THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LI. STUDIES ON THE PHOSPHATIDE OF THE HUMAN TUBERCLE BACILLUS*

BY R. J. ANDERSON, W. C. LOTHROP,† AND M. M. CREIGHTON‡

(From the Department of Chemistry, Yale University, New Haven)

(Received for publication, June 22, 1938)

In a previous report from this Laboratory it was shown that the phosphatide fraction from the human type of tubercle bacillus, Strain H-37, when hydrolyzed with dilute sulfuric acid, yielded some 66 per cent of fatty acids and about 33 per cent of water-soluble compounds (1). The water-soluble compounds, in addition to inorganic phosphoric acid and glycerophosphoric acid, consisted of inosite (2), mannose (3), and some other hexose, presumably glucose because it gave a typical glucosazone (4). Saponification of the phosphatide with dilute alcoholic potassium hydroxide led to quite different cleavage products (5). The alcoholic solution contained the potassium salts of the fatty acids, while an alcohol-insoluble substance remained which was found to consist of an organic phosphoric acid and a phosphorus-containing polysaccharide. The polysaccharide, which was named manninositose, gave on hydrolysis with dilute sulfuric acid phosphoric acid, mannose, and inosite. Since this was the first time that a substance had been described which gave inosite and mannose on hydrolysis, we were interested in studying more fully the properties of this unique compound and in determining whether

* The present report is a part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.
† Holder of a National Tuberculosis Association Fellowship in Chemistry at Yale University, 1937-38.
‡ Holder of a Sterling Research Fellowship in Chemistry at Yale University, 1937-38.
it was a glycoside or whether the inosite and mannose were combined as esters of phosphoric acid.

In the work (5) reported several years ago the phosphatide had been saponified by prolonged heating with alcoholic potassium hydroxide and this drastic treatment might have changed the carbohydrate molecule.

In the experiments forming the subject of the present report very mild alkali treatment at room temperature was employed, but the cleavage products were essentially the same as were found in the first experiment. The fatty acids were split off and an organic phosphoric acid together with a phosphorus-containing polysaccharide separated from the solution. The phosphorus was split off by heating a solution of the polysaccharide in dilute ammonium hydroxide in a sealed tube to 170° for 8.5 hours. The dephosphorylated substance gave no reduction with Fehling’s solution until after it had been boiled with dilute acid, thus indicating that it was a glycoside. When the glycoside was hydrolyzed by boiling with dilute sulfuric acid, the maximum reduction, as determined by the Shaffer-Hartmann method (6), was attained in 2.5 hours and amounted to 63 per cent, calculated as glucose. The only cleavage products that could be isolated were mannose and inosite and the amounts obtained would indicate that the glycoside, for which we retain the name manninositose, is a triglycoside containing 2 molecules of mannose combined with 1 molecule of inosite.

A substance very similar to and probably identical with manninositose was isolated from the polysaccharide of the avian tubercle bacillus by du Mont and Anderson (7). The avian tubercle bacillus glycoside was present, however, in the free state and not combined with phosphoric acid.

In the present study no evidence whatever was obtained of the presence of any hexose except mannose. However, in earlier analyses in which the phosphatide had been hydrolyzed with dilute sulfuric acid three carbohydrates, namely inosite, mannose, and some other hexose which gave a glucosazone after the mannose had been removed as phenylhydrazone, were always found (5). It is evident therefore that direct acid hydrolysis of the phosphatide yields different hexoses than are obtained after the phosphatide has been saponified with dilute potassium hydroxide.
EXPERIMENTAL

The phosphatide had been prepared from the human type of tubercle bacillus and purified as described in a former paper (8). The substance was a white amorphous powder containing about 3.0 per cent of phosphorus and 0.3 per cent of nitrogen. It was readily dispersed in water, forming colloidal solutions. The concentrated solutions were cloudy but became perfectly clear on sufficient dilution with water. The aqueous solutions gave no reduction when boiled with Fehling’s solution but after they were heated for some time with dilute acid reducing sugars were liberated.

