THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

THIRD PAPER.

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In the preceding papers of this series (1) the significance of the so called "soluble specific substance" of Pneumococcus (2) was discussed and methods were given for the isolation of the specific substances of Types II and III pneumococcus. In the present communication refinements of these methods are described. With their aid it has been possible to obtain both of these substances free from nitrogen and possessing much greater activity when tested with the homologous immune sera than did the products previously isolated. Further data are presented concerning the nature of the polysaccharides with which these substances appear to be identified.

The isolation of a specific substance from Type I pneumococcus is also described, and it is shown that this substance, while apparently also a sugar derivative, contains nitrogen and differs also in other respects from the specific substances isolated from the other two fixed types of pneumococci.

EXPERIMENTAL.

1. The Soluble Specific Substance of Type I Pneumococcus.

The original method of purification of the soluble specific substance was modified after numerous trials to fit the properties of the Type I substance.

8 day cultures of Type I pneumococcus in meat infusion phosphate broth are concentrated in 21 liter lots on the water bath to a volume of 1.2 to 1.5 liters. The concentrate is precipitated with 1.4 volumes of alcohol and put through the

initial 3 layer separation as described in Paper I. The middle layer[1] is dissolved as well as possible in 200 to 300 cc. of water and the mixture is centrifuged and the precipitate washed twice with small amounts of water. The clear solution and washings are kept on ice and combined with subsequent lots until 300 to 325 liters have been worked up. After solution of the deposit of salts the total concentrate, at a volume of about 4.5 to 5.0 liters, is treated with 1:1 hydrochloric acid until strongly acid to Congo red. The resulting precipitate is allowed to settle for several hours in the cold, after which as much of the supernatant liquid as possible is siphoned off and the remainder cleared by centrifugation. The precipitate is washed once with 0.01 normal hydrochloric acid and again separated by repeated centrifugation at high speed. The clear solution and washings, at a volume of 5 to 6 liters, are now precipitated with 7 to 8 liters of chilled alcohol and allowed to stand overnight in the cold. A test portion of the supernatant liquid, when neutralized and boiled down to small volume, should give no immediate precipitate with Type I antiserum. As much as possible is siphoned off and the crude specific substance separated from the remainder by centrifugation. The precipitate is then washed in the centrifuge bottles with 0.5 normal acetic acid, in which the isoelectric Type I substance is insoluble, while most of the accompanying glycogen or erythrodextrin dissolves. After several hours in the cold the mixture is centrifuged and the precipitate taken up in water. The specific substance is dissolved by addition of sodium hydroxide until the mixture is faintly alkaline to litmus, the volume is adjusted to about 700 cc., and the mixture is centrifuged at high speed until clear. The precipitate is washed with 50 cc. of water and again centrifuged. To the solution and washings are added 20 gm. of sodium acetate, and when this is dissolved 400 cc. of alcohol are added, with vigorous stirring, and the mixture is allowed to stand overnight in the cold. In relatively concentrated solution and in the presence of sufficient salts the Type I specific substance is quantitatively precipitated by 0.5 volume of alcohol in the cold. The use of sodium acetate is advantageous as it is not thrown out by alcohol at the concentrations used. The precipitate is centrifuged off, water is added up to a volume of about 500 cc., the mixture is made slightly alkaline again if necessary, and is finally centrifuged to remove any insoluble material. The solution is then stirred mechanically and the specific substance is rendered insoluble by the careful addition of 16 cc. of glacial acetic acid. After several hours in the cold it is centrifuged off and redissolved with the aid of sodium hydroxide as before, except that the volume should now be about 400 cc. 15 gm. of sodium acetate are added, and the specific substance is precipitated in the cold with 200 cc. of alcohol. After having stood overnight it is centrifuged off and redissolved in enough water to bring the volume to about 250 cc., using enough sodium hydroxide to make

[1] Centrifuged precipitates containing the soluble specific substances are usually compact and gummy, and care must be taken to smooth out all lumps when the deposit is redissolved.

the reaction slightly alkaline. The solution is chilled, stirred mechanically, and treated with an equal volume of barium hydroxide solution in equilibrium with the crystalline phase at about 50°. Most of the specific substance separates at once, and the mixture may be centrifuged after several hours in the ice box. Under optimal conditions the precipitation is complete, but occasionally up to about 15 per cent of the specific substance may remain in solution. If this is to be recovered it is best worked up separately, as coloring matter and other impurities are eliminated with it. The barium hydroxide precipitate is taken up in about 400 cc. of water and treated with 5 normal sulfuric acid until, after all lumps are smoothed out, the reaction remains acid to Congo red. The mixture is then centrifuged, and the precipitate of barium sulfate washed twice with very dilute sulfuric acid. The supernatant solutions are neutralized, and if opalescent, may be passed first through a Berkefeld V candle and then through the W grade. The solution is concentrated *in vacuo* to about 200 cc., made faintly alkaline with sodium hydroxide, and precipitated in the cold with 100 cc. of alcohol after the addition of 10 gm. of sodium acetate. The next day the precipitate is collected in the usual way, redissolved in water, and at a volume of about 200 cc. is again thrown out at the isoelectric point with 7 cc. of glacial acetic acid, using the same precautions as before. The precipitate is taken up in a little water and redissolved by addition of 1:1 hydrochloric acid. The volume is made up to about 125 cc. with water and hydrochloric acid, and the specific substance is then precipitated as the hydrochloride by the addition, with mechanical stirring, of 250 cc. of chilled redistilled alcohol. The centrifuged product is redissolved and reprecipitated in the same way and is then taken up in as small a volume as possible of cold water. The extremely viscous solution is then dialyzed in collodion bags in the ice box against successive changes of distilled water. The Type I specific substance is a weak base, and as the excess of hydrochloric acid diffuses out the hydrochloride is hydrolyzed and the isoelectric substance precipitates. Completion of the process and removal of the last traces of chlorine ion are accelerated by mixing the contents of the bags each time the water is changed. The mixture is finally centrifuged, the solution, which contains a little active material, is added to the next preparation, and the precipitate is washed with acetone, filtered on a hardened paper in a Büchner funnel, and dried. The yield should be from 2 to 3 gm.

