THE SOLUBLE SPECIFIC SUBSTANCE OF FRIEDLÄNDER'S
BACILLUS.

III. ON THE ISOLATION AND PROPERTIES OF THE SPECIFIC CARBOHYDRATES FROM TYPES A AND C FRIEDLÄNDER BACILLUS.

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The parallelism between capsular synthesis and virulence among pathogenic bacteria is one which has been extensively investigated, but researches concerning the chemical nature of encapsulating substances have indeed been very few. Fürst (1) has described the encapsulating substance of the Friedländer bacillus. He believed it to be a mucoprotein-like substance. Hamm (2) contended that the capsule substance contained nucleoprotein. Preisz (3) has attempted unsuccessfully to immunize animals with the capsule substance from the anthrax bacillus. More recently Toenniessen (4) succeeded in isolating a nitrogen-free polysaccharide from the Friedländer bacillus, which he believed to be identical with the capsule material of this microorganism. Similar polysaccharides have been isolated from cultures of the bacilli of the aerogenes group (5), from Streptococcus hornensis (6), and even from certain yeasts (7).

Strange as it may seem, none of these earlier investigators ascribed immunological significance to these bacterial carbohydrates. The so-called soluble specific substance of the pneumococcus, first observed by Dochez and Avery (8), has been identified with the polysaccharide portion of the organism. An extensive investigation into the chemical and immunological nature of carbohydrates from the pneumococcus group has been carried out in this laboratory (9). Similar investigations have recently been extended to the group of Friedländer bacilli.

From a strain of Friedländer's bacillus, Mueller, Smith, and Litarzczek (10) have recorded the isolation of carbohydrate-containing material which at high dilutions reacted specifically with homologous antibacterial serum. It has been shown by Julianelle (11)
that bacilli of this group are separable immunologically into sharply defined specific types. These types have been designated as Type A, Type B, and Type C; the remaining unclassified strains were placed in a heterologous group X. The method of isolation of the specific carbohydrate of Type B Friedländer bacillus has been given in detail (12). The present communication, which concludes a systematic description of the specific polysaccharides from the three fixed types of Friedländer's bacillus deals with a description of the polysaccharides from the Type A and Type C microorganisms. A more detailed study concerning the nature of the hydrolytic products of the Type A specific carbohydrate is reported separately (13).

EXPERIMENTAL.

I.

1. Isolation of the Specific Carbohydrate of Type A Friedländer Bacillus.

Autoclaved washings from 50 large Blake bottles of 72 hour cultures of Type A Friedländer bacillus, grown on solid agar containing 0.5 per cent dextrose at pH 7.6, were treated with a sterile solution of purified trypsin. After standing overnight at 37° the mucoid-like washings (containing the specific carbohydrate) became nearly clear. The faintly alkaline solution was treated with 50 gm. of sodium acetate and 2 volumes of alcohol. A flocculent precipitate, containing all the specific carbohydrate in an impure state, settled out on standing. The supernatant liquid which gave no test for specific substance with antiserum, was discarded and the precipitate was separated by centrifugation. After dissolving the precipitate in a liter of water an opalescent solution was secured. By adding acetic acid it was found that a small amount of non-specific nitrogenous material could be separated by centrifugation. The solution of impure specific carbohydrate thus obtained was again treated with 2 volumes of alcohol and the resulting precipitate of specific carbohydrate was again separated by centrifugation. This process of alcoholic precipitation (a process which was found to eliminate nitrogenous impurities) was repeated five or six times until finally a snow-white product was secured, which gave a perfectly clear solution when dissolved in water. This solution, now at a volume of 500 cc., was treated with 50 cc. of 1:1 hydrochloric acid and was dialysed in collodion bags against successive changes of distilled water.

1 8 gm. of commercial trypsin were dissolved in 200 cc. of water, acidified with N/1 hydrochloric acid until Congo red paper turned a faint purple. The solution was centrifuged, and the supernatant liquid was neutralized with N/1 sodium hydroxide, and again centrifuged.
until free from Cl ion both within and without the bag. The dialysed solution of the carbohydrate was concentrated to 200 cc. in vacuo, and was then treated with 400 cc. of redistilled glacial acetic acid. After standing 2 or 3 hours a flocculent precipitate settled out of the solution. This non-specific precipitate was separated by centrifugation and was discarded. The clear supernatant liquid, which contained all the specific material, was concentrated to small volume in vacuo, and was then poured into 10 volumes of redistilled acetone containing a trace of hydrochloric acid. Between 5 and 6 gm. of polysaccharide were recovered.

