THE SOLUBLE SPECIFIC SUBSTANCE OF A STRAIN OF FRIEDLÄNDER'S BACILLUS.

PAPER I.

BY MICHAEL HEIDELBERGER, PH.D., WALTHER F. GOEBEL, PH.D., AND OSWALD T. AVERY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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In 1921 Toenniessen (1), working on the capsular material of a strain of Friedländer's bacillus, reported the isolation of a snow-white, non-reducing, substantially nitrogen-free polysaccharide which gave a red color with iodine. After hydrolysis the resulting reducing sugars were found to yield an osazone which Toenniessen believed to be that of galactose. These findings were subsequently confirmed by Kramár (2). More recently (3) the so called "soluble specific substance" of the Pneumococcus, first observed by Dochez and Avery (4), has been identified with the polysaccharide portion of the organism. It seemed not unlikely, therefore, that Toenniessen's carbohydrate from the Friedländer bacillus would also possess specific properties, and an investigation of this point was undertaken. Meanwhile the correctness of this surmise has been indicated in a preliminary report by Mueller, Smith, and Litarczek (5), who isolated carbohydrate-containing material with a nitrogen content of 1.3 per cent from a strain of Friedländer's bacillus, and showed that this substance at high dilutions caused precipitation of homologous immune serum.

In the present work a strain of Friedländer's bacillus recovered from a spontaneous guinea pig infection was used. This will be referred to as the E strain, its laboratory designation. From this strain, by a procedure essentially the same as that used in the case of the Pneumococcus (3, 6), a nitrogen-free polysaccharide with specific properties of a most unusual nature has been obtained.

EXPERIMENTAL.

1. Isolation of the Soluble Specific Substance of Friedländer's Bacillus.

Autoclaved washings from 60 to 70 Blake bottles of 48 hour cultures of the E strain of Friedländer's bacillus on solid agar at pH 7.6 were diluted to 3 liters and run through a Sharples centrifuge to remove bacterial debris. The resulting liquid, made slightly alkaline, was treated with 60 gm. of sodium acetate and 4.5 liters of alcohol. After several hours the flocculent precipitate was separated by centrifugation and was dissolved in 2 liters of water. The solution was again centrifuged and reprecipitated as before. With each alkaline precipitation protein material, but no polysaccharide,[1] was eliminated, until finally a biuret-free product was secured. Usually from four to six such precipitations were necessary. At this point the solution of the substance, at a volume of 2 liters, was filtered through a Berkefeld V candle, evaporated to 500 cc., and precipitated with an excess of hot barium hydroxide solution saturated at 60–70°.

The barium salt was suspended in 500 cc. of water and treated with a slight excess of sulfuric acid. The barium sulfate was centrifuged off, washed with very dilute sulfuric acid, and the clear supernatants were neutralized and concentrated to 100 cc. *in vacuo*. The polysaccharide was then precipitated at 0° by the addition of 35 cc. of 1:1 hydrochloric acid and 225 cc. of redistilled alcohol. After standing for 1 hour the precipitate was centrifuged off in the cold, redissolved in 100 cc. of water, and reprecipitated under the same conditions. The final product was washed free from chloride ion with acetone on a hardened filter paper and dried over sulfuric acid *in vacuo*. The yield was 2 to 2.5 gm.

A number of preparations were isolated in this way. The properties of the polysaccharide thus obtained are shown in Table I. The methods of purification employed were the same in all preparations except in the case of Nos. 103 I and 103 II, in which, instead of using agar media, 2 day cultures grown in glucose-aminoid-peptone broth at pH 7.6 were employed. In these cases the initial steps were identical with those employed in the preparation of the soluble specific substances of Types II and III pneumococcus. One volume of alcohol was found to be sufficient for the initial precipitation of the soluble substance. The usual three layer separation was obtained by centrifugation and only the middle layer contained active material. The remainder of the process of purification was as outlined above.

[1] In this preparation complete precipitation of the polysaccharide was followed by tests of portions of the hydrolyzed mother liquors with Fehling solution.

The properties of the various preparations were remarkably uniform, as shown by Table I.

The soluble specific substance of the E strain of Friedländer's bacillus is a white fluffy amorphous powder with acid properties strong enough to turn wet Congo red paper blue when a few particles are dusted upon it. After the substance is dry it dissolves with difficulty in water, but passes readily into solution on neutralization with dilute sodium hydroxide. A 1:200 solution is not precipitated by solutions of silver nitrate, copper sulfate, or phosphotungstic acid,

TABLE I.

Soluble Specific Substance of the Friedländer Bacillus (E.)