The Type I specific substance obtained by this method contains 5 per cent of nitrogen and is often ash-free and colorless, although some preparations are still grayish in color and contain a little ash. In its isoelectric form the product is very sparingly soluble, but may be dissolved with the aid either of alkali or mineral acid. Concentrated solutions are extremely viscous. The specific optical rotation, +300°, is about the same on either side of the isoelectric point, which is

in the vicinity of pH 4. When boiled with a sufficient excess of mineral acid the substance is slowly hydrolyzed with formation of reducing sugars, which it has not yet been possible to identify fully. Reducing sugars also appear when the substance is treated with nitrous acid in the cold. One-half of the nitrogen is evolved at the same time, with simultaneous disappearance of the specific reaction. Since the specific substances of Types II and III pneumococcus are unaffected by nitrous acid under these conditions, it would appear that this half of the nitrogen, at least, is an integral part of the specific substance and is either linked to the reducing group of a sugar derivative, as Karrer believes is the case with the polyglucosamines (3), or else is so placed in the complex molecule that its removal causes some other type of scission. About one-half of the remaining nitrogen in the substance is given off within 15 minutes when the reaction mixture, freed from nitrous acid, is made alkaline and distilled with steam. The substance contains no sulfur or phosphorus.

When a concentrated aqueous solution of the hydrochloride of the specific substance is carefully treated with 1:1 hydrochloric acid a point is reached, at an acidity of 1.3 normal, at which the substance again precipitates, redissolving again if still more acid is added. Whether this indicates lactam formation, or some other reversible intramolecular change, is not yet clear.

$\frac{1}{2}$ per cent solutions, prepared by dissolving the specific substance in dilute acid and bringing it back past the isoelectric point with dilute sodium hydroxide, give precipitates with solutions of the following reagents: barium hydroxide, copper sulfate, silver nitrate, neutral and basic lead acetates, uranyl nitrate, and phosphotungstic acid. There is no color developed with iodine-potassium iodide solution, nor does the unhydrolyzed substance show reduction with Fehling's solution. The biuret, tannic acid, xanthroproteic, and ninhydrin tests are negative. With Millon's reagent a jelly is formed in the cold, but the precipitate redissolves on heating and no color is developed. Potassium permanganate is not immediately reduced.

After hydrolysis with nitric acid and subsequent oxidation mucic acid was obtained.

1.5 gm. of air-dry Preparation 39 were dissolved in 200 cc. of hot 1.4 normal nitric acid and boiled under a reflux condenser for 4 hours. The resulting green-

ish yellow solution was concentrated *in vacuo* to about 10 cc., and 4 cc. of concentrated nitric acid were added. The next day the mixture was boiled for a few moments and then stirred on a large clock-glass over boiling water until dry. The process was repeated with a few cc. of 1:1 nitric acid, after which the dry, white residue was taken up in acetone and allowed to stand overnight. The crystalline residue was filtered off, washed with a little acetone, and taken up in 5 cc. of normal ammonium hydroxide. The insoluble portion, apparently calcium oxalate, was discarded. Addition of 0.3 cc. of concentrated nitric acid to the filtrate resulted in the deposition of 0.21 gm. of mucic acid melting and decomposing at 216° with preliminary softening and blackening. The acetone mother liquors above were concentrated to dryness and taken up in water, when a second fraction of mucic acid was left behind. This was purified in the same way, yielding 0.07 gm., melting and decomposing at 215°. For analysis the two fractions were combined and recrystallized from water.

0.1008 gm., anhydrous, ash-free substance gave 0.1253 gm. CO_2 and 0.0442 gm. H_2O.

Calculated for $C_6H_{10}O_8$: C, 34.27 per cent; H, 4.80 per cent. Found: C, 33.9 per cent; H, 4.9 per cent.

From the aqueous mother liquors from which the second crop of mucic acid was obtained was isolated an acid potassium salt in a yield of 0.191 gm. and containing 22.3 per cent of potassium.

A repetition of the oxidation in the cold with 1 gm. of Preparation 41 gave after 1 week a deposit of mucic acid weighing 0.115 gm. after two recrystallizations.

