THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

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In 1917 Dochez and Avery (1) showed that whenever pneumococci are grown in fluid media, there is present in the cultural fluid a substance which precipitates specifically in antipneumococcus serum of the homologous type. This soluble substance is demonstrable in culture filtrates during the initial growth phase of the organisms; that is, during the period of their maximum rate of multiplication when little or no cell death or disintegration is occurring. The formation of this soluble specific material by pneumococci on growth *in vitro* suggested the probability of an analogous substance being formed on growth of the organism in the animal body. Examination of the blood and urine of experimentally infected animals gave proof of the presence of this substance in considerable quantities in the body fluids following intraperitoneal infection with pneumococcus. In other words, this soluble material elaborated at the focus of the disease readily diffuses throughout the body, is taken up in the blood, passes the kidney, and appears in the urine unchanged in specificity. Similarly, a study of the serum of patients suffering from lobar pneumonia has revealed a substance of like nature in the circulating blood during the course of the disease in man. Furthermore, examination of the urine of patients having pneumonia due to pneumococci of Types I, II, and III has shown the presence of this substance in some stage of the disease in approximately two-thirds of the cases. Recently from filtered alkaline extracts of pulverized bacteria of several varieties, including pneumococci, Zinsser and Parker (2) have prepared substances which appear free from coagulable protein. These substances, called "residue antigens,"

are specifically precipitable by homologous antisera. These observers consider these acid- and heat-resistant antigenic materials analogous to the soluble specific substance of pneumococcus described by Dochez and Avery (1). In spite of the fact that these "residue antigens" are precipitable by homologous sera produced by immunization with the whole bacteria, Zinsser and Parker have so far failed to produce antibodies in animals by injecting the residues.

In the earlier studies by Dochez and Avery certain facts were ascertained concerning the chemical characteristics of this substance. It was found that the specific substance is not destroyed by boiling; that it is readily soluble in water, and precipitable by acetone, alcohol, and ether; that it is precipitated by colloidal iron, and does not dialyze through parchment; and that the serological reactions of the substance are not affected by proteolytic digestion by trypsin. Since the substance is easily soluble, thermostable, and type-specific in the highest degree, it seemed an ideal basis for the beginning of a study of the relation between bacterial specificity and chemical constitution. The present report deals with the work done in this direction.

EXPERIMENTAL.

The organism used in the present work was Pneumococcus Type II. The most abundant source of the soluble specific substance appeared to be an 8 day autolyzed broth culture; hence this material was used as the principal source of supply. For comparison dissolved pneumococci and lots of urine containing the specific substance were also worked up, with essentially the same results, as will be seen from Table I.

The process for the isolation of the soluble specific substance consisted in concentration of the broth, precipitation with alcohol, repeated re-solution and reprecipitation, followed by a careful series of fractional precipitations with alcohol or acetone after acidification of the solution with acetic acid, and, finally, repeated fractional precipitation with ammonium sulfate and dialysis of the aqueous solution of the active fractions.

Five lots of 15 liters each of 8 day cultures of Pneumococcus Type II in meat infusion phosphate broth are each concentrated on the water bath in large evapo-

rating dishes to 1,000 to 1,200 cc. and precipitated in a separatory funnel by the gradual addition, with vigorous rotation, of 1.2 volumes of 95 per cent alcohol. The mixture separates into two layers, and is allowed to stand over night, or for several hours. The upper layer, which is almost black and comprises the largest part of the mixture, contains only traces of the soluble specific substance, and is siphoned off and discarded. The lower, more viscous layer is run into a 250 cc. centrifuge bottle (occasionally a second will be required), capped, and rotated at high speed for ½ hour. Three layers are formed, of which the uppermost is merely a further amount of the liquid previously discarded. The middle layer consists of a compact, greenish cake of insoluble matter and gummy material, and contains most of the soluble specific substance. The bottom layer, from which salts often separate, is a brownish syrup rich in salts and nitrogenous matter and relatively poor in specific substance, and can, by careful manipulation, be poured off to a large extent. Although a small proportion of the specific substance is lost if this syrup is discarded, its elimination represents so considerable a purification as to warrant the sacrifice of the active material contained. The gummy cake remaining in the centrifuge bottle, together with adhering salts and syrup, is now rinsed out and ultimately combined with similar material from the other lots. All of this is then dissolved as completely as possible in water, care being taken to break up the many lumps of gummy material, diluted to 1 liter, and again precipitated with alcohol. In this case about 1.3 liters are required to precipitate all but the last traces of active material from the upper layer. This is again discarded and the lower layer treated as before. At this stage there is relatively less of the bottom layer, and it is more difficult to separate it from the cake containing the specific substance, but as much as possible is removed. The remaining material is smoothed out with water, diluted to about 500 cc., and centrifuged. The precipitate is washed twice with water, and the washings are combined with the main solution. The still turbid liquid, the volume of which should be about 750 cc., is put through the alcohol purification process a third time, about 1.1 liters of alcohol being required. After having been centrifuged, the active material is again dissolved in water, made definitely acid to litmus with acetic acid, and again centrifuged. The precipitate is washed three times with water acidulated with acetic acid, and the filtrate and washings are combined in a separatory funnel and diluted again if necessary to 750 cc. Acetone (redistilled) is now added until a permanent precipitate forms, about 250 cc. being necessary. The precipitate is allowed to settle, whereupon the lower part of the mixture containing the precipitate is drawn off and centrifuged. The clear supernatant fluid is restored to the main solution, while the precipitate, which consists largely of insoluble material and gives an aqueous solution almost devoid of activity, is discarded. Fractional precipitation is continued, and even when the specific substance appears in quantity in the precipitate, it is occasionally possible to separate a lower, inactive, syrupy layer, as in the previous purifications by alcohol. Addition of acetone is continued until a test portion, heated on the water bath to remove acetone, diluted with saline, and neutralized, no longer gives a pre-