Saponification of the Phosphatide—The phosphatide, 11.4 gm., was dissolved in 100 cc. of benzene, in which it gave a perfectly clear, faintly yellowish solution, and 1.5 gm. of potassium hydroxide dissolved in 10 cc. of absolute alcohol were added. On standing at room temperature the solution turned cloudy and a gelatinous precipitate separated slowly. After 24 hours the precipitate was filtered off and washed carefully with benzene and with ether and dried in vacuo. The filtrate on standing for another 24 hours deposited a small amount of insoluble matter which was filtered off, washed as before, and dried in vacuo. The total yield of benzene-insoluble matter was 6.48 gm. or 56.8 per cent of the phosphatide.

Examination of the Benzene-Insoluble Substance—The materials which had separated from the benzene solution were dissolved in 100 cc. of water, giving a cloudy solution which was strongly alkaline in reaction. Acidification with acetic acid gave a heavy precipitate which was removed by filtration, washed with water, and dried in vacuo. It formed a somewhat sticky mass which weighed 1.6 gm. and was found to consist of fatty acids.

The filtrate and washings were concentrated in vacuo to a thick syrup and the syrup was dehydrated by grinding under absolute alcohol in a mortar until a fine white powder was formed. This product, which represents the crude polysaccharide, weighed 4.6 gm., corresponding to 40.3 per cent of the phosphatide.

Isolation of the Fatty Acids—The benzene solution was freed of excess potassium hydroxide by means of carbon dioxide and the potassium carbonate was filtered off, washed with benzene, and discarded. The filtrate was concentrated to dryness in vacuo
and the residue was dissolved in ether. The ethereal extract was washed, first with dilute hydrochloric acid and then with water, after which it was dried over sodium sulfate, filtered, and evaporated to dryness. The oily residue weighed 5.7 gm. This material was combined with the fatty acid fraction mentioned above, namely 1.6 gm., thus giving a total of 7.3 gm. of fatty acids corresponding to 64 per cent of the phosphatide. Both of these fractions of fatty acids were tested for phosphorus with completely negative results. The fatty acids were reserved for a future study.

Examination of the Crude Polysaccharide—A portion of the crude polysaccharide was hydrolyzed with dilute sulfuric acid, after which the solution was extracted with ether. The ethereal extract on evaporation to dryness left the merest trace of a residue, thus indicating that all the fatty acids had been split off by the alkali treatment.

The crude polysaccharide on analysis was found to contain 6.20 per cent of phosphorus, 0.12 per cent of nitrogen, and 29.6 per cent of ash.

Separation of an Organic Phosphoric Acid from the Crude Polysaccharide—The remaining portion of the crude polysaccharide, 4.5 gm., was dissolved in 50 cc. of water and a solution of neutral lead acetate was added until no further precipitate occurred. The precipitate was filtered off, washed with water, and decomposed with hydrogen sulfide. The lead sulfide was filtered off; the filtrate was concentrated in vacuo to a small volume and neutralized with barium hydroxide. The solution was filtered and the barium salt was precipitated with alcohol. After the substance had been reprecipitated in the same manner, 0.21 gm. of a white amorphous powder was obtained.

For analysis the substance was dried in vacuo over dehydrite. Found, Ba 40.41, 40.11; P 9.23, 9.47.

While the analytical values agree approximately with the calculated composition of barium glycerophosphate containing 2 molecules of water of crystallization, the substance was not pure glycerophosphate, because after hydrolysis with dilute sulfuric acid the solution was found to contain 26 per cent of reducing sugar calculated as glucose. The small amount of available material prevented the identification of the sugar but it is very probable
that the substance was identical with the organic phosphoric acid obtained from the polysaccharide after treatment with hot alcoholic potassium hydroxide, as will be described later.