Since the Type I specific substance gives a positive test for glucuronic acid with naphthoresorcinol it is possible that galacturonic acid is present as a part of the molecule, for this would yield mucic acid on oxidation. That the mucic acid does not arise from oxidation of galactose itself is indicated by the difficulty with which an osazone is formed from the products of hydrolysis, although crystallization of the osazone formed might also be hindered by the complexity of the reaction mixture.

While the Type I specific substance as at present obtained probably does not represent a single, chemically pure compound, it would nevertheless seem as if the major portion of the impurities had been removed. As will be seen from Table I, in which the figures given are calculated to the ash-free basis, the total nitrogen, the nitrogen and reducing sugars liberated on treatment with nitrous acid, and the optical rotation are remarkably constant in the majority of instances, although the preparations listed were isolated in different ways.

29B was prepared from Type I pneumococci themselves, instead of the autolyzed culture fluid, by solution in diluted bile, removal of the "nucleoprotein" with acetic acid, and adsorption of the specific substance on alumina, as described in the case of Type II pneumococcus (1).

In 36 and 37 the use of barium hydroxide was omitted, necessitating a more laborious fractionation. 37A was prepared from 37 by adsorption on a particularly active form of alumina (Alumina A) prepared according to the directions of Willstätter and Kraut (4).

41B_2 was obtained from 41 by interaction with neutralized formalin in slightly alkaline solution. Discharge of the phenolphthalein color indicated that the specific substance combined with the aldehyde. However, fractional precipitation of the resulting substance with alcohol (B_2 being the second fraction) failed to yield a product with different properties. That the combination with formaldehyde was a labile one was indicated by the fact that the product recovered, when once more treated as before with formalin, again showed an increase in the acid reaction, indicating that any formaldehyde combined with the specific substance had been eliminated in the process of isolation (addition of cold dilute hydrochloric acid and precipitation with alcohol).

43A was obtained by digestion of the product of the third alcoholic precipitation (see method) with an amount of ice-cold water sufficient to dissolve only a portion of the specific substance. The mixture had a total volume of 300 cc. in this case, and the specific substance was reprecipitated with alcohol and then purified with the aid of barium hydroxide. The residue from the A fraction was worked up in the usual way as 43B, while the part of it not precipitated by barium hydroxide, but separating when the alkaline mother liquors were acidified with acetic acid, is represented by 43C. It will be seen from Table I that each of these fractions gave the same analytical values, and the physical properties corresponded equally closely.

Finally, 44A, after precipitation with barium hydroxide, removal of the barium, and precipitation with alcohol, was purified twice over the sparingly soluble hydrochloride mentioned above. It was hoped that precipitation in this way by hydrochloric acid without the assistance of alcohol would result in the removal of nitrogenous or carbo-

TABLE I.

Preparation No.	Specific rotation.	Total N.	Amino N.	Reducing sugars on acid hydrolysis.*	Reducing sugars by HNO_2.	C	H	Ash.	Precipitation with anti-pneumococcus serum.†
Type I pneumococcus.									
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
29B	+301°	4.8	2.7	31.8		43.3	5.8		1:4,000,000
36	+304.5°	4.6‡	2.4	31.6				1.5	1:6,000,000
37	+295.5°	5.1	2.6	27.2				0.7	1:6,000,000
37A	+287°	4.1‡	2.5		31.7			7.9	1:6,000,000
38A	+310°	5.0	2.6	28.6	28.5			1.8	1:6,000,000
39	+304°	4.4‡	2.5		27.1			1.6	1:6,000,000
41	+303°	5.0	2.6		26.0			0.0	1:8,000,000
$41B_2$	+279°	4.4‡	2.5		23.9				
43A	+303°	5.0	2.5		28.8			1.2	1:6,000,000
43B	+300°	4.9	2.5	28.4	28.5			0.0	1:6,000,000
43C	+300°	5.0	2.6		28.0			0.0	1:6,000,000
44A	+300°	5.0	2.6		28.5	43.3	5.5	0.5	1:8,000,000
Type II pneumococcus.									
					Acid equivalent.				
25	+63.2°	0.18		80.3				1.9	1:3,000,000
25A	+70.2°	0.0		68.4	1302	45.8	6.4	0.35	1:6,000,000
25B	+56.7°	0.0			946				1:2,000,000
25C	+72.2°	0.12		67.6	1190				1:5,000,000
26A	+75.2°	0.0		69.1	1258			1.6	1:6,000,000
$26A_1$	+72.8°	0.0		68.2	1252			0.0	1:5,000,000
26B	+54.4°			56.0	1105				
Type III pneumococcus.									
30	−37.3°			73.0	330				1:6,000,000
30I	−30.9°	0.0		73.3	347				1:5,000,000
30A	−35.1°	0.0		71.0	339			0.0	1:6,000,000
33	−30.5°	0.0		73.0	343			0.0	
33I	−32.5°	0.0		74.5	341			0.0	
33II	−34.0°	0.0		75.5	340	42.7	5.3		
33B III	−30.8°	0.0		72.5	358			0.0	1:6,000,000

* Calculated as glucose.

