THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXVII. THE LIPIDS OF THE HUMAN TUBERCLE BACILLUS H-37 CULTIVATED ON A DEXTROSE-CONTAINING MEDIUM*

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The high percentage of lipids differentiates the tubercle bacillus and acid-fast bacteria in general from other microorganisms. However, the amount of lipids in acid-fast bacteria varies not only from strain to strain but also with the composition of the medium (1). Results obtained in this laboratory indicate that the lipids of the human tubercle bacillus, Strain H-37, when grown under identical conditions but at different times on the Long synthetic medium (2) show wide variations in the amounts of various lipid fractions and also in chemical constants (3, 4). Similar variations were found in this laboratory in a comparative investigation of five different strains of human tubercle bacilli, four of which had been recently isolated from human cases of tuberculosis (4).

In other investigations1 conducted in this laboratory on the chemistry of the pathogenic microorganism Phytomonas tumefaciens it was found, when the bacteria were cultivated in a synthetic medium in which glycerol was the chief source of carbon, that the growth was slight and the bacterial cells contained only 2 per cent of lipids. However, when sucrose was added to the medium in place of glycerol, the bacterial growth was more luxuriant and the lipid content of the bacteria amounted to about 6 per cent (5).

In view of the results with Phytomonas tumefaciens it appeared of interest to study the lipid production and the chemical composition of the lipid fractions of the human tubercle bacillus when cultivated on a modified Long synthetic medium in which glycerol was replaced by dextrose. Through the cooperation of Sharp and Dohme, Glenolden, Pennsylvania, we were provided with a lot of tubercle bacilli, Strain H-37, which had been cultivated on the modified Long synthetic medium. The bacilli were extracted and the lipid fractions were separated essentially as described in previous studies (4).

According to the results of our earlier investigations the alcohol-ether

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1939–40.

1 These experiments were conducted in collaboration with The International Cancer Research Foundation, Philadelphia, and Dr. A. J. Riker of the University of Wisconsin.
extracts of tubercle bacilli contain phosphatide, acetone-soluble fat, and a wax fraction which melts at about 50°. The phosphatide, as shown by Dr. F. R. Sabin and collaborators (6), possesses important biological properties. In view of the general occurrence of phosphatides in all living cells it is believed that this class of compounds is of vital importance in the life of the cells.

In the present investigation of the alcohol-ether extract of the bacilli grown on the dextrose-containing medium it was surprising that no phosphatide could be found. The only substances contained in the alcohol-ether extract were acetone-soluble fat and a low melting wax which had the same properties as the wax previously obtained from the mother liquors in the purification of the phosphatides (4). The chloroform-soluble wax isolated from the bacilli after the alcohol-ether extraction also had a low melting point and we were unable to find any high melting wax corresponding to the so called "purified wax" which melts with decomposition at about 205° (7).

The firmly bound lipid isolated after the partly defatted bacilli had been treated with dilute hydrocholoric acid was a wax-like substance with a low melting point.

The results obtained in the present investigation indicate that the composition of the medium on which the bacilli are grown exerts a remarkable influence on the nature of the lipids that are elaborated. The presence of glycerol in the medium is apparently necessary for the production of certain lipid fractions. The importance of this fact must be considered in future investigations.

In view of the differences in lipid components mentioned above it appeared of interest to analyze the acetone-soluble fat and the low melting wax in order to determine whether these fractions contained the characteristic specific constituents previously found in tubercle bacilli grown on the glycerol-containing Long medium. The acetone-soluble fat was found to contain tuberculostearic acid and phthioic acid and the pigment phthiocerol, compounds identical with those previously isolated. The fat did not contain any glycerol but a carbohydrate was isolated among the water-soluble constituents. The amount of carbohydrate was too small to permit definite identification but it was probably trehalose.

The low melting wax fractions yielded on saponification a polysaccharide which gave pentose color reactions. The principal ether-soluble component was mycolic acid. There were also found dextrorotatory fatty acids analogous to phthioic acid and also the alcohol phthiocerol.