Preparation No.	$[\alpha]_D$	Acid equivalent.	Ash.	C	H	N	Percentage of reducing sugars on hydrolysis.	Highest dilution giving a precipitate with immune serum.	
								Anti-Friedländer serum E.	Antipneumococcus serum Type II.
101	+100.0°	670	0.0	44.6*	6.1	0.0	73.0	1:2,000,000	1:2,000,000
103 I	+102.5°	674	0.0			0.0	72.4	1:2,000,000	1:2,000,000
103 II	+100.0°	704	0.0			0.0	70.0	1:2,000,000	1:2,000,000
104	+100.0°	722	0.0			0.06	73.0	1:2,000,000	1:2,000,000
104 A	+100.0°	685	0.0			0.0	72.0		
105 A	+100.0°	706	0.0			0.66	72.0	1:2,000,000	1:2,000,000
105 B†	+101.5°	674	0.0			0.2	73.0		
105 Ad	+100.0°	716	0.0			0.0	78.0		
25 A‡	+ 70.2°	1302	0.35	45.8	6.4	0.0	68.4		1:6,000,000

* Theory for $(C_6H_{10}O_5)_x$, C = 44.4, H = 6.2.

† This represented a residue which failed to pass through a Berkefeld filter.

‡ A preparation of Type II pneumococcus soluble specific substance given for comparison.

but yields precipitates with barium hydroxide and with both neutral and basic lead acetate. It gives no color with iodine-potassium iodide solution. In several of the preparations the polysaccharide was obtained free from nitrogen. When any traces of nitrogen remained these could be removed by the additional methods of purification outlined below. Micro Kjeldahl determinations (Pregl) were made on samples as large as 30 to 40 mg.

In many of its precipitation reactions and in yielding glucose on hydrolysis (see below) the substance resembles the soluble specific

substance of Type II pneumococcus, although in its present state of purity there are points of difference between the two products (compare with 25 A, Table I; also reactions with barium hydroxide and neutral lead acetate). This similarity in chemical properties led to a test of the specific reaction of the substance with Type II antipneumococcus serum. Under these conditions a "specific" immune precipitate resulted, while no reaction occurred in the presence of antipneumococcus sera Types I and III. This unusual phenomenon and its immunological significance are treated in detail in the following paper.

2. Attempts at Further Purification of the Specific Substance.

Since the specific substance yielded a precipitate with uranyl nitrate, a property common to the analogous substances of the three types of Pneumococcus, a portion was precipitated with this salt to see whether additional purification could be effected.

To a neutralized solution of 0.4 gm. of Preparation 104 in 80 cc. of water 20 cc. of 5 per cent uranyl nitrate solution were added. The heavy precipitate which formed was separated after 24 hours by centrifugation. The supernatant fluid gave a negative specificity test and only a faint reaction for uranyl ions. The precipitate was suspended in water and dissolved by addition of the least possible amount of normal hydrochloric acid. Molar potassium dihydrogen phosphate solution was then added until no further precipitate occurred. The uranyl phosphate was centrifuged off and the supernatant liquid, containing the specific substance, was concentrated *in vacuo* to 50 cc. and precipitated by the addition of 1 gm. of sodium acetate and 100 cc. of alcohol. The precipitate was dissolved in 20 cc. of water and the solution cooled to 0° and treated with 3 cc. of hydrochloric acid and 60 cc. of redistilled alcohol. After 1 hour the substance was separated from the slightly active supernatant liquid by centrifugation, redissolved in 20 cc. of water, and thrown into 200 cc. of acetone. The yield was 0.27 gm.

A comparison of this preparation (104 A, Table I) with the original material (104) shows that the only significant change was the removal of the last traces of nitrogen.

The highly adsorbent Alumina A prepared according to Willstätter and Kraut (7) was also used for further purification.

A solution of 0.5 gm. of Preparation 105 A was shaken with 1.5 gm. (calculated as Al_2O_3) of Type A alumina in a volume of 250 cc. at pH 5.0. At the end of 1

hour the alumina, containing the adsorbed polysaccharide, was centrifuged off from the now specifically inactive liquid. The precipitate was extracted once with 250 cc. of N/100 sodium hydroxide for 2 hours and a second time with 200 cc. of N/5 sodium carbonate solution. After the extracts were neutralized with acetic acid they were concentrated *in vacuo* to a volume of 50 cc. and the polysaccharide was precipitated with 100 cc. of alcohol. The specific substance was centrifuged off, dissolved in 25 cc. of water, and the solution was centrifuged from a small amount of insoluble alumina and finally treated at 0° with 5 cc. of 1:1 hydrochloric acid and 60 cc. of redistilled alcohol. After 2 hours the precipitate was centrifuged off, dissolved in 15 cc. of water, and poured into 10 volumes of cold acetone. The product was finally filtered and washed with acetone until free from chloride ion. 0.32 gm. of dry material was recovered.

A comparison of the product (105 Ad, Table I) with the original material (105 A) shows that adsorption and recovery of the polysaccharide had little effect other than elimination of the nitrogen present.