† 2 hours at 37° and overnight in the cold.

‡ Micro Kjeldahl determinations using aeration method for collecting NH_3. Results about 10 per cent low. In the other cases Pregl's method was used.

hydrate material that would otherwise be precipitated by the alcohol customarily added. However, this preparation checked closely with the others in optical rotation, carbon, hydrogen, and nitrogen, and in the other quantitative and qualitative tests used.

2. Type II Pneumococcus.

A. Further Purification of the Type II Soluble Specific Substance.

The specific substance from 312 liters of broth culture was isolated according to the method given in Paper II. After the third precipitation with ammonium sulfate the deposited material was redissolved in hot water, run through a Berkefeld W candle to remove opalescence, and concentrated to 150 cc. The solution was cooled to 0°, acidified with 35 cc. of 1:1 hydrochloric acid, stirred mechanically, and precipitated by the addition of 350 cc. of chilled redistilled alcohol. After 2 hours in the ice box the precipitate was centrifuged off in the cold and redissolved in 75 cc. of cold water. The precipitation process was then repeated with 18 cc. of 1:1 hydrochloric acid and 300 cc. of alcohol. The precipitate, after centrifuging, was redissolved in 100 cc. of water, rinsed into a collodion bag with 5 cc. of normal hydrochloric acid, and dialyzed in the cold against distilled water until free from chlorine ion. The solution was concentrated to 50 cc. *in vacuo* and stirred into 600 cc. of redistilled acetone. After having stood overnight the precipitate was collected on hardened paper, washed with redistilled acetone, and dried. The yield was 3.3 gm. in the case of Preparation 26A, and 2.4 gm. for 25A.

The supernatant from the initial acid-alcohol precipitation still reacted strongly with Type II antiserum and was accordingly precipitated with 0.5 volume of ether. The material deposited was dissolved in 20 cc. of water and, after addition of a little hydrochloric acid, was dialyzed as in the previous instance, concentrated to 20 cc., and poured into 12 volumes of acetone. 0.6 gm. was recovered (26B) in one case and 0.5 in another (25B).

In an attempt to push the purification further a portion of 26A was again put through the acid-alcohol precipitation process. The product, $26A_1$, gave, however, the same analytical values as that from which it was derived.

From a comparison of Preparation 25, Table I, in which these additional steps in the purification process were omitted, with 25A, 26A, and $26A_1$, it will be seen that the remaining nitrogen in the specific substance has been eliminated, all but traces of ash have been removed, a fraction (25B and 26B) has been separated containing impurities of lower optical rotation, and the highest dilution at which the substance reacts with its homologous serum has been increased. In order to reduce the analytical error to a minimum micro Kjeldahl determinations were run on 25 mg. of substance in the case of 26A.

B. Confirmation of Glucose as the Chief Sugar Constituent of the Type II Specific Substance.

In the previous papers it was shown that glucosazone could be obtained from the hydrolysis products of the Type II soluble specific substance. Although the hydrolyzed material showed a specific rotation of about +55°, indicating that the resulting hexose was probably glucose, the possibility that the sugar might have been mannose or fructose was by no means excluded, owing to the possible presence of other optically active substances.

In order definitely to determine this point, a portion of the specific substance was hydrolyzed and subsequently oxidized.

0.5 gm. of Preparation 25A was dissolved in 25 cc. of water and boiled for 5 hours under a reflux condenser with an equal volume of normal nitric acid. The solution was evaporated *in vacuo* to 3 to 4 cc., treated with 2 cc. of concentrated nitric acid, and allowed to stand at room temperature overnight. The mixture was then boiled for 2.5 minutes over a flame, and poured on to a large watch-glass, stirred, and quickly evaporated over a boiling water bath. A thick paste was obtained and this was twice evaporated with water to expel the last traces of nitric acid, dissolved in 2 cc. of water, made strongly alkaline with 40 per cent potassium hydroxide solution, and reacidified with glacial acetic acid. The mixture was cooled in ice water, and after 24 hours in the cold the crystals of potassium acid saccharate which had separated were filtered off. 0.1 gm. was recovered. This was recrystallized from 1.5 cc. of water, yielding 0.062 gm. of the purified salt.

0.0602 gm. substance gave 0.0210 gm. K_2SO_4.

Calculated for $KOOC(CHOH)_4COOH$: K, 15.75 per cent. Found: 15.65 per cent.

The isolation of potassium acid saccharate through the oxidation of the hydrolytic products of the Type II soluble specific substance, together with the isolation of glucosazone previously reported, leaves little doubt that the units from which the polysaccharide is built up are those of glucose.

C. Preparation of a Triacetate of the Type II Soluble Specific Substance.

If the true soluble specific substance were actually a polysaccharide it should be possible to convert it into an acetyl derivative just as the celluloses and starches may be acetylated. On the other hand,

if the polysaccharide present were an impurity the altered solubilities of an acetyl derivative might be expected to facilitate its removal from the actual specific substance.

