type I pneumococcus.

SUMMARY.

1. The method for the concentration and purification of the soluble specific substance of Pneumococcus has been improved.

2. Highly purified specific substance of Type II pneumococcus of polysaccharide nature is shown to be recovered essentially unchanged after precipitation by immune serum, by uranyl nitrate, by basic lead acetate, or by safranine.

3. Marked chemical differences are shown to exist between the specific substances of Type II and Type III pneumococcus, although both react as polysaccharides.

4. The weight of evidence is considered to be in favor of the view that the specific substances of Pneumococcus Types II and III are actually polysaccharide derivatives.

5. The immunological significance of the foregoing view is discussed.

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