THE SOLUBLE SPECIFIC SUBSTANCE OF FRIEDLÄNDER'S BACILLUS.

IV. ON THE NATURE OF THE HYDROLYTIC PRODUCTS OF THE SPECIFIC CARBOHYDRATE FROM TYPE A FRIEDLÄNDER BACILLUS.

BY WALTHER F. GOEBEL.

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

(Received for publication, June 25, 1927.)

The methods of isolation and the immunological significance of specific carbohydrates from the three fixed types of Pneumococcus and of Friedländer's bacillus have been described in a series of communications from this laboratory (1). These unusual polysaccharides, which are believed to be identical with the capsular material of the microorganisms from which they are derived, have been isolated as nitrogen-free,1 amorphous compounds possessing marked acidic properties. They are complex carbohydrates built up apparently from molecules of hexose and hexuronic acids in varying proportions. Although no contention has been made that these polysaccharides are pure distinct chemical individuals, a fair amount of evidence has been gathered which supports this view.

These carbohydrates have many physical and chemical characteristics in common; they show distinct differences, however, in the degree to which they rotate the plane of polarized light, in their acid equivalent values, and in the selective specificity which they show toward antibacterial serum.

The properties of these carbohydrates are briefly given in Table I.

The polysaccharide from the Type A Friedländer bacillus resembles the Pneumococcus Type III specific carbohydrate in some of its chemical properties, though immunologically the two compounds are totally unrelated. Both polysaccharides have

1 With one exception.
low acid equivalent values and both give a strong naphthoresorcinol test, a test which indicates the presence of glucuronic acid or an isomer within the molecule. A sugar acid, termed aldobionic acid, has been isolated from the hydrolytic products of

### Table I

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<tr>
<th>Soluble Specific Substances of the Three Fixed Types of Pneumococcus and of Friedländer's Bacillus.</th>
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<td><strong>Pneumococcus type.</strong></td>
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<td>I</td>
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<td><strong>Friedländer type.</strong></td>
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* Compounds given in parentheses have not been completely identified, but evidence of their presence has been obtained.
† Rabbit serum.

the Pneumococcus Type III polysaccharide (2). This acid appears to be built up from 1 molecule of glucuronic acid and 1 molecule of glucose. The Friedländer Type A specific carbohydrate is in itself a strong acid and since it gives a characteristic
test for glucuronic acid, it was thought that the hydrolytic products of the Friedländer carbohydrate might contain an aldobionic acid either identical or isomeric with the aldobionic acid of the Pneumococcus Type III soluble specific substance.

An investigation into the nature of the hydrolytic products of the Friedländer Type A specific carbohydrate was therefore undertaken with the hope of isolating this acid and of throwing light on the nature of the polysaccharide molecule as a whole.

**EXPERIMENTAL.**

*Hydrolysis of the Specific Polysaccharide.*

34 gm. of dry specific polysaccharide prepared from cultures of the A strain of Friedländer bacillus by a method previously described (1, b) were dissolved in 1 liter of normal sulfuric acid and the solution was boiled for 5 hours under a reflux. The sulfuric acid was quantitatively removed with barium hydroxide, and the solution was filtered from the barium sulfate. The clear yellow filtrate was boiled with a little norit and an excess of calcium carbonate and was filtered. This filtrate, after concentration to 50 cc. *in vacuo*, was poured into 10 volumes of methyl alcohol. The precipitate, the calcium salts of sugar acids, was filtered from the alcoholic solution of true sugars. The alcoholic filtrate was evaporated to dryness *in vacuo*. 13.0 gm. of dry calcium salt and 19.0 gm. of sugars were recovered.

**A. Properties and Identification of Components of the Sugar Acid Fraction.**

1. *Purification of the Calcium Aldobionate and Properties of the Aldobionic Acid.*

The calcium salt, a yellow amorphous powder, was dissolved in 1½ times its weight of water and was precipitated with an equal volume of absolute alcohol. The mixture was centrifuged. A pale yellow supernatant fluid was separated by decantation from a deep yellow oily lower layer. The latter was redissolved in an equal volume of water and was reprecipitated with 1½ volumes of alcohol. The suspension was centrifuged and the supernatant liquid was added to the supernatant liquid from the first purifica-
tion. The deep yellow lower layer was discarded. The combined supernatant liquids from the purification were evaporated to a syrup in vacuo and were finally poured into methyl alcohol. 9.2 gm. of dry substance were recovered.