0.5 gm. of Preparation 25A was accordingly dried to constant weight *in vacuo* and shaken overnight at room temperature with a mixture of 2.5 cc. of dry acetic anhydride and 6 cc. of dry pyridine. Since no reaction appeared to have taken place the tightly stoppered bottle containing the mixture was heated in a water oven for 24 hours. The resulting suspension, which had darkened somewhat, was poured into ice water but most of the solid failed to dissolve. It was therefore filtered off, macerated with water until the washings failed to give a specific test with Type II antiserum, and shaken with 200 cc. of water and a few glass beads until it had disintegrated into a very fine powder. After centrifugation the supernatant fluid gave only a weak test with immune serum. The residue was next washed with 50 cc. of methyl alcohol, which removed most of the coloring matter, and was dried *in vacuo*. 0.7 gm. of an amorphous, fluffy, tan powder was recovered.

0.1009 gm. substance gave 0.1864 gm. CO_2 and 0.0503 gm. H_2O.

Calculated for $[C_6H_7O_5(OCOCH_3)_3]_x$: C, 49.95 per cent; H, 5.60 per cent. Found: C, 50.4 per cent; H, 5.6 per cent.

0.1084, 0.1190 gm. substance hydrolyzed with 0.5 N NaOH, neutralized 9.70, 10.90 cc. 0.1 N NaOH respectively. Calculated: 11.30, 12.40.

The triacetate was found to be practically insoluble in water and the common organic solvents. It dissolved, however, when warmed with dilute sodium hydroxide, the neutralized solution giving a heavy precipitate with antiserum. On account of the extreme insolubility of the triacetate it was difficult to determine definitely whether or not it was specific in itself. However, the large yield and the fact that it again yields a specifically reacting substance on hydrolysis showed that no separation of the polysaccharide from the actual specific substance had been effected.

D. Recovery of the Type II Specific Substance from Its Precipitate with Homologous Antibody.

In Paper II it was shown that the Type II specific substance could be recovered from its precipitate with immune serum. Owing to the difficulty met with in separating the polysaccharide from large amounts of serum protein it seemed advisable to repeat this experiment, using instead of the serum an antibody solution purified essentially according to Felton (5).

1.5 liters of Type II antipneumococcus serum were gradually added with stirring to 28.5 liters of well chilled N/100 acetic acid-sodium acetate buffer at pH 5. After having stood overnight the precipitate was centrifuged off, dissolved in 750 cc. of 0.85 per cent salt solution, centrifuged once more, and again added to 19 volumes of the same buffer solution. The resulting precipitate, which contained a large proportion of the antibodies in the original serum, was finally collected as before and dissolved in 1.5 liters of saline.

To this solution was slowly added 0.2 gm. of Preparation 25A dissolved in 500 cc. of saline. After 2 hours in the incubator at 37° and standing in the ice box overnight the precipitate was centrifuged off and was washed with 150 cc. of saline, centrifuged again, and suspended in 150 cc. of a citrate-phosphate-borate buffer solution prepared according to Northrop (6). The mixture was digested at 37° with 1 gm. of trypsin added in small portions at intervals until a clear solution was obtained. This was then treated with 2 volumes of alcohol and the resulting precipitate was suspended in water and treated with dilute sodium hydroxide solution until it just dissolved. The solution was heated to boiling and neutralized with dilute acetic acid, causing the separation of a coagulum. The supernatant liquid containing the specific substance was evaporated to complete dryness and taken up in boiling water. A small amount of insoluble protein material was centrifuged off and the supernatant liquid was dialyzed until free from salts and repeatedly evaporated and extracted with water. With each evaporation a small amount of insoluble protein material was eliminated. The extract was finally saturated with ammonium sulfate, whereby a heavy yellow protein precipitate separated. This was centrifuged off, redissolved in a little water, reprecipitated with ammonium sulfate, and again centrifuged. The supernatant liquid containing the soluble substance was again dialyzed until free from sulfate. The solution was then concentrated to 5 cc. and precipitated with 10 cc. of alcohol and a few crystals of sodium acetate. The inactive supernatant liquid was discarded and the precipitate was twice reprecipitated by alcohol from a volume of 5 cc. The substance was finally precipitated from the same volume at 0° by the addition of 1.5 cc. of 1:1 hydrochloric acid and 2 volumes of alcohol. It was then dissolved in a few cc. of water and added to a large excess of acetone. The yield was 0.12 gm.

The recovered specific substance (25C) failed to give the biuret reaction and contained only 0.12 per cent of nitrogen, whereas in the original experiment with serum the nitrogen content was 1.0 per cent. In its other properties it agreed well with the highly purified material used to precipitate the antibody solution.

3. The Soluble Specific Substance of Type III Pneumococcus.

A. Further Attempts at Purification.

A preparation of the Type III specific substance which had been twice purified by precipitation with hydrochloric acid (see Paper II) was reprecipitated three times in this manner in order to determine whether any additional impurities could be eliminated. However, the preparations, 33, 33I, and 33II checked well in analytical values with each other and with previous preparations, except that no nitrogen was found, although in the case of 33I 35.5 mg. of substance were used for each micro Kjeldahl determination.

Since it had been found that the Type III substance gave a precipitate when barium hydroxide in excess was added, an additional purification of the substance was attempted over the barium salt.