3. Isolation of Glucosazone and Potassium Hydrogen Saccharate from the Hydrolysis Products of the Specific Substance.

1 gm. of Preparation 101 was dissolved in 60 cc. of normal sulfuric acid and the solution was boiled for 5 hours, until no further increase in reducing sugars could be observed. After dilution to 250 cc. the sulfuric acid was quantitatively removed by barium hydroxide solution. The barium sulfate was centrifuged off and the supernatant fluid was concentrated to 30 cc., boiled with Norite, filtered, and finally treated with 1.4 gm. of phenylhydrazine acetate. After heating on the water bath for 1 hour the crystalline osazone which had formed was filtered off. A second fraction was obtained on heating the solution further. Since both portions appeared to be quite pure they were combined, washed with a small amount of methyl alcohol to remove tar, filtered off, and dried. 0.25 gm. of substance was recovered, melting at 201° with decomposition. After recrystallization from 60 per cent ethyl alcohol the decomposition point was 203–204°. A mixture of this compound with the osazone prepared from pure glucose also melted at 203–204°.

0.1000 gm. of substance gave 13.40 cc. of N_2 at 23°C., 761.7 mm.

Calculated for $C_{18}H_{22}O_4N_4$; N, 14.45 per cent. Found: N, 14.49 per cent.

0.0576 gm. of the compound dissolved in 5 cc. of pyridine-alcohol mixture had an initial $[\alpha]_D$ of $-67.9°$ which decreased after standing 4 days to $-24.2°$.

From the analysis, melting point, and direction of mutarotation (8) the substance is undoubtedly glucosazone. The identification of glucosazone limits the sugar formed on hydrolysis of the specific substance either to glucose, fructose, or mannose.

In order to ascertain which of these sugars was actually present 1 gm. of Preparation 103 II was boiled for 5½ hours under a reflux condenser with 100 cc. of one-half normal nitric acid. The hydrolysis products had a specific rotation of +75°. The solution was concentrated *in vacuo* to 5 cc. and to it was added 1 cc. of concentrated nitric acid. After 24 hours at room temperature the mixture was boiled for 2.5 minutes and then quickly evaporated, with stirring, on a large watch-glass over a boiling water bath. A thick paste was obtained which was twice evaporated with a small quantity of water, to expel the last traces of nitric acid, and was finally dissolved in 5 cc. of water. The solution was made strongly alkaline with 40 per cent potassium hydroxide solution and then acidified with glacial acetic acid. After 5 hours the crystals of potassium acid saccharate which had separated were filtered off and recrystallized from 2 cc. of boiling water.

0.0494 gm., ignited with sulfuric acid, yielded 0.0173 gm. K_2SO_4.

Calculated for $COOH(CHOH)_4COOK$; K, 15.75 per cent. Found: 15.70 per cent.

It is thus evident that as in the case of the specific substance of the Type II pneumococcus glucose is the principal sugar from which the specific substance of Friedländer's bacillus (Strain E) is built up.

DISCUSSION.

It is clear from the foregoing that the E strain of Friedländer's bacillus yields, on fractionation, a nitrogen-free polysaccharide with specific properties of the order possessed by the soluble specific substances of the three fixed types of Pneumococcus. It is a strong acid with an equivalent value of about 685, sparingly soluble in water after drying, but yielding soluble alkali salts. The specific optical rotation is +100°. The polysaccharide itself is non-reducing, but on hydrolysis with mineral acid yields reducing sugars, among which glucose has been shown to be present. In the present instance, as in those of the pneumococci, carbohydrate and specific function are apparently inseparable, and the isolation of a fourth specific substance of this nature adds additional weight to the growing mass of evidence that the soluble specific substances of microorganisms are often actually polysaccharides (*cf.*(5) also Mueller and Tomcsik (9)[2]).

[2] These investigators isolated specific polysaccharide material from Friedländer's bacillus and yeast. Since the present communication was accepted for publication Laidlaw and Dudley (*Brit. J. Exp. Path.*, 1925, vi, 197) have described a specifically precipitating, non-nitrogenous carbohydrate isolated from tubercle bacilli.

While the specific substance of the E strain, as purified up to the present, has properties which set it apart from the three analogous substances of the fixed types of Pneumococcus there is nevertheless a certain resemblance to that of the Type II pneumococcus, a resemblance extending even to precipitation with Type II antipneumococcus serum. The immunochemical relationships of these otherwise widely different microorganisms are treated in the succeeding paper.

Work on the specific substance of the E strain is being continued and the soluble specific substances of other strains of the Friedländer bacillus are also under investigation.

SUMMARY.

1. A method is given for the isolation of a specifically reacting nitrogen-free polysaccharide from the so called E strain of Friedländer's bacillus.

2. The properties of this polysaccharide are described.

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