The salt was next dissolved in water and was treated with slightly less than the theoretical amount of oxalic acid. The calcium oxalate was filtered off, and the filtrate was evaporated to dryness in vacuo. The free sugar was dissolved in methyl alcohol and was separated from a slight amount of insoluble calcium salt by centrifugation. The methyl alcoholic solution of the sugar acid was evaporated to dryness in vacuo, and the residue was dissolved in 100 cc. of water. A pale yellow solution was obtained. This solution was chilled to 0° and to it were added 10 cc. of 25 per cent basic lead acetate solution. A deep yellow precipitate was formed which was removed by centrifugation. The precipitate was discarded. The colorless supernatant liquid was treated with basic lead acetate solution until no further precipitation resulted. The precipitate was separated by centrifugation. The lead salt of the sugar acid was next suspended in water, and was treated with hydrogen sulfide. After filtering off the lead sulfide, the free sugar acid was obtained from the filtrate by evaporation to dryness in vacuo. The free sugar acid, as obtained in this manner, gave a strong naphthoresorcinol test and reduced Fehling’s solution vigorously. 0.3124 gm. when dissolved in 15 cc. of water showed an optical rotation of -2.25° in a 2 dm. tube. This is equivalent to [α]₀ = -54°. 0.1000 gm. of substance neutralized 3.95 cc. of N/14 sodium hydroxide, an acid equivalent of 354. Its reducing power as measured both by the Shaffer-Hartmann (3) and by the Willstätter-Schudel (4) method, is just 50 per cent that of glucose.

The substance is apparently similar to the aldobionic acid obtained from the hydrolysis of the Pneumococcus Type III specific polysaccharide and seems to be built up from two sugars, a hexose and a hexose-uronic acid, in such a way that the carboxyl and one aldehyde group remain free in the molecule.

0.1013 gm. substance: 0.1496 gm. CO₂ and 0.0492 gm. H₂O.
Calculated for C₁₁H₁₉O₁₀COOH. C 40.45 per cent, H 5.66 per cent.
Found. " 40.31 " " 5.40 ""
2. Identification of Components of the Aldobionic Acid.

(a) Identification of the Sugar Half of the Molecule.—1.0 gm. of aldobionic acid was dissolved in 50 cc. of N sulfuric acid and the solution was boiled for 15 hours under a reflux. At the end of this time the sulfuric acid was quantitatively removed with barium hydroxide. The filtrate was boiled with calcium carbonate, filtered, and the solution was evaporated to dryness in vacuo. The residue was shaken with methyl alcohol and again filtered. In this manner the alcohol-insoluble calcium salt of unhydrolyzed sugar acid was separated from the hexose liberated by hydrolysis. The free sugar, of course, was in the alcoholic filtrate. This alcoholic solution was evaporated in vacuo to dryness. The residue was taken up in water and was diluted to 50 cc. In a 2 dm. tube the solution gave a rotation of + 0.57°, or \([\alpha]_b = +47.5^\circ\). An analysis by the Shaffer-Hartmann method showed the solution to contain 0.30 gm., calculated as glucose. The remaining solution was treated with 3.5 mols of phenylhydrazine acetate and was heated 1 hour on the water bath. The crystalline osazone which formed was filtered off and washed with a few drops of methyl alcohol. The yield was 0.16 gm. The product melted at 203-204°. Its initial \([\alpha]_b\) was \(-57.2^\circ\), mutarotating to \(-24^\circ\) after 48 hours.

From the melting point of the osazone, its direction of mutarotation, and finally from the specific rotation of the sugar solution itself, it is justifiable to conclude that this product of the hydrolysis of the aldobionic acid is glucose, and that the hexose half of the molecule is therefore glucose.

(b) Identification of the Sugar Acid Half of the Molecule—2.0 gm. of aldobionic acid were boiled under a reflux with 50 cc. of \(N\) hydrobromic acid and 0.5 cc. of bromine. The bromine was replaced from time to time. At the end of 15 hours the hydrobromic acid and bromine were removed as completely as possible by distillation in vacuo. The remaining traces of hydrobromic acid were removed with silver sulfate, the excess silver ion was removed with hydrogen sulfide, and, after filtration, the sulfate ion was removed quantitatively with barium hydroxide. The aqueous residue, containing no inorganic constituents, was evaporated to 2 cc., and was then made alkaline with 50 per cent potassium
hydroxide. After acidification with glacial acetic acid, crystals of potassium acid saccharate separated from the solution. 0.2 gm. was recovered. After recrystallization from water the substance had the following analysis.

