THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

IV. CONCERNING THE SO CALLED TUBERCLE BACILLI WAX.
ANALYSIS OF THE PURIFIED WAX.*

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INTRODUCTION.

The literature dealing with the chemistry of the tubercle bacillus contains many references to the presence of a large amount of a wax-like substance in the lipid fraction. The nature of this waxy material, its properties, and functions have been the subject of several studies. It seems evident from published data that the substance is very stable and is saponified with great difficulty, but after complete saponification it presumably yields higher alcohols and fatty acids.

Attention was first called to this wax by Aronson (1) and all subsequent investigators have corroborated Aronson’s observation. Kresling (2) reported that the unsaponifiable matter isolated from tubercle bacilli fat consisted of some higher alcohol. Similar observations have been reported by Bulloch and Macleod (3), Dorset and Emery (4), Auclair and Paris (5), Fontes (6), Panzer (7), and by Kozniewski (8). The chemical composition and properties of this alcohol were studied by Tamura (9) who described it as a monohydric alcohol of the formula C_{29}H_{56}O to which he gave the name mykol. Mykol was acid-fast and was

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probably responsible for the acid fastness of the bacilli. Two higher alcohols which differed in composition and properties from mykol have been described by Bürger (10). Although these products were not studied very completely, Bürger assigned the formula $C_{19}H_{36}O$ to one substance and $C_{18}H_{28}O$ to the other one. The work of Tamura has been accepted generally by subsequent investigators and Goris (11) in his work states that he isolated substances corresponding in properties to mykol. Although mykol is stated not to give any of the sterol color reactions, it is referred to by Wells, Dewitt, and Long (12), as follows: "Mykol may thus be considered an alcohol, of the general group of sterols, of which cholesterol is the best known member."

In an earlier work on the separation of the lipoids of tubercle bacilli (13), we encountered a large amount of a wax-like material in the chloroform extract. It represented about 46 per cent of the total lipids and 11 per cent of the dried bacteria. The crude wax was easily soluble in chloroform, benzene, and toluene and it dissolved in less than its own weight of ether, but it was practically insoluble in alcohol, methyl alcohol, or acetone. The substance contained a small amount of phosphorus and nitrogen and on combustion it left about 1 per cent of ash.

An analysis of crude wax is given by Lewkowitsch (14) and from the data published by Goris (11) it is evident that the material which he examined contained some ash together with phosphorus and nitrogen. The occurrence of the two latter elements has been regarded as evidence of the presence of contaminating phosphatides or lecithin.

For the present investigation the crude wax was purified by precipitation from ether and toluene by methyl alcohol. This treatment yielded a white amorphous powder which represented about 78 per cent of the original material and which we designate as "purified wax." The more soluble portion of the crude wax was obtained from the mother liquors and since it formed a yellowish salve-like mass at room temperature it was called "soft wax." The wax fractions mentioned above have been analyzed and the present report deals with the composition of the purified wax.

All of the lipoid fractions that we prepared were tested biologically in Dr. Sabin’s laboratory at The Rockefeller Institute.
for Medical Research and in these experiments it was found that
the purified wax elicited a reaction similar to that of the phos-
phatide (15). It has been shown, however, that the active
principle in the phosphatide is associated with a new saturated
liquid fatty acid called phthioic acid (16).

It was of more than passing interest, therefore, to find an acid
similar to phthioic acid among the mixed fatty acid obtained
from the purified wax and that this substance also caused the
formation of artificial tubercular tissue.

The purified wax differed decidedly in composition and proper-
ties from usual waxes. The material melted with decomposition
between 200–205°. On hydrolysis it yielded nearly 40 per cent of
water-soluble substances among which we found glycerophos-
phoric acid, reducing sugars which gave pentose reactions, and
some nitrogen-containing compound that could not be identified.
The ether-soluble constituents on the other hand contained a
large amount of unsaponifiable wax-like material and a small
amount of fatty acids.