cipitate with immune serum, after which the upper layer may be discarded. The active precipitates are then redissolved in water, centrifuged again, and the supernatant liquid is diluted to 375 cc., reacidified with acetic acid, and again fractionated with acetone. If inactive fractions are obtained, the process is again repeated until no further purification results. Alcohol may be used for these fractionations instead of acetone, the only difference being that a somewhat larger proportion is required. The active material is then dissolved in about 150 cc. of water and again made definitely acid with acetic acid. The solution is treated with solid ammonium sulfate until the first slight precipitate forms. This is generally inactive, and if so, may be discarded. Finally, ammonium sulfate is added to saturation, completely precipitating the specific substance if the volume of the solution is not too great. The mixture is allowed to stand for several hours and is then centrifuged and the precipitate washed with a little saturated ammonium sulfate solution. It is redissolved in about 75 cc. of water acidified with acetic acid, centrifuged if necessary, and again precipitated by saturation with ammonium sulfate. Finally, the specific substance so obtained is dissolved in water and dialyzed first against running tap water in the presence of chloroform and toluene, and finally against distilled water until tests for sulfate and phosphate ion are negative. Addition of acetic acid during the early stages of the dialysis assists in the removal of calcium, which otherwise forms a large part of the ash.

The dialyzed solution is concentrated to dryness on the water bath and the residue redissolved in hot water. If the solution is not perfectly clear, it is centrifuged again before being evaporated to dryness, and the whole process is repeated as long as insoluble material separates. Toward the end of the final concentration absolute alcohol may be added to assist in the precipitation of the substance.

Variations in the exact volumes given are often necessary with different lots of broth, but this will occasion little difficulty if all fractionations are controlled by the specific precipitin test.

As so obtained the soluble specific substance forms an almost colorless varnish-like mass which may be broken up and dried to constant weight at 100°C. *in vacuo*. The yield from 75 liters of broth averages about 1 gm., although it varies within rather wide limits in individual lots.

By the method outlined above all substances precipitable with phosphotungstic acid or capable of giving the biuret reaction were eliminated. The residual material (Preparation 17, in Table I), for which no claim of purity is made, as efforts at its further purification are still under way, contained, on the ash-free basis, 1.2 per cent of nitrogen. It was essentially a polysaccharide, as shown by the formation of 79 per cent of reducing sugars on hydrolysis, and by the isolation and identification of glucosazone from the products of hydrolysis.

0.4 gm. of Preparation 17 was heated under a reflux condenser with 40 cc. of 0.5 N HCl for 7 hours. The filtered solution was treated with 0.4 cc. of phenylhydrazine, followed by saturated sodium acetate solution until Congo red paper was no longer turned blue. After 1 hour in the water bath the crystalline osazone was filtered off, washed with water, purified by stirring well with dry methyl alcohol, and again filtered. 0.037 gm. was obtained, melting and decomposing at 196–197°C. with preliminary darkening and softening. A second fraction of 0.026 gm. of even purer osazone was obtained by heating the mother liquors of the first fraction 2 hours longer after addition of 0.2 cc. more of phenylhydrazine and purifying the crude product with methyl alcohol. This portion melted quite sharply at 205–206°. The true melting point of pure glucosazone is 208°.