0.0500 gm. substance: 0.0176 gm. K₂SO₄.
Calculated for HOOC(CHOH)₄COOK. K 15.75 per cent.
Found. " 15.78 " "

It has been shown that the hexose half of this aldobionic acid molecule is glucose. Glucose does not yield saccharic acid under the conditions of the above experiment. One must therefore assume that the saccharic acid owes its origin to the hexose-uronic acid half of the aldobionic acid molecule, and that the hexose-uronic acid is therefore glucuronic acid.

(c) Oxidation of the Aldobionic Acid with Barium Hypoiodite.—0.6 gm. of aldobionic acid was oxidized to the dibasic sugar acid by means of barium hypoiodite (5). The glucuronogluconic acid was isolated as its calcium salt.

0.0861 gm. substance: 0.1096 gm. CO₂, 0.0338 gm. H₂O.
Calculated for C₁₀H₁₅O₇(CO₂)₄Ca. C 35.12 per cent, H 4.42 per cent.
Found. " 34.71 " " 4.65 " "

When analyzed by the method of Pervier and Gortner (6), the substance yielded 15 per cent of furfural. Since glucuronic acid yields about one-third of the amount of furfural liberated by pentoses under corresponding treatment, this figure would correspond roughly to 50 per cent of glucuronic acid within the molecule. The saccharobionic acid, when isolated as its calcium salt, is a non-reducing, water-soluble, amorphous compound which gives a strong naphthoresorcinol test.

B. Properties and Identification of Components of the Sugar Fraction.

The so called sugar or alcohol-soluble fraction which composed approximately two-thirds of the total hydrolytic products of the Type A specific polysaccharide was an optically inactive amorphous substance having a reduction value equal to 75 per cent that of glucose. The material gave only a faint naphthoresorcinol test; it gave no orcinol test.
1. Identification of Glucose.

1.0 gm. of substance was dissolved in 50 cc. of water and was heated with 3.5 mols of phenylhydrazine acetate. After 1 hour on the water bath an osazone was filtered off which was washed with a few drops of methyl alcohol. 0.25 gm. was recovered. This osazone had an initial $[\alpha]_0 = -56.5^\circ$, mutarotating to $-23^\circ$ after 48 hours. The melting point was 204-205$^\circ$. The osazone was obviously glucosazone.

When oxidized with nitric acid in the usual manner, 1.0 gm. of substance yielded 0.38 gm. of potassium acid saccharate.

0.0495 gm. substance: 0.0174 gm. $K_2SO_4$.
Calculated for HOOC(CHOH)$_2$COOK. K 15.75 per cent.

From these experiments one may conclude that part of the mixture is glucose.

2. Properties of the Non-Fermenting Sugar.

It was observed that the glucose part of the sugar fraction could be fermented away with yeast; consequently 10.0 gm. of sugar were dissolved in 100 cc. of water and the solution was treated with 20 gm. of Fleischmann's yeast. After 12 hours at 37$^\circ$ the yeast was centrifuged off and to the supernatant liquid was added a small amount of alumina to clear the solution. The mixture was again centrifuged and the clear supernatant fluid was concentrated to small volume in vacuo. The soluble yeast dextrins were precipitated with neutral lead acetate. The supernatant liquid was then treated with an excess of basic lead acetate and the precipitate was centrifuged off. The supernatant liquid from this precipitation contained no reducing sugars and was discarded.

The lead salt of the sugar acid thus obtained was suspended in water, and the lead was removed with hydrogen sulfide. After filtering off the lead sulfide, the filtrate yielded 3.5 gm. of an acidic reducing substance. This material had an $[\alpha]_0 = -58.8^\circ$ and a reduction value of 40 per cent calculated as glucose.

The substance appears to be an impure disaccharide acid. From its optical rotation one might suspect the material to be
identical with the aldobionic acid described above, for the optical rotations of both substances are approximately the same. However, this second compound does not give a naphthoresorcinol test, and it therefore cannot be identical with the aldobionic acid previously described.

1.0 gm. of the sugar fraction was dissolved in 50 cc. of water and was treated with 2.0 gm. of yeast. After 12 hours the yeast was removed and the solution was diluted to 100 cc. in a volumetric flask. A similar blank experiment was made, substituting pure glucose for the unknown sugar. After fermentation the first solution showed a reduction of 27 per cent (calculated as glucose on the basis of total weight); the blank solution showed no reduction.

This experiment demonstrates that half of the total sugars in the sugar fraction is fermentable by yeast. The sugar fraction appears, therefore, to be made up from equal parts of glucose (reduction value 100 per cent) and a second disaccharide acid (reduction value 50 per cent).

DISCUSSION.