It is evident from the products that are obtained on hydro-
lyzing the purified wax that the substance is not a wax in the
ordinary meaning of that term but that it represents a complex
phosphatide containing a large proportion of carbohydrates com-
bined in its molecule.

The fraction which we have designated as unsaponifiable matter,
above, may be the substance that has been called wax by earlier
investigators and it seems self-evident that an identical product
must have been obtained by those who have employed prolonged
extraction of the bacilli with the so called Aronson's mixture,
since the latter consists of 1 per cent hydrochloric acid in alcohol.
The nature of this "unsaponifiable wax" has not yet been eluci-
dated but further data regarding its composition and properties
will be published shortly.

Not only the tubercle bacilli wax but the mykol of Tamura
have been described as being acid-fast and the wax has been
regarded as the carrier of acid fastness of the organism (17). It
is interesting in this connection to note that the purified wax
described in this paper, although it yields on hydrolysis 56 per
cent of the unsaponifiable wax, has been found to be non-acid-fast,
while the unsaponifiable wax was strongly acid-fast. This result
seems hardly possible unless the acid fastness should be found to
depend upon the presence of free hydroxyl groups in the substance
here termed unsaponifiable wax. We are indebted to Professor
W. L. Kulp of the Department of Bacteriology, Yale University,
and to Dr. M. C. Kahn of Cornell University Medical College
for the determinations on acid-fastness.

EXPERIMENTAL.

The crude wax was of light yellow color and it resembled
beeswax in appearance but was more brittle. The substance was
readily soluble in ether, chloroform, benzene, and toluene, but it
was practically insoluble in alcohol, methyl alcohol, and in cold
acetone. Heated in a capillary tube, it melted at 50–51°. Quali-
tative reactions indicated the presence of phosphorus and nitrogen
and on combustion it left 1.02 per cent of ash.

Purification of the Crude Wax.

The wax which weighed 427 gm. had been obtained as described
in a former paper (13). It was dissolved in 1 liter of anhydrous
ether¹ and yielded a faintly cloudy solution which could not be
cleared by filtration. The addition of 1 liter of cold methyl
alcohol caused a heavy nearly white precipitate. After cooling
in ice water the precipitate was filtered on a Buchner funnel and
washed with methyl alcohol. The substance was again precipi-
tated two times from 1.5 liters of ether by adding 1.5 liters of
methyl alcohol. The product formed a nearly white amorphous
powder. It was distinctly less soluble in ether than in the begin-
ning. The ethereal solutions were cloudy and it was impossible
to obtain clear solutions either by filtration or by centrifuging.
The amount of the insoluble matter was, however, very small and
it was in such a fine colloidal condition that nothing settled out
on standing. The substance was more soluble in chloroform,
but even this solution was faintly cloudy.

For further purification the substance was dissolved in 500 cc.
of toluene and except for the presence of a few paper fibers the

¹ Throughout all operations air was excluded as far as possible by the use
of carbon dioxide. All solvents were saturated with this gas and all flasks,
desiccators, etc., were filled with carbon dioxide before any substance was
introduced into them.
solution appeared to be perfectly clear. After filtering, the substance was precipitated by adding 2 volumes of methyl alcohol. It was precipitated a second time from toluene by adding acetone. After the mixture had been cooled in ice water the precipitate was filtered off, washed with methyl alcohol, and dried in a vacuum desiccator.

The purified wax obtained after the operations described above formed a nearly white powder and the yield was about 78 per cent of the original material. When heated in a capillary tube the substance turned yellow between 180–190° and melted with decomposition between 200–205°.

Analysis of the purified wax: P 0.407, 0.41; N 0.77; ash, 1.39 per cent.

Recovery of Wax Residue from Mother Liquors.