Isolation of the Polysaccharide—The filtrate from the neutral lead acetate precipitate was freed from excess lead with hydrogen sulfide and the filtrate was concentrated to a thick syrup. The syrup was dehydrated by grinding under absolute alcohol as described before. The yield was 4.33 gm. of a white powder.

Attempts to Dephosphorylate the Polysaccharide—Several experiments were carried out to determine the best procedure by which to dephosphorylate the polysaccharide. Heating the substance with 14 per cent ammonium hydroxide in a sealed tube to 150-155° for 6 hours or to 160° for 7.5 hours led to incomplete removal of the phosphorus, but heating to 170° for 8.5 hours removed all of the phosphorus but no reducing sugars were liberated.

Examination of the reaction products formed in the preliminary experiments showed that inorganic phosphoric acid, an alcohol-insoluble solid polysaccharide, and an alcohol-soluble syrup had been produced. The alcohol-soluble syrup contained glycerol, the latter being identified as glyceryltribenzoate. The results obtained indicated that some glycerol was combined in the polysaccharide.

In order to split off the glycerol-containing substance, 1.9 gm. of the polysaccharide were refluxed for 2 hours with 1 per cent alcoholic potassium hydroxide. The polysaccharide which remained as an insoluble mass was recovered and dissolved in water. The solution was neutralized with acetic acid and neutral lead acetate was added until no further precipitate occurred. The precipitate was filtered off and washed with water.

The polysaccharide was isolated from the filtrate, as will be described later.

Isolation of the Organic Phosphoric Acid—The lead salt was decomposed with hydrogen sulfide in the usual manner and the filtrate was concentrated to dryness in vacuo. The residue, which was insoluble in alcohol, indicating that it was not glycero-phosphoric acid, was dissolved in water and the solution was neutralized with barium hydroxide. The barium salt was precipitated with alcohol and gave 0.55 gm. of a white amorphous powder.
For analysis the substance was dried at 78° in vacuo over dehydrite. Found, Ba 40.79, 40.18; P 8.28, 8.36.

The composition is very similar to that of the barium salt described above and the analytical values agree approximately with the calculated composition of a barium salt of the formula, C₉H₁₆O₄P₂Ba₂ (684.8). Calculated Ba 40.13, P 9.05, corresponding to an acid having the formula C₅H₉O₄P₂. Such an acid would correspond to mannose-glycerol diphosphoric acid. A similar barium salt was isolated in the original study of manninositose (5).

Hydrolysis of the Barium Salt—The balance of the barium salt, 0.529 gm., was dissolved in water and the barium was precipitated by adding sufficient sulfuric acid to make a 5 per cent solution. The barium sulfate was removed and the filtrate was refluxed for 3 hours, after which the sulfuric acid was removed quantitatively with barium hydroxide. After filtration the solution was concentrated in vacuo to a volume of 25 cc., neutralized with barium hydroxide, and diluted with an equal volume of alcohol but only a slight precipitate occurred, thus indicating the presence of a small amount of unhydrolyzed organic phosphoric acid. The precipitate was filtered off and the solution was freed of a trace of barium with sulfuric acid and concentrated in vacuo to a volume of 3 cc. To this solution, which gave a strong reduction when heated with Fehling's solution, were added 0.25 gm. of phenylhydrazine hydrochloride and 0.2 gm. of sodium acetate. Almost immediately a crystalline precipitate began to separate.

After the crystals had been filtered off, the filtrate was heated in a water bath for an hour but no osazone separated, thus indicating that other hexoses such as glucose or fructose were absent.

The crystalline product mentioned above was recrystallized from 60 per cent alcohol. The crystal form, melting point, and mixed melting point were identical with those of mannose phenylhydrazone.

The only cleavage product definitely identified was mannose phenylhydrazone but indication of the presence of an organic phosphoric acid was obtained and the latter may have been glycerophosphoric acid. It is probable that the original product represented a barium salt of a mannose-glycerol diphosphoric acid.
but the definite identification of such an acid will be a problem for future investigation.