**EXPERIMENTAL**

The tubercle bacilli, Strain H-37, were cultivated according to the standard procedure in 1 liter Pyrex bottles for a period of 8 weeks. The medium...
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contained the same ingredients as the Long synthetic medium (2) except that the glycerol was replaced by 10 per cent of dextrose. The cultures, 77 in number, were filtered off and washed with water, after which the bacterial cells were suspended in 3.5 liters of alcohol and shipped to the Sterling Chemistry Laboratory.

To the bacterial suspensions were added 2 liters of alcohol² and the mixture was shaken thoroughly. The cells settled rather quickly and after the mixture had stood overnight the clear supernatant was siphoned off. The cells were further dehydrated by treatment with 2 liters of alcohol and the supernatant was siphoned off after the cells had settled. The cells were next extracted four times with 2 liters of alcohol-ether, equal parts, for each extraction. The bacterial cells were then filtered off, washed with alcohol-ether, and extracted with chloroform as will be described later. The alcoholic and alcohol-ether extracts were combined and concentrated under reduced pressure until the ether and most of the alcohol had been removed. The lipids that remained in the aqueous suspension were extracted with ether and the ethereal solution was concentrated to a volume of about 1 liter. The solution was forced through a Chamberland filter under carbon dioxide pressure and the filter was washed thoroughly with ether. The clear filtrate was concentrated to a volume of about 400 cc. and mixed with 500 cc. of acetone. The precipitate which should consist of crude phosphatide was filtered off and washed with acetone.

The precipitate was reprecipitated ten times from ether solution with acetone and was finally obtained as a white powder which weighed 1.5265 gm. The substance melted at 50–56°. Analysis, found, P 0.58, N 0.28 per cent.

The properties of this fraction resemble those of the low melting wax that we have previously found in the mother liquors from the purification of the phosphatide (4). Attempts to isolate a substance from this material corresponding in properties to the usual phosphatide were unsuccessful. The substance was separated into several fractions by treatment with ethyl acetate and precipitations from ether solution with acetone but all the fractions had low melting points and the phosphorus content was low. It is evident therefore that a substance corresponding in properties to the usual tubercle bacillus phosphatide was not present in this lot of bacilli.

Isolation of a Low Melting Wax from the Mother Liquors—The ether-acetone mother liquors resulting from the above operations were concentrated to a volume of about 300 cc. and cooled in ice water, whereupon a white precipitate separated. The precipitate was filtered off, washed with acetone, and dried. The filtrate was concentrated to about 150 cc. and

² All solvents had been carefully purified and redistilled before they were used. The alcohol had been distilled over potassium hydroxide. The ether was freed from peroxides, dried over calcium chloride, and distilled over potassium hydroxide.
again cooled in ice water. The slight precipitate that separated was filtered off, washed with acetone, and dried. The two precipitates mentioned above were combined, giving a total weight of 8.0763 gm. The material was a nearly white powder which melted at 41-44°. It contained a trace of phosphorus.

The Acetone-Soluble Fat—The acetone mother liquor from the low melting wax was evaporated to dryness and the residue consisting of acetone-soluble fat was further dried in vacuo. It formed a soft brown salve-like mass with a perfume-like odor and it weighed 7.4733 gm.

The Chloroform-Soluble Wax—The bacterial residue from the alcohol-ether extraction was extracted four times with chloroform-ether, equal parts. 2 liters of solvent were used for each extraction. The bacterial cells were filtered off and washed with chloroform-ether and dried. The dry bacterial residue weighed 86 gm.

The chloroform-ether extracts were combined and evaporated to dryness in vacuo. The residue was dissolved in ligroin and the solution was forced through a Chamberland filter under carbon dioxide pressure. The filter was washed three times with ligroin. No unfiltrable lipid was noticed on the filter.