The mother liquors from the precipitations mentioned above were concentrated by distillation, a stream of carbon dioxide being passed through the liquid, and the dark brown residue was dried in a current of carbon dioxide. The material was dissolved in toluene, and the solution was filtered. The addition of 2 volumes of methyl alcohol caused the separation of an oily precipitate. The mixture was shaken and cooled in ice water when the precipitate solidified. It was filtered, washed with methyl alcohol, and dried in a vacuum desiccator. At room temperature the substance formed a light brown salve-like mass that weighed 82 gm. Hence this fraction will be designated as “soft wax.”

The final mother liquor, after it had been concentrated and dried in a current of carbon dioxide, formed a brown oil that weighed 10 gm.

Analysis of the soft wax: The substance contained only a trace of nitrogen and about 0.08 per cent of phosphorus.

Saponification of the Purified Wax.

While we were under the impression that we were dealing with an ordinary wax, a preliminary saponification was made by boiling the purified substance for 8 hours with alcoholic potassium hydroxide.2 When the substance was treated in this manner, two striking phenomena were observed: (a) The solid white powder gradually decomposed, forming two separate constituents

2 The air was displaced by nitrogen during the saponification.
that were insoluble in the boiling alcoholic solution. One of these constituents formed a colorless oil that solidified to a hard white wax-like cake when allowed to cool to room temperature. (b) The other portion remained as a solid, somewhat sticky mass on the bottom of the flask.

The two constituents were separated mechanically by decanting the hot alcoholic solution containing the oily substance in suspension. The residue in the flask was treated repeatedly with boiling alcoholic potassium hydroxide, but it did not dissolve. It was also insoluble in ether, but it was easily soluble in water.

**Examination of Ether-Soluble Constituents after Saponification of the Purified Wax.**

The hot alcoholic solution obtained by decantation contained a large amount of an insoluble colorless oil. To insure complete saponification the mixture was again refluxed for 12 hours. On cooling, the oily material formed a hard white cake on the bottom of the flask. The alcoholic solution was decanted and the solid wax washed several times with alcohol.

The alcoholic solution and washings were concentrated by distillation to a small volume and then diluted with water which caused a small amount of insoluble matter to separate. The mixture was extracted several times with ether and the ethereal solution was washed with water.

The alkaline aqueous solution containing the water-soluble soaps was united with the washings from the ethereal solution. The ethereal extract mentioned above was used to dissolve the solid wax-like cake that had separated on cooling the saponification mixture. The substance dissolved slowly in the ether on being warmed gently. The ethereal solution was filtered, concentrated by distillation, and the warm residue was dried in a current of carbon dioxide. The substance was again boiled for 3.5 hours with alcoholic potassium hydroxide, diluted with 1 liter of hot water, and extracted with hot benzene. The benzene solution was washed with water, dried with sodium sulfate, filtered, concentrated by distillation to about 100 cc., and mixed with 350 cc. of acetone. A heavy amorphous precipitate was produced which, after cooling in ice water, was filtered on a Buchner funnel and washed with acetone. The substance formed
a white powder and it had been precipitated almost completely since the mother liquor yielded a very small amount of material when evaporated to dryness. For further purification the substance was precipitated once more from benzene and twice from chloroform by adding 3 volumes of acetone. The product which represents what would usually be designated as unsaponifiable matter, was a snow-white powder, and it had no sharp melting point. When heated in a capillary tube it sintered at about 60° and formed a slightly opaque melt at 64°. The substance was readily soluble in chloroform and in benzene, but it was not very soluble in cold ether and the ethereal solution was faintly cloudy. In warm ether it dissolved more readily and the solutions were clear. The ethereal solutions gelatinized on cooling, forming a semitransparent jelly. On warming, the mixture liquified, forming a clear solution. A 3 per cent solution in warm ether set to such a solid on cooling that the flask could be inverted without breaking the gel.

On combustion the substance left about 4.2 per cent of a fused colorless ash consisting of potassium carbonate. It seemed evident therefore that the material was either a potassium salt of some higher fatty acid or that it was contaminated with soaps. But every effort to fractionate the substance into different constituents proved fruitless.

Removal of Potassium from the Unsaponifiable Matter.