Dephosphorylation of the Polysaccharide—The filtrate from the lead precipitate mentioned above was freed from excess lead by hydrogen sulfide and, after removal of the lead sulfide, the clear solution was concentrated in vacuo to a thick syrup. The syrup was dissolved in 15 cc. of 14 per cent ammonium hydroxide and the solution was heated in a sealed tube to 170° for 8.5 hours. The contents of the tube had a slight straw color and a small amount of precipitate had separated. The solution was filtered and concentrated in vacuo to remove the ammonia, after which it was diluted with water and an excess of barium hydroxide was added which caused a heavy precipitate of barium phosphate. The latter was filtered off and discarded.

The filtrate after removal of excess barium was concentrated in vacuo to a thick syrup. Since it was impossible to induce crystallization, the substance was precipitated by pouring the syrup into absolute alcohol. The product, a white amorphous powder weighing 1.3 gm., was free from phosphorus and it did not contain any reducing sugar. Attempts to crystallize the substance were unsuccessful.

Acetylation of the Polysaccharide—The product described above was treated with 25 cc. of pyridine and 6 cc. of acetic anhydride. Practically all of the substance dissolved on standing at room temperature for 3 days. The solution, after it had been concentrated in vacuo to about 5 cc., was poured into dilute sulfuric acid, the reaction product was extracted with chloroform, and the extract was washed with water until the washings were neutral to litmus. The acetyl derivative obtained on evaporation of the solvent was found to be easily soluble in the usual organic solvents and it did not crystallize. The concentrated alcoholic solution of the substance on being mixed with water gave a white amorphous powder which weighed 1.4 gm.

The melting point was not sharp. When heated in a capillary tube, it began to sinter at about 98°, became transparent at 108°, and fused at 112°. The melt did not crystallize on cooling but remained as a transparent mass.

Rotation—0.1425 gm. of substance dissolved in methyl al-
Lipids of Tubercle Bacilli. LIII

cohol and diluted to 10 cc. gave in a 1 dm. tube a reading of $\alpha = +0.694^\circ$; hence $[\alpha]_D^{25} = +48.7^\circ$.

**Analysis**

\[ \text{C}_{18}\text{H}_{20}\text{O}_{16}(\text{COCH}_3)_{12} \ (1008). \]  
Calculated. C 50.00, H 5.55  
Found. " 49.48, " 5.97  
Mol. wt. (East), 907, 983, 940, 966

**Saponification of the Acetyl Derivative**—Since the acetyl derivative could not be obtained in crystalline form, it was saponified and the free glycoside was isolated. For saponification 1.0 gm. of the acetyl derivative was refluxed for 4 hours with 120 cc. of methyl alcohol and 50 cc. of a saturated aqueous solution of barium hydroxide. The amount of barium hydroxide neutralized during the saponification was equivalent to 11.30 cc. of \( \text{N CH}_3\text{COOH} \), corresponding to 67.78 per cent of acetic acid.

**Isolation of Manninositose**—After the methyl alcohol had been distilled off under reduced pressure and the barium had been precipitated quantitatively with sulfuric acid, the filtrate was evaporated in vacuo to dryness. The residue was a thick syrup which was very soluble in water but insoluble in alcohol and which could not be induced to crystallize. The syrup was dissolved in 10 cc. of water and the solution was poured with constant stirring into 200 cc. of absolute alcohol, whereupon a finely divided precipitate separated. The precipitation was completed by adding 100 cc. of ether, after which the precipitate was collected, washed with absolute alcohol, and dried in vacuo. The white amorphous powder weighed 0.45 gm. The substance had no definite melting point. When heated in a capillary tube, it began to fuse at about 215\(^\circ\), showed slight effervescence at about 250\(^\circ\), and turned faintly yellowish in color.