The clear filtrate on concentration to dryness left a nearly white waxy residue which weighed 7.8 gm. For purification the substance was dissolved in 100 cc. of ether and precipitated by the addition of 250 cc. of cold acetone. The substance was filtered off and reprecipitated in the same manner. The final purification was carried out by dissolving the precipitate in 125 cc. of ether and adding 250 cc. of cold methyl alcohol. The precipitate was filtered off, washed with methyl alcohol, and dried in vacuo. The product was a white powder which weighed 7.1 gm. It melted at about 53° and contained a trace of phosphorus and 0.39 per cent of nitrogen.

The Firmly Bound Lipids—The bacterial residue was examined for firmly bound lipids as follows: 10 gm. of the dried cells were treated with 100 cc. of a mixture of alcohol and ether, equal parts, containing 1.25 cc. of concentrated hydrochloric acid at 45-50° for 2 hours. After the mixture had cooled, the cells were filtered off and washed with alcohol. The cell residue was next extracted under a reflux with 100 cc. of chloroform-ether, equal parts, at 45-50° for 2 hours. After the mixture had cooled, the cells were filtered off and washed with chloroform-ether. The extractions were repeated three times. After the cell residue had been dried, it weighed 8.5164 gm.

The lipids recovered from the acid alcohol-ether extract were a yellowish solid wax-like mass which weighed 0.2127 gm. This fraction was not further examined.

The chloroform-ether extracts were combined and concentrated under
reduced pressure to a volume of about 150 cc. To the solution was then added 1.0 gm. of solid sodium bicarbonate in order to remove any hydrochloric acid. After the mixture had stood overnight, the solution was filtered and the filtrate was evaporated to dryness. The residue which weighed 0.8903 gm. was dissolved in 10 cc. of ether and precipitated by addition of 30 cc. of cold acetone. The precipitate was filtered off, washed with acetone, and dried. The substance was a white powder which weighed 0.8392 gm. and it melted at about 44°. It was free from phosphorus.

**Table I**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Description</th>
<th>M.p. °C.</th>
<th>N per cent</th>
<th>P per cent</th>
<th>Weight gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Low melting wax</td>
<td>50-56</td>
<td>0.26</td>
<td>0.58</td>
<td>1.5265</td>
</tr>
<tr>
<td>II</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>41-44</td>
<td>Trace</td>
<td></td>
<td>8.0763</td>
</tr>
<tr>
<td>III</td>
<td>Acetone-soluble fat</td>
<td>53</td>
<td>0.39</td>
<td>&quot;</td>
<td>7.4733</td>
</tr>
<tr>
<td>IV</td>
<td>CHCl₃-soluble wax</td>
<td>44</td>
<td></td>
<td>None</td>
<td>7.8000</td>
</tr>
<tr>
<td>V</td>
<td>Bound lipids</td>
<td></td>
<td></td>
<td></td>
<td>9.4858*</td>
</tr>
</tbody>
</table>

Total lipids....................................................... 34.3619

* Calculated value.

**Table II**

<table>
<thead>
<tr>
<th>Description</th>
<th>gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipids</td>
<td>34.3619</td>
</tr>
<tr>
<td>Polysaccharide</td>
<td>1.0887</td>
</tr>
<tr>
<td>Extracted bacterial residue</td>
<td>76.5142*</td>
</tr>
<tr>
<td>Total dry bacterial mass</td>
<td>111.9648</td>
</tr>
</tbody>
</table>

* Calculated value.

The total bound lipids from 10 gm. of partly defatted bacilli amounted to 1.103 gm., corresponding to 11.03 per cent of the bacterial residue.

The lipid fractions isolated are summarized in Table I.

**Isolation of Polysaccharide**—The polysaccharide contained in the aqueous solution which remained after the lipids had been extracted from the concentrated alcoholic and alcohol-ether extracts as previously mentioned was isolated in the usual manner by means of basic lead acetate and ammonia.
The lead precipitate was decomposed with hydrogen sulfide and after the lead sulfide had been filtered off, the filtrate was concentrated under reduced pressure to a thick syrup which was dehydrated by grinding under absolute alcohol. The substance weighed 1.0887 gm. but it was not further examined.

The total yield of material obtained from the 77 cultures that were provided for this investigation is summarized in Table II.