0.5 gm. of Preparation 33 was finely pulverized and suspended in 25 cc. of water. The mixture was heated to boiling under a reflux condenser and to it was added enough 0.2 normal barium hydroxide solution to keep the reaction just alkaline to phenolphthalein. The solution was centrifuged from a few particles which had failed to dissolve, and the supernatant liquid, which contained practically all of the substance as a soluble barium salt, was treated with a slight excess of saturated barium hydroxide solution. After the resulting heavy flocculent precipitate was centrifuged off the supernatant liquid gave only a slight precipitate with immune serum. The barium salt was washed once with water containing a small amount of barium hydroxide and was then dissolved in about 350 cc. of hot water and treated cautiously with sulfuric acid until the supernatant liquid showed neither barium nor sulfate ions. The barium sulfate was centrifuged off and the supernatant liquid concentrated *in vacuo* to small bulk and dialyzed in a collodion bag. After further concentration *in vacuo* the solution was added to 10 volumes of redistilled acetone. The precipitate was sucked dry on a filter, washed with acetone, and dried *in vacuo* over phosphorus pentoxide until constant in weight, a process which rendered the recovered Type III acid insoluble. The yield was 0.43 gm.

A comparison of the properties of Product 33B III with those of 33 (Table I) indicates that precipitation of the Type III acid as the barium salt does not effect a fractionation.

An attempt was also made as in the case of Type I to remove possible impurities by adsorption of the specific substance on the highly reactive Type A alumina prepared according to the method of Willstätter and Kraut (4).

As a result of preliminary experiments it was found that at a pH below 6.0 1 gm. of gel (calculated as Al_2O_3) adsorbed approximately 0.35 gm. of polysaccharide. At a pH higher than 7.0 the adsorption of the Type III substance did not reach completion.

0.5 gm. of Preparation 30 was dissolved in the equivalent amount of N/14 sodium hydroxide. To the solution was added a suspension of 1.5 gm. of alumina and the volume was made up to 750 cc. The pH was adjusted with N/5 hydrochloric acid to 5.0 and the suspension was shaken for 1 hour, after which the supernatant fluid gave a negative precipitin test. The mixture was centrifuged and the precipitate washed once with water. It was then extracted twice with N/5 sodium carbonate solution and the extract, after neutralization, was concentrated *in vacuo*, dialyzed in parchment, again concentrated *in vacuo*, and dialyzed in collodion until free from chloride ion. The solution was then further concentrated *in vacuo* and precipitated from a volume of 15 cc. by 5 cc. of 1:1 hydrochloric acid and 40 cc. of redistilled alcohol at 0°. The specific acid was washed free from chlorides with 50 per cent alcohol and finally with acetone. 0.36 gm. of dry material was recovered.

A comparison of Product 30A with the starting material shows that adsorption of the specific substance on alumina did not result in the separation of any significant amount of impurity, a result also given by the other methods of purification attempted.

The variations shown by the optical rotation of the Type III substances listed in Table I may perhaps be explained by the fact that in each instance the insoluble acid was dissolved in a slight excess of dilute alkali. It was found that alkaline solutions of the substance suffered a slow decrease in optical rotation, followed by a slow return to the original value after acidification. For instance, in one case a portion of Preparation 33II, with an initial $[\alpha]_D$ of $-34.1°$ showed a drop in 0.7 per cent sodium hydroxide solution to a value of $-21.2°$, this returning to $-36.0°$ after acidification.

B. Hydrolytic Products of the Type III Specific Acid.

As a preliminary step in the study of the products of hydrolysis, the relation between the specificity, the time of hydrolysis, the percentage of reducing sugars, and the optical rotation was studied.

0.0836 gm. of dried Preparation 30I, of which the $[\alpha]_D$ was $-30.9°$, was dissolved in 0.55 cc. of concentrated sulfuric acid at $-10°$ and allowed to stand at this temperature for 1 hour. The viscous solution was then diluted to 21 cc.

to give an approximately normal solution of acid, and, before hydrolysis on boiling, its optical rotation, reducing power, and specificity were tested. The values observed are to be found in the following table.

Period of boiling.	$[\alpha]_D$	Reducing sugars (calculated as glucose).	Reaction with Type III antipneumococcus serum.
min.		*per cent*	
0	−15.9°	0.0	++
140	+8.8°	54.8	±
180	+15.4°	61.0	−
300	+22.6°	69.4	−
360	+23.4°	65.5	−

It is thus evident that in boiling normal sulfuric acid the specific reaction of the Type III acid persists up to the 3rd hour, and that the maximum reducing power is attained in about 5 hours.