2. Properties of the Soluble Specific Substance of Type A Friedländer Bacillus.

The soluble specific substance of the strain of Type A Friedländer's bacillus is a white amorphous powder with marked acidic properties.

<table>
<thead>
<tr>
<th>Preparation No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>117-A</td>
</tr>
<tr>
<td>118</td>
</tr>
<tr>
<td>120</td>
</tr>
<tr>
<td>122</td>
</tr>
<tr>
<td>123</td>
</tr>
</tbody>
</table>

the acid equivalent being approximately 430. A 1:200 solution turns Congo red paper blue. Such solutions are not precipitated by copper or silver ions. The polysaccharide is incompletely precipitated by solutions of phosphotungstic acid, barium hydroxide, and uranyl nitrate, but is completely precipitated by both neutral and basic lead acetate solutions. The nitrogen-free product, when oxidized with sodium peroxide, gives no test for sulfur or phosphorus. The polysaccharide gives no color test with potassium iodide-iodine solution. Acid hydrolysis of the material gives a reducing solution which shows a strong naphthoresorcinol test. Attempts were made to purify the specific substance by partial precipitation with barium hydroxide, uranyl nitrate, and by adsorption on alumina, but in each
instance a substance with properties similar to the original material was recovered. The properties of the various preparations were remarkably uniform as shown by Table I.

II.

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

The specific carbohydrate of the strain of Type C Friedländer's bacillus was obtained from autoclaved washings of cultures grown on dextrose agar media exactly as was the carbohydrate from Type A. The product was isolated in an ash-free form by the addition of 2 volumes of alcohol to a 1:50 solution of carbohydrate, containing 10 cc. of 1:1 hydrochloric acid. After standing at 0° for 1 hour the polysaccharide separated as a flocculent precipitate which was filtered on a hardened paper and washed free from chloride with absolute alcohol. The yield from 100 Blake bottles was about 3 gm.

2. Properties of the Soluble Specific Substance of Type C Friedländer Bacillus.

The material thus isolated was an amorphous water-soluble powder, free from nitrogenous impurities and ash. The substance is a strong acid, for it turns moist Congo red paper blue. A 1:200 solution is not precipitated by solutions of silver nitrate, copper sulfate, phoshotungstic acid, or ammonium molybdate, but is precipitated by solutions of uranyl nitrate, neutral and basic lead acetates, and by concentrated solutions of barium hydroxide. The carbohydrate gives no color with iodine-potassium iodide solution. The substance has a specific rotation of +100° and its acid equivalent is approximately 680. It reacts with immune rabbit serum at a dilution of 1:2 million.

The properties of various preparations are shown in Table II.

3. Hydrolysis of the Type C Specific Substance.

1.5 gm. of Preparation 3-A were boiled for 5 hours with 50 cc. of n/1 sulfuric acid under a reflux. At the end of this time the sulfuric acid was quantitatively removed with barium hydroxide, the solution was boiled with an excess of calcium carbonate and a small amount of norit, and was filtered. The clear filtrate was evaporated to dryness in vacuo, and the residue was extracted with methyl alcohol, the alcoholic solution was filtered and was then evaporated to dryness in vacuo. (In this manner the free sugars were separated from the sugar acids, which were
present as their calcium salts and which were insoluble in methyl alcohol.) The residue of free sugars was dissolved in water and diluted to 100 cc. in a volumetric flask. An analysis by the Shaffer-Hartmann method showed 0.84 gm. of reducing sugars calculated as glucose. On the basis of this weight the solution showed a specific optical rotation of +48.2°.

**TABLE II.**

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>$\alpha$</th>
<th>Acid equivalent</th>
<th>Ash</th>
<th>$N$</th>
<th>Reducing sugars on hydrolysis (as glucose)</th>
<th>Highest dilution giving a precipitate with immune rabbit serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$+90.0^\circ$</td>
<td>610</td>
<td>0.0</td>
<td>0.5</td>
<td>75.0</td>
<td>1:2,000,000</td>
</tr>
<tr>
<td>1-A</td>
<td>$+100.0^\circ$</td>
<td>680</td>
<td>0.0</td>
<td>0.0</td>
<td>73.1</td>
<td>1:2,000,000</td>
</tr>
<tr>
<td>2</td>
<td>$+100.0^\circ$</td>
<td>681</td>
<td>0.0</td>
<td>0.0</td>
<td>--</td>
<td>1:2,000,000</td>
</tr>
<tr>
<td>3</td>
<td>$+85.0^\circ$</td>
<td>575</td>
<td>0.0</td>
<td>0.0</td>
<td>--</td>
<td>1:2,000,000</td>
</tr>
<tr>
<td>3-A</td>
<td>$+101.0^\circ$</td>
<td>674</td>
<td>0.0</td>
<td>0.0</td>
<td>75.0</td>
<td>1:2,000,000</td>
</tr>
<tr>
<td>3-B</td>
<td>$+58.0^\circ$</td>
<td>794</td>
<td>0.0</td>
<td>0.3</td>
<td>55.0</td>
<td>1:1,000,000</td>
</tr>
</tbody>
</table>