It will be seen from the data in Table II that the total lipids amounted to 30.6 per cent of the dry bacilli. Although no phosphatide could be found, it is evident that the total lipid content was about the same as that obtained when the bacilli are cultivated on the ordinary glycerol-containing Long medium.

**Analysis of the Acetone-Soluble Fat**

**Constants of the Fat**—The acetone-soluble fat had the following constants: iodine No. (Rosenmund-Kuhnenn method) 55.5, saponification No. 137, acid No. 68, ester No. 69, Reichert-Meissl No. 6.5, Polenské No. 5.7, unsaponifiable matter 7.36 per cent.

**Saponification of the Fat**—The fat, 5.84 gm., was saponified by refluxing with 100 cc. of 4 per cent alcoholic potassium hydroxide for 5 hours in an atmosphere of nitrogen. The solution was concentrated by distillation to a volume of about 50 cc., diluted with water, and the unsaponifiable matter was extracted with ether. The unsaponifiable matter obtained on evaporation of the ethereal solution was again refluxed for 2 hours with alcoholic potassium hydroxide, after which the unsaponifiable matter was isolated as mentioned above. The alkaline solutions were combined and examined for water-soluble components and fatty acids.

The unsaponifiable matter formed a thick amber-colored mass that weighed 0.4303 gm., corresponding to 7.36 per cent of the fat. The substance had a pleasant odor and the iodine number was 127.8.

**Isolation of the Fatty Acids**—The alkaline soap solution was acidified with hydrochloric acid and the fatty acids were extracted with ether. In order to remove phthiocol and certain acids the ethereal solution was extracted with five portions of 1 per cent aqueous sodium bicarbonate solution. The ethereal solution was then dried over sodium sulfate, filtered, and the ether was distilled off. The residue consisting of higher fatty acids weighed 4.06 gm., corresponding to 69.5 per cent of the fat.

**Determination of Phthiocol**—The sodium bicarbonate extract which was dark red in color was acidified with hydrochloric acid and extracted with ether. The ethereal solution was dried over sodium sulfate, filtered, and the ether was distilled off. The dark colored residue weighed 0.57 gm. The phthiocol contained in this material was isolated as described previously (4) but the amount was too small to be obtained in pure crystalline
form. The phthiocol was therefore estimated by the colorimetric method (8) and amounted to about 2.5 mg. The substance showed the reactions of phthiocol. It was easily soluble in dilute sodium bicarbonate, with a bright red color and on acidification the color turned yellowish. A solution in dilute methyl alcohol deposited a few small yellow crystals.

Examination of the Aqueous Solution—The acidified aqueous solution from which the fatty acids had been extracted was examined for trehalose and glycerol by the method formerly described (4). A carbohydrate fraction which gave a Molisch reaction was isolated by means of basic lead acetate and ammonia. The carbohydrate weighed 63 mg. An attempt was made to acetylate this product in pyridine with acetic anhydride and a water-insoluble acetyl derivative was obtained but the amount was too small to permit of its isolation in crystalline form.

The filtrate from the basic lead acetate precipitate was examined for glycerol but none was found.

Examination of the Fatty Acids—The fatty acids, 4.06 gm., were separated by means of the lead salt-ether procedure and gave 0.765 gm. of solid acids and 3.278 gm. of liquid fatty acids. The solid acids were not further examined. The liquid fatty acids were esterified and gave 3.315 gm. of methyl esters which were distilled in a high vacuum at a temperature between 140-210° through a modified Widmer column. The distillate was a yellow oil which weighed 2.093 gm. and the iodine number was 21. The residue in the distilling flask was a thick dark oil, 1.20 gm., and the iodine number was 46. This fraction was not further examined.

The distilled esters after reduction with hydrogen in the presence of platinum oxide were completely saturated, as the iodine number was 0. The hydrogenated esters were saponified with alcoholic potassium hydroxide. The free acids were isolated, after which the lead salt-ether separation was repeated. The solid reduced acid weighed 0.281 gm. and was not further examined. The liquid saturated acids isolated from the ether-soluble lead salt weighed 1.788 gm.; $[\alpha]_D = +4.57^\circ$; mol. wt. 362.