6 gm. of Preparation 30I with a water content of 7.6 per cent, were finely pulverized and slowly added to a mixture of 30 gm. of concentrated sulfuric acid and 10 cc. of water at 0°. The acid was stirred slowly with a turbine and after a short time 10 cc. more of the sulfuric acid mixture were added. After 2 hours only a few lumps remained and the mixture was allowed to stand in the ice box overnight. It was then poured into 800 cc. of water and boiled under a reflux condenser for 5 hours, at which time the reducing power showed a constant value. The sulfuric acid was removed quantitatively with barium hydroxide. The barium sulfate was centrifuged off, and the liquid concentrated *in vacuo* to 100 cc., boiled with calcium-free Norite, and filtered. The colorless filtrate was treated with an excess of basic lead acetate and centrifuged. The precipitate was washed once with water containing a little basic lead acetate, three times with alcohol, and was then filtered off, washed with acetone, and dried. 10.7 gm. of the lead salt were recovered in this way.

The supernatant from the basic lead acetate precipitate contained a strongly reducing substance (0.96 gm. calculated as glucose) and was accordingly treated with hydrogen sulfide to remove lead, filtered, and concentrated, finally, *in vacuo* to dryness in a desiccator. After a few days crystals could be seen with a hand lens but on account of the difficulty in isolating them in appreciable quantities the whole was treated with 6 cc. of 1:1 nitric acid and oxidized as described previously in this paper.

0.15 gm. of recrystallized potassium acid saccharate was readily obtained.

0.0804 gm. substance gave 0.0282 gm. K_2SO_4.

Calculated for $COOK(CHOH)_4COOH$: K, 15.75 per cent. Found: K, 15.73 per cent.

A similar sugar fraction from the hydrolysis of 4.0 gm. of Type III acid, containing 0.6 gm. of sugar calculated as glucose was de-leaded with hydrogen sul-

fide and treated in a volume of 50 cc. with 1.3 gm. of phenylhydrazine dissolved in 3 cc. of 50 per cent acetic acid. After warming for 1 hour on the water bath the copious deposit of yellow crystals was filtered off, washed with 10 cc. of methyl alcohol to remove tar, filtered, and dried. 0.40 gm. of osazone was obtained, melting and decomposing at 203–204°. A solution of 0.0760 gm. in 7.6 cc. of pyridine-alcohol mixture showed, in a 0.5 dm. tube, a rotation of −0.33°. After 2 days the reading had decreased to −0.12°, mutarotation in this direction being a characteristic of glucosazone (7). Recrystallized for analysis, the osazone decomposed at 206–210°.

Calculated for glucosazone, $C_{18}H_{22}O_4N_4$: N, 15.45 per cent. Found: 15.42 per cent.

The salt formed as described above by the addition of basic lead acetate to the hydrolysis product was suspended in about 300 cc. of water and treated with hydrogen sulfide. The lead sulfide was removed and the filtrate concentrated *in vacuo* to small volume, boiled with Norite, filtered, and the filtrate evaporated to complete dryness over phosphorus pentoxide. 3.1 gm. of a white glassy mass remained. It was readily soluble in water, difficultly soluble in alcohol, and gave a strong glucuronic acid test with naphthoresorcinol. However, its non-crystalline nature, its insolubility in alcohol, and the difficulty of isolating from it a bromophenylosazone did not support its being glucuronic acid itself. Moreover its $[\alpha]_D$ was +12.3° (0.3372 gm. in 20 cc. of water), whereas the specific rotation of glucuronic acid is +19.1°. It also had a reducing power of only 48.0 per cent, calculated as glucose. This value could be increased about 10 per cent by boiling with 0.5 normal hydrochloric acid, while boiling with hydrobromic acid of the same strength increased the reducing power about 15 per cent and concentrated hydrochloric acid in the cold caused an increase of only 7 to 8 per cent. Saccharic acid could not be isolated from the product by oxidation with bromine. The reducing group was also readily oxidized by nitric acid, but the product again showed remarkable stability toward further hydrolysis.

It is thus quite certain that the second component of the hydrolysis products of the Type III acid is not glucuronic acid itself, although there is evidence that it consists of glucuronic acid combined with some other substance, perhaps a hexose derivative. It is hoped to clear up this point when more material becomes available.

As to the portion of the hydrolysis products not precipitated by basic lead acetate, however, there can be no doubt that the principal substance present is glucose, owing to the high yield of glucosazone obtained, and to the isolation of acid potassium saccharate on oxidation of the sugar.

C. Conversion of the Insoluble Type III Acid into a Soluble Form.

When the insoluble form of the Type III acid, obtained in the usual way, is boiled with water it gradually enters into solution.

0.1 gm. of Preparation 30I was shaken for 1 hour with 200 cc. of water at 50°. Since only a swelling of the granules of the specific substance occurred, the mixture was diluted to a volume of 500 cc. and boiled under a reflux condenser for 1½ hours. The resulting clear solution was filtered as a precaution and concentrated, finally, in a vacuum desiccator until the volume was 5 cc. The solution, which was still clear, was thrown into 100 cc. of acetone and filtered. The precipitate was washed with acetone and absolute alcohol and was dried at room temperature over sulfuric acid at atmospheric pressure until a constant weight of 0.09 gm. was reached. The substance thus recovered was a white amorphous powder soluble in water, but after it was again dried *in vacuo* it gradually passed into the insoluble derivative. $[\alpha]_D$ of the soluble form was $-31.9°$, and the acid equivalent (352) was in agreement with the values obtained by solution of the insoluble form in dilute alkali and subsequent neutralization.