The total purified material which weighed about 27 gm. was dissolved in 500 cc. of warm ether. Dry hydrochloric acid was passed into this solution, when a white precipitate of potassium chloride separated. The precipitate was filtered off and the ethereal solution was washed with water until the hydrochloric acid was removed. The solution was filtered, concentrated by distillation to about 400 cc., and mixed with 300 cc. of acetone. On cooling in ice water a snow-white precipitate separated which was filtered off and washed with acetone. The substance was again twice precipitated from 300 cc. of ether by adding 200 cc. of acetone and cooling in ice water. It was finally twice precipitated from 200 cc. of ether by cooling in a freezing mixture of ice and salt. After the snow-white amorphous powder had been dried in a vacuum desiccator over sulfuric acid it weighed 24.5
gm. The substance separated from the solutions mentioned above in a manner characteristic of crystalline bodies but when examined under the microscope, it was found to be non-crystalline, consisting of small transparent globular particles that varied considerably in size. When heated in a capillary tube, the substance softened at 56° and melted at 57–58°. When 0.4897 gm. of the substance was burned in a platinum crucible, it left no weighable ash. For analysis the substance was dried at 61° in vacuo over phosphorus pentoxide but there was no loss in weight.

\[
\begin{align*}
0.1654 \text{ gm. substance} & : 0.2014 \text{ gm. H}_2\text{O and } 0.4960 \text{ gm. CO}_2, \\
0.1180 \text{ “ } & : 0.1441 \text{ “ ” } 0.3532 \text{ “ ” } 0.3532 \text{ “ ” }
\end{align*}
\]

\[
\begin{align*}
\text{Found. } & \text{ C 81.78, 81.63. } \\
& \text{ H 13.62, 13.66. }
\end{align*}
\]

The analytical results indicate that the relation between carbon and oxygen is in the ratio of 23.5:1. But since the substance possesses acid properties and also probably two free hydroxyl groups, it is evident that these numbers would have to be multiplied by 4 to account for the simplest formula containing one carboxyl and two hydroxyl groups. The calculation leads to the unusual and improbable formula of \( \text{C}_{91}\text{H}_{188}\text{O}_4 \). Since the substance does not crystallize and since it does not yield any crystalline derivatives, it is impossible to present any convincing evidence concerning the molecular magnitude of this compound. Attempts to determine its molecular weight by the boiling point or freezing point methods have led to abnormal results. For instance in the boiling point method, with benzene or ether as solvents, a depression of the boiling point was observed rather than a rise, and in the freezing point method, with naphthalene or camphor as solvents, molecular weights varying from 1046 to 1824 were obtained.

Values obtained on titrating the alcoholic solution of the substance with 0.1 N potassium hydroxide, or by analyses of the potassium salt, the silver salt, and of the acetyl derivatives all agree with the formula \( \text{C}_{91}\text{H}_{188}\text{O}_4 \). Further work is in progress in an attempt to elucidate the nature of this interesting compound.

**Properties of So Called Unsaponifiable Matter.**

The ash-free substance was readily soluble in benzene, toluene, chloroform, ligroin, petroleum ether, and in ether. On cooling
these solutions in ice water the substance separated as a dense white amorphous powder. The addition of cold alcohol or acetone caused a similar appearing precipitate. It was quite soluble in boiling acetone but on cooling the substance separated almost completely. It was very slightly soluble in alcohol; 1 part required about 500 parts of boiling absolute alcohol. In methyl alcohol or in glacial acetic acid it was very slightly soluble. It was apparently saturated since a chloroformic solution showed no visible absorption of bromine. The chloroformic solution of the potassium salt decolorized bromine and at the same time a white precipitate separated which evidently consisted of potassium bromide. When an ethereal solution of the substance was allowed to stand for 16 hours in contact with a large excess of bromine, a small amount of halogen was absorbed, apparently through substitution. The reaction product contained about 2 per cent of bromine. In the Liebermann-Burchard reaction no coloration whatever was produced.