50 mg. of the combined fractions, dissolved in 3 cc. of alcohol and 2 cc. of pyridine, gave, in a tube 0.5 dm. long, an initial rotation of −0.20° and a final rotation of −0.08°, while Levene and La Forge (3) give −0.62° and −0.35° for a solution of twice the concentration of recrystallized glucosazone. The only other hexose whose osazone rotates to the left is altrose, and this is known only as a laboratory product. Moreover, its osazone melts at 178° and decomposes at 189°.[1]

The aqueous solution of the substance gave the Molisch reaction out to the limit of delicacy of the test. Reduction of Fehling's solution occurred only after hydrolysis. Phosphorus was present only in traces; sulfur and pentoses were absent. A 1 per cent solution gave no biuret reaction, no precipitate with phosphotungstic acid, mercuric chloride, or neutral lead acetate, precipitated heavily with basic lead acetate, and gave a faint turbidity with tannic acid. Calcium is very tenaciously retained, but does not appear to be an essential part of the molecule, as the specific reaction was also given by calcium-free preparations. No color is given by iodine solution.

The soluble specific substance is remarkably stable to acids. A solution in 0.5 N hydrochloric acid maintained its activity undiminished and failed to reduce Fehling's solution after 36 hours at room temperature, but showed reducing sugars and absence of precipitation with immune serum after transfer to the water bath.

The limits of delicacy of the specific precipitation of the soluble substance were titrated, as shown in Table I, and it is seen that Preparation 17 still gave a specific reaction in a dilution as high as 1:3,000,000.

Table I represents a summary of the reactions of some of the earlier preparations worked with, as well as the later ones. Prep-

[1] We are greatly indebted to Dr. P. A. Levene for valuable aid and advice on this phase of the work.

aration 4 was obtained from the urine of a patient with a Type II pneumococcus infection, while No. 8 was obtained from an antiformin solution of the pneumococci. In both of these cases, as well as in Nos. 9, 11, and 15, the method of purification given above had not been fully worked out.

Attempts to stimulate antibody production by the immunization of animals with the purified substance yielded negative results.

TABLE I.

Summary of the Properties of Various Preparations of the Soluble Specific Substance of Pneumococcus Type II.

Preparation No.	Total N.	Hydrolysis.			S	P	Specific rotation $[\alpha]_D$	Precipitation with immune serum.*	Molisch reaction.†
		NH_2 N	NH_3 N	Reducing sugars.					
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
4‡	6.1	3.5			1.5		Too dark.	1:80, 000	
4A§	4.7			63.0		1.0‖	−20.6°	1:640,000	1:320, 000
8¶	2.9			++			+19.8°	1:1, 250, 000	1:640, 000
9	6.6				1.8		−8.6°	1:640,000	
11	2.1	0.9	1.3				+31.6°	1:2, 500, 000	1:1, 250, 000
15	2.0			49.0		0.9‖	+30.8°	1:2, 500, 000	1:1, 250, 000
17	1.2	C = 46.2 per cent.	H = 6.1 per cent.	79.0	None.	Tr.	+55.7°	1:3, 000, 000	1:1, 500, 000

* After 2 hours at 37°C. and over night at 4°.

† Unless the α-naphthol solution is fresh, other colors will mask the purple at high dilutions. The figures represent the dilution of the preparation itself; in other words, the highest dilution giving the reaction in the previous column.

‡ From urine.

§ Preparation 4 repurified.

‖ Due to incomplete dialysis.

¶ From dissolved pneumococci.

DISCUSSION.

While it has long been known that the capsular material of many microorganisms consists, at least in part, of carbohydrates (4), any connection between this carbohydrate material and the specificity relationships of bacteria appears to have remained unsuspected. While it cannot be said that the present work establishes

this relationship, it certainly points in this direction. Evidence in favor of the probable carbohydrate nature of the soluble specific substance is the increase in specific activity with reduction of the nitrogen content, the increase in optical rotation with increase in specific activity, the parallelism between the Molisch reaction and specific activity, the high yield of reducing sugars on hydrolysis, and the actual isolation of glucosazone from a small quantity of the material. The small amounts of substance available up to the present have hindered the solution of the problem, and it is hoped that efforts at further purification of the soluble specific substance, now in progress with larger amounts of material, will definitely settle the question.

SUMMARY.

1. A method is given for the concentration and purification of the soluble specific substance of the pneumococcus.

2. The material obtained by this method is shown to consist mainly of a carbohydrate which appears to be a polysaccharide built up of glucose molecules.

3. Whether the soluble specific substance is actually the polysaccharide, or occurs merely associated with it, is still undecided, although the evidence points in the direction of the former possibility.

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