Whether the reversible equilibrium between the soluble and insoluble forms is one involving simple hydration and dehydration, or one depending on the opening and closing of a lactone or anhydride grouping cannot be decided with the evidence at present available.

DISCUSSION.

The following data are given in order to summarize briefly the properties of the soluble specific substances of the three fixed types of Pneumococcus, in the state of purity thus far attained.

The soluble specific substance of Type II pneumococcus is apparently a weakly acidic, nitrogen-free polysaccharide made up chiefly of glucose units. Its acid equivalent is about 1250 and the specific optical rotation is about $+74°$. It is not precipitated by barium hydroxide or heavy metal salts with the exception of basic lead acetate and uranyl compounds. It reacts at a dilution of 1:5,000,000 with Type II antipneumococcus serum but does not precipitate Type I and Type III antisera at a concentration of 1:400. The substance is converted by acetic anhydride and pyridine into a very sparingly soluble triacetyl derivative.

The Type III soluble substance, while also isolated as a nitrogen-

free polysaccharide, is a strong acid with an acid equivalent of about 340, and is made up not only of glucose units but also those of either glucuronic acid or a derivative of this acid. It rotates the plane of polarized light about 33° to the left. It is precipitated by barium hydroxide in excess and by heavy metal salts, and is also rendered insoluble by the addition of strong hydrochloric acid. In as high a dilution as 1:6,000,000 it still reacts with Type III antipneumococcus serum.

The Type I soluble specific substance also appears to be a sugar derivative, but differs from the other two substances in its lower percentage of sugar liberated on hydrolysis and in containing nitrogen as an apparently essential component. It rotates the plane of polarized light about 300° to the right, is a strong acid and a weak base, and is very sparingly soluble at its isoelectric point, which lies at about pH 4. In spite of a nitrogen content of 5.0 per cent the substance gives none of the usual protein color tests. One-half of the nitrogen is liberated on treatment with nitrous acid and reducing sugars appear at the same time, while the specific reaction vanishes. Under the same conditions the Type II and Type III substances are unaffected by nitrous acid. The substance gives the color reaction for glucuronic acid with naphthoresorcinol, but yields mucic acid on oxidation, indicating a relationship to galactose. Since the carbon and hydrogen contents of the substance are close to the theoretical values for polysaccharides it appears possible that in it a nitrogenous sugar derivative is linked to galacturonic acid through the reducing group of the latter. Further evidence on this point will be sought. The Type I substance is precipitated by barium hydroxide in excess, by heavy metal salts, and by phosphotungstic acid. In the specific precipitin reaction with homologous Type I antipneumococcus serum it can be detected in dilutions as great as 1:6,000,000 while at a concentration of 1:400 it gives a faint cloud with Type III antiserum.

The three polysaccharides contain no sulfur or phosphorus and differ from the starch-glycogen group of carbohydrates in giving no color with iodine and in their resistance to the ordinary carbohydrate-splitting enzymes.

It would be, of course, idle to assume that in their present state of purity, each of the specific substances represents a definite chemical

compound. However, in the case of the three fixed types of Pneumococcus three totally distinct substances have been isolated from cultures grown in the same medium. Successive preparations of the specific substance have in each instance been quite uniform regardless of the widely different methods employed in the process of purification. Moreover, substances reactive to the same degree with homologous antisera have been derived both from the microorganisms themselves and from autolyzed broth cultures.

It is thought that these and other considerations based on the data presented warrant the belief that the three polysaccharides isolated represent the actual specific substances, stripped of at least a large portion of accompanying impurities, and that they do not merely represent inert material carrying an extremely minute amount of the true specific compounds.

If this be accepted it may be concluded that the soluble specific substance of each of the three fixed types of Pneumococcus is a distinct chemical substance, differing in many striking particulars from the corresponding product elaborated by the other two types, but having in common the properties of polysaccharide structure and of resistance to enzyme action. Each substance breaks down on hydrolysis into reducing sugars, a part of which, at least, is peculiar to itself. The Type I substance differs sharply from the other two in containing nitrogen and in possessing basic as well as acid properties, while of the other two substances, the Type II is a dextrorotatory weak acid and the Type III a levorotatory strong acid. Especially striking is the occurrence of specific substances of such widely differing properties in microorganisms as closely related as the three fixed types of Pneumococcus.

The immunological significance of the specific substances has been discussed by the writers in a recent paper (8) and will therefore not be touched upon in the present communication.

Many of the questions raised in the course of the work are still under investigation and the specific substances of other microorganisms are being studied.

SUMMARY.

1. Refinements in the methods for the isolation of the soluble specific substances of Types II and III pneumococcus are described.

These improvements have resulted in the isolation of the end-products in a form free from nitrogen and of enhanced activity with immune serum.

2. The soluble specific substance of Type I pneumococcus is described and shown to differ sharply from the corresponding substances of the other two types, each of which, in turn, differs from the other.

3. Progress is reported on the identification of the sugar units from which the polysaccharides are built up.

4. The evidence so far accumulated is believed to favor strongly the view that the polysaccharides isolated are the actual specific substances of Pneumococcus.

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