The liquid saturated fatty acids were esterified with diazomethane and were fractionated and re-refractionated through a modified Widmer column into four fractions. Fractions I and II, total weight 0.63 gm., were optically inactive but the free acids, 0.60 gm., obtained on saponification of the esters were partly solid. The lead salt-ether separation was therefore repeated. The solid acid obtained from the ether-insoluble lead salt weighed 98 mg. The liquid acid isolated from the ether-soluble lead salt weighed 0.497 gm. This acid was a liquid at room temperature. It was optically inactive and the molecular weight determined by titration was 308. This acid would correspond therefore to somewhat impure tuberculostearic acid.
Fraction III of the esters had \([\alpha]_D = +7.7^\circ\) and the free acid, 0.73 gm., obtained on saponification was a thick oil with a neutral equivalent of 396.

Fraction IV of the esters had \([\alpha]_D = +6.5^\circ\). The free acid, 0.286 gm., obtained on saponification was a thick oil and had a neutral equivalent of 436.

No attempt was made to purify these acids further but the properties and dextrorotation would indicate the presence of phthioic acid.

**Examination of the Wax Fractions**—The wax fractions Nos. I, II, and IV, Table I, had similar properties and were combined. For saponification 15.5 gm. of this material were dissolved in 120 cc. of benzene and 2.5 gm. of potassium hydroxide dissolved in 20 cc. of methyl alcohol were added. The solution turned cloudy and after a short time it gelatinized. After the mixture had stood overnight, it was heated to boiling, whereupon most of the precipitate dissolved but a hard insoluble mass remained on the bottom of the flask. The yellowish supernatant solution was decanted and the flask was rinsed several times with hot benzene.

The benzene-insoluble material was examined for polysaccharide as will be described later.

To the benzene solution were added 2 gm. of potassium hydroxide dissolved in 3 cc. of water and 30 cc. of methyl alcohol and the solution was refluxed in a water bath for about 7 hours. The solution was concentrated by distillation to a volume of about 50 cc. and transferred to a separatory funnel with ether, after which it was shaken with an excess of dilute hydrochloric acid in order to remove the potassium salts. The solution after it had been washed with water until the washings were neutral to litmus was dried over sodium sulfate, filtered, and concentrated to dryness under reduced pressure. The residue was a yellowish oil when warm and a solid wax-like mass at room temperature. The product was dissolved in 50 cc. of ether and diluted with 100 cc. of acetone. On cooling in ice water, a white precipitate separated which was filtered off and washed with acetone. The substance was designated Fraction I. It weighed 6.8 gm.

The filtrate was concentrated to a small volume and diluted with 50 cc. of alcohol. The white precipitate that separated was filtered off, washed with alcohol, and dried. This material, Fraction II, weighed 1.8 gm.

The filtrate from Fraction II was neutralized with alcoholic potassium hydroxide and an alcoholic solution of lead acetate was added in excess. The lead salt that separated was filtered off, mashed with alcohol, and dried, thus giving Fraction III.

The filtrate from above was concentrated under reduced pressure to dryness, after which it was transferred with ether and water to a separatory funnel. The aqueous layer was acidified with acetic acid and the mixture was thoroughly shaken, after which the aqueous portion was drawn off and
discarded. The ethereal solution after it had been washed free of acetic
acid with water was extracted with 2.5 per cent aqueous potassium hydrox-
ide in order to remove any fatty acids that had not been precipitated as
lead salts. The alkaline extract yielded Fraction IV.

The ethereal solution was next washed with water, dried over sodium
sulfate, filtered, and the ether was distilled off. The residue consisting of
neutral material was designated Fraction V.

Fraction I. Crude Mycolic Acid—The substance was dissolved in 50 cc.
of ether and the solution was diluted with acetone. On cooling in ice water,
a fine globular precipitate separated which was filtered off, washed with
acetone, and dried in air. The substance weighed 6.2 gm. and melted at
55–56°. Its properties were similar to those of mycolic acid. The molec-
ular weight determined by titration was 1445. In chloroform solution
the substance showed no optical rotation.