**Rotation**—0.2207 gm. of substance dissolved in water and diluted to 10 cc. gave in a 1 dm. tube a reading of $\alpha = +1.637^\circ$; hence $[\alpha]_D^{25} = +74.1^\circ$. The solution showed no mutarotation.

**Hydrolysis of Manninositose**—When the glycoside was refluxed with 5 per cent sulfuric acid, the maximum reduction as determined by the Shaffer-Hartmann method (6) was attained in about 2.5 hours and amounted to 63 per cent calculated as glucose. For the determination of the cleavage products 0.4 gm. of the glycoside was refluxed with 60 cc. of 5 per cent sulfuric
acid for 4 hours, after which the sulfuric acid was removed quantitatively with barium hydroxide and filtered from barium sulfate. The filtrate was concentrated in vacuo to a volume of 8 cc.

Separation of Mannose As Phenylhydrazone The solution mentioned above was mixed with 0.4 gm. of phenylhydrazine dissolved in 1.0 cc. of alcohol. The mannose phenylhydrazone began to crystallize almost immediately and after the solution had stood overnight the crystals were filtered off, washed with water and with alcohol, and dried in vacuo. The faintly yellow-colored crystals weighed 0.3465 gm., corresponding to 0.2310 gm. of mannose, equivalent to 57.75 per cent of the glycoside. After recrystallization from 60 per cent alcohol large, nearly colorless crystals were obtained which melted when rapidly heated at 194–195° with decomposition and did not depress the melting point of pure mannose phenylhydrazone which melted at the same temperature.

Isolation of Inosite—The filtrate from the mannose phenylhydrazone was freed of excess phenylhydrazine by means of benzaldehyde in the usual manner and the excess of benzaldehyde was removed by extraction with chloroform. The solution, after being decolorized with norit and concentrated to about 4.0 cc., was mixed with alcohol until it turned slightly cloudy. On standing, prismatic crystals separated and were collected, washed with alcohol, and dried in vacuo. Addition of ether to the mother liquor caused another small crop of crystals to separate which were filtered off, washed, dried, and combined with the first lot. The total yield of crystalline inosite was 0.1105 gm., corresponding to 27.62 per cent of the glycoside.

The mother liquor on evaporation to dryness left a solid residue which weighed about 50 mg. When this material was dissolved in a little water and tested with Fehling's solution, a slight reduction was obtained. The substance yielded neither an insoluble phenylhydrazone nor an osazone on treatment with phenylhydrazine. Undoubtedly some inosite was present because inosite does not separate quantitatively from a solution.

The inosite on recrystallization from water by the addition of alcohol separated in characteristic needle-shaped prisms. It gave the reaction of Scherer, melted at 225°, and caused no depression of the melting point when mixed with pure inosite.

The amounts of the cleavage products obtained show that
about 2 parts of mannose and 1 part of inosite were recovered and the total recovery amounted to 85.37 per cent of the glycoside. These values would indicate that manninositose is a triglycoside containing 2 molecules of mannose combined with 1 molecule of inosite.

SUMMARY

The phosphatide of the human tubercle bacillus contains at least two types of carbohydrates and both contain phosphorus in organic combination.

One carbohydrate appears to be a mannose-glycerol diphosphoric acid which gives a water-insoluble lead salt.

The other carbohydrate is a phosphorus-containing glycoside, manninositose phosphoric acid, which is not precipitated from aqueous solution by lead acetate.

Manninositose phosphoric acid on being heated with dilute ammonium hydroxide to 170° yields inorganic phosphoric acid and the glycoside manninositose.

Manninositose on hydrolysis with dilute sulfuric acid gives only mannose and inosite and approximately in the ratio of 2:1.

BIBLIOGRAPHY

THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI: LIII. STUDIES ON THE PHOSPHATIDE OF THE HUMAN TUBERCLE BACILLUS
R. J. Anderson, W. C. Lothrop and M. M. Creighton


Access the most updated version of this article at http://www.jbc.org/content/125/1/299.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/125/1/299.citation.full.html#ref-list-1