The aldobionic acid which forms approximately one-third of the hydrolytic products of the Type A Friedländer specific carbohydrate, corresponds to the formula \( \text{C}_{12}\text{H}_{20}\text{O}_{12} \). The acid has a reduction value of 50 per cent that of glucose; it contains one carboxyl and one aldehydic group in the molecule. The reducing group is aldehydic as shown by the fact that it may be quantitatively estimated by the method of Willstätter and Schudel. The acid yields glucose on hydrolysis, but when hydrolyzed in the presence of an oxidizing agent saccharic acid is obtained. Since glucose, the hexose half of the aldobionic acid molecule, does not yield saccharic acid under such conditions, one must conclude that this substance is derived from the -uronic acid half of the molecule. This -uronic acid must necessarily be glucuronic acid. The aldobionic acid may be considered therefore as a compound built up from 1 molecule of glucose and 1 molecule of glucuronic acid in such a manner that one aldehyde group and the carboxyl group remain free.

When the aldehydic group of the aldobionic acid is oxidized to a
carboxyl group by means of barium hypoiodite, a dibasic sugar acid is obtained which still contains an intact molecule of glucuronic acid. The conclusion which may be drawn is obvious; namely, that the reducing group of the glucuronic acid is protected by chemical combination. The aldobionic acid may therefore be considered as a glucoside of glucuronic acid and glucose. The glucosidic linkage is through the reducing group of the hexuronic acid to one of the carbon atoms of glucose.

The formula

\[
\begin{align*}
\text{COOH} & \quad \text{H} \quad \text{CH} \\
\text{HOCH} & \quad \text{O} \quad \text{HCOH} \\
\text{CH} & \quad \text{HOCH} \\
\text{O} & \quad \text{HCOH} \\
\text{C} - \text{O} - \text{CH} & \quad \text{C} - \text{C} - \text{C} - \text{C} - \text{C}
\end{align*}
\]

would satisfy the properties of the aldobionic acid which has been described. Whether the linkage is on carbon atom (6) of the glucose molecule or on one of the other carbon atoms is at present unknown.

The hexose and uronic acids as well as their mode of linkage in this new aldobionic acid are the same as in the case of the aldobionic acid from the Pneumococcus Type III specific carbohydrate. The two acids differ, however, in their specific optical rotations and in their stability toward mineral acid hydrolysis. These differences may be attributed to differences in the position of the linkage between the glucose and glucuronic acid. The connecting bond in each case probably lies on a different carbon atom of the glucose molecule.

It seems to be extremely significant that two sugar acids which differ only in their spatial configuration, have been isolated from the capsular material of two totally unrelated microorganisms. The significance of these acids will be discussed in a future communication.
The so-called sugar fraction which forms the remaining two-thirds of the hydrolytic products of the Type A Friedländer specific carbohydrate, has a reduction value of 75 per cent that of glucose. Half of the sugar which composes this fraction may be fermented away with yeast. The non-fermenting sugar which remains has a reduction value of approximately 50 per cent that of glucose. This sugar appears to be a lactone of a second disaccharide acid. The compound has not been investigated in detail as yet. The fermentable sugar is undoubtedly glucose, since both glucosazone and saccharic acid were isolated in excellent yields.

In conclusion it may be said that the hydrolytic products of the Type A Friedländer soluble specific substance appear to be three sugars; namely, an aldobionic acid, glucose, and a second disaccharide acid. These three sugars are found approximately in the ratio of 1:1:1. If one considers the polysaccharide as a whole, as built up from 2 molecules of aldobionic acid (1 of which is in the form of a lactone) and 1 molecule of glucose, the complex molecule may be represented by the gross formula \((\text{C}_{30}\text{H}_{41}\text{O}_{26})_x\). Such a compound should have an acid equivalent of 410 and a carbon and hydrogen content of 43.9 per cent and 5.4 per cent respectively. A comparison of these figures with the observed values in Table I shows a remarkably close approximation.

SUMMARY.

1. The soluble specific substance of the Type A Friedländer bacillus yields on hydrolysis an aldobionic acid, glucose, and a second unidentified sugar acid. These compounds occur approximately in the ratio of 1:1:1.

2. A detailed chemical study of the new aldobionic acid has been made, showing it to consist of a molecule of glucuronic acid linked through its reducing group to a molecule of glucose. It is isomeric with an acid derived similarly from the soluble specific substance of Type III pneumococcus.

3. The polysaccharide appears to be a condensate of 2 molecules of aldobionic acid and 1 molecule of glucose.
BIBLIOGRAPHY.