Fraction II was probably mainly crude mycolic acid but it was not
examined.

Fraction III—The dried lead salt was treated with ether and the insol-
uble portion was filtered off and discarded. The ethereal solution was
freed from lead by means of dilute hydrochloric acid, after which it was
washed with water, dried over sodium sulfate, filtered, and evaporated to
dryness. The residue was a slightly yellowish thick oil at room tempera-
ture which weighed 1.4 gm.

Titration—0.3372 gm. of acid dissolved in 30 cc. of ether plus 10 cc. of
alcohol required 7.94 cc. of 0.1 N KOH. Found, mol. wt. 424.

Rotation—0.5321 gm. of acid dissolved in chloroform and diluted to 10 cc.
gave in a 1 dm. tube \( \alpha = +0.44° \); hence, \([\alpha]_b = +8.2°\).

Judging by the rotation and molecular weight the acid is somewhat
impure phthioic acid.

Fraction IV—The alkaline solution was acidified with dilute hydrochloric
acid and extracted with ether. The ethereal solution was washed with
water, dried over sodium sulfate, filtered, and evaporated to dryness. The
residue was a thick yellowish oil which partly solidified at room tempera-
ture.

Rotation—0.3616 gm. of acid dissolved in chloroform and diluted to 10 cc.
gave in a 1 dm. tube \( \alpha = +0.20° \); hence \([\alpha]_b = +5.5°\).

Judging by the rotation and properties this acid also contained phthioic
acid.

Fraction V—the neutral fraction weighed 0.3 gm. It was a slightly
yellowish solid which showed crystalline structure. The substance crystal-
lized from ethyl acetate in aggregates of small prismatic crystals. After
two recrystallizations from ethyl acetate 50 mg. of colorless crystals were
obtained. The crystals melted at 73–74°. In crystal form and melting
point the substance was identical with phthiocerol.
The Polysaccharide—The insoluble product which separated from the alkaline benzene solution of the wax weighed 1.6 gm. It was treated with a small amount of water and gave a cloudy strongly alkaline solution. The solution was slightly acidified with acetic acid, which caused a precipitation of fatty acid. The addition of neutral lead acetate gave a further precipitate which was filtered off and washed with water. The polysaccharide contained in the filtrate was isolated in the usual manner by means of basic lead acetate and ammonia. The lead salt was decomposed with hydrogen sulfide and the filtrate from the lead sulfide was concentrated to a thick syrup which was dehydrated by grinding under absolute alcohol until a white powder was obtained. After the powder had been filtered off, washed with absolute alcohol, and dried in vacuo, it weighed 0.5 gm. The substance gave the usual color reactions for pentose with phloroglucinol and orcinol. This polysaccharide is therefore similar to the polysaccharide contained in the wax of the tubercle bacillus cultivated on glycerol-containing medium.

SUMMARY

1. The total lipids of the human tubercle bacillus, Strain H-37, cultivated on a modified Long synthetic medium in which dextrose replaced glycerol, amounted to 30.6 per cent, which is comparable to the lipids obtained from tubercle bacilli grown on the regular Long medium.
2. The lipids did not contain any phosphatide.
3. The only lipid fractions that could be isolated were acetone-soluble fat and a low melting wax.
4. The acetone-soluble fat on analysis was similar in composition to the fat elaborated on a glycerol-containing medium. Tuberculostearic acid and phthioic acid were present and also the pigment phthiocol. The fat contained no glycerol but a carbohydrate which could not be definitely identified.
5. The wax fractions differed from the waxes isolated from the tubercle bacillus cultivated on a glycerol-containing medium in that no high melting wax could be found.
6. The low melting wax, however, gave on analysis certain of the characteristic components of the tubercle bacillus wax; namely, mycolic acid, the alcohol phthiocerol, dextrorotatory fatty acids analogous to phthioic acid, and a polysaccharide that contained pentose.

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