Preparation 1 was the first preparation isolated and had a rather high nitrogen content. The nitrogen was removed (Preparation 1-A) by precipitating the substance in a 1:100 solution with barium hydroxide saturated at 60° and then recovering the polysaccharide from the precipitate, by treating it with an excess of sulfuric acid, centrifuging off the barium sulfate, and reprecipitating the polysaccharide, in the presence of acid, with alcohol.

Preparation 3 was obviously impure. It was separated into two fractions by treating a 1:100 solution of the carbohydrate with a slight excess of barium hydroxide saturated at 50°. The precipitate was centrifuged off and the carbohydrate was recovered in the usual way (Preparation 3-A). The supernatant liquid from this barium hydroxide precipitation still contained specific material. This was removed by precipitation with alcohol. The precipitate, Preparation 3-B, was obviously an impure substance still containing specific material. Attempts at further purification of the preparations by adsorption on alumina, precipitation with uranyl nitrate, etc., yielded products with properties identical with the starting material.

Half of the solution was treated with 3.5 mols of phenylhydrazine acetate and an osazone was isolated in the usual manner. 0.1 gm. was recovered. The substance melted at 201-203° and had an $[\alpha]_D = -54.3^\circ$ mutarotating to $-20.0^\circ$ after 48 hours.

The second half of the solution was evaporated to 1 cc. and was oxidized with
3 cc. of 1:1 nitric acid. 0.070 gm. of potassium acid saccharate was obtained which was recrystallized from 1 cc. of water. 0.030 gm. was recovered.

0.005326 gm. substance gave 0.001853 gm. K₂SO₄.

Calculated for COOH (CHOH)₄COOK, K 15.75 per cent.

Found K 15.58 per cent.

It is evident that this sugar, which represents the greater part of the hydrolytic products of this soluble specific substance, is glucose. 0.5 gm. of residue was left from the methyl alcoholic extract of the original total hydrolysate. This material appeared to be the calcium salt of a sugar acid. It was an impure substance, judging from its color. It gave a strong naphthoresorcinol test and a strong reduction test. It is possibly the salt of an aldobionic acid such as has been found among the hydrolysis products of other specifically reacting polysaccharides. The substance will be investigated further when more material is available.

DISCUSSION.

It is clear from the foregoing experiments that strains of Friedländer's bacillus of Types A and C yield, on fractionation, two chemically distinct nitrogen-free polysaccharides with highly specific properties. Both are strong acids and both contain glucuronic acid, or an isomer, within their molecules as shown by the naphthoresorcinol test. The polysaccharides themselves are non-reducing, but on hydrolysis with mineral acids they yield reducing sugars. In both instances, as in the case of the pneumococci, specific function and carbohydrate are apparently inseparable.

On comparing the specific carbohydrates from Type B and Type C Friedländer bacillus, an unusual similarity in properties is to be observed. These substances, however, possess two distinct differences. Immunologically they show no cross-relationship. Their solubility in water in the pure state and their whole behavior during purification are entirely different. In pure form the Type B substance is difficultly soluble in water whereas the Type C substance is readily soluble. The Type B carbohydrate may be readily precipitated by alcohol in the presence of hydrochloric acid. The Type C substance, on the other hand, precipitates completely only after standing at 0° for an hour or more. The fact that two substances
so alike in physical properties are totally dissimilar in immunological reactions, may possibly be explained on the basis of slight differences in the intramolecular linkages of sugar to sugar, or of sugar to sugar acid.

SUMMARY.

1. Methods are given for the isolation of specifically reacting nitrogen-free polysaccharides from Type A and Type C Friedländer's bacillus.

2. The properties of these specific carbohydrates have been outlined.

BIBLIOGRAPHY.