No effect could be observed on shaking the substance with cold concentrated sulfuric acid. When treated with hot concentrated sulfuric acid, it melted and turned yellowish brown.

When boiled with a large excess of acetic anhydride, about 1:200, the substance dissolved completely, but on cooling it separated as small oily drops that solidified to a white hard cake when cold. When acetylated in this manner the substance took up acetic acid corresponding to two acetyl groups. When acetylated by the method of Einhorn and Hollandt (18), the presence of only one acetyl group could be demonstrated.

From the method by which it is obtained the substance would ordinarily be classified as unsaponifiable matter. The formation of acetyl derivatives would indicate that it possesses properties of a higher alcohol but as has been shown above it also possesses distinct acid properties. It might be argued that it is a mixture of higher alcohols and higher fatty acids, which mixtures, as is well known, are separated with great difficulty. However, all attempts to separate the substance into such constituents have proved entirely unsuccessful. Attempts have been made to separate acid constituents by means of the potassium or silver salts and laborious fractionations have been carried out with the ash-free substance without resulting in any noteworthy change.
in properties. The substance is extremely stable. It may be boiled for many hours with alcoholic potassium hydroxide or with sodium ethoxide without any decomposition or change being noticed.

For the present we are undecided whether to classify the material as unsaponifiable matter, as a higher hydroxy fatty acid, or simply as tubercle bacilli wax. The unique properties of the substance would seem to justify either or all of these classifications.

Isolation of Fatty Acids Occurring as Water-Soluble Soaps after Saponification of the Purified Wax.

The alkaline aqueous solutions of the saponification mixture after extraction with ether and benzene were acidified with hydrochloric acid. The fatty acids were extracted with ether, the ethereal solution was washed with water, filtered, and the ether was distilled. The oily residue was dried in a current of carbon dioxide. On cooling it formed a yellowish crystalline solid which obviously contained some oily material. It was combined with other similar fractions and reserved for complete analysis, as will be described later.

Examination of Water-Soluble Constituents after Saponification of the Purified Wax.

It has been mentioned earlier that a notable amount of material, insoluble in alcohol and in ether, remained at the bottom of the flask after saponifying the purified wax. This material was easily soluble in water. The aqueous solution was extracted with ether to remove a small amount of wax-like substance. The solution gave a white precipitate on the addition of alcohol. On boiling with Fehling’s solution no reduction occurred, but after the solution had been boiled for a few minutes with dilute hydrochloric acid it gave a heavy reduction on boiling with Fehling’s solution. It is evident, therefore, that the alcohol-insoluble material represented a carbohydrate complex which gave reducing sugars on hydrolysis. Long boiling with alcoholic potassium hydroxide would undoubtedly alter or decompose any sugars that might be present in the compound. In order to obtain any evidence regarding the nature of the carbohydrates present in the purified wax, it would be necessary to employ some other method of hydrolysis.
Hydrolysis of the Purified Wax by Means of Acids.

The purified wax was not wetted by water and the substance could not be brought into a state of suspension by continued grinding with water in a mortar. After a sample had been treated in this manner the wax particles were filtered off and the filtrate was tested for phosphorus and for carbohydrates, but only the faintest traces of these components could be detected. Evidently both phosphorus and carbohydrate were present in the wax in some combination that was insoluble in water.

A sample of the purified wax was boiled under a reflux condenser with 4 per cent sulfuric acid for 12 hours. Except for a slight yellow coloration of the powder that floated on top of the boiling acid, there was no evidence of any decomposition. The mixture was next heated in an autoclave to 120° for 2 hours. During this process the wax turned quite brown and it agglutinated to a hard, compact mass that apparently had not been hydrolyzed. The aqueous acid solution was faintly yellowish in color, but it contained only very slight traces of phosphorus or reducing sugars.

2 gm. of the wax were refluxed with 200 cc. of alcohol containing 5 per cent of sulfuric acid. After the mixture had been boiled for 1 hour evidence of hydrolysis could be observed and as the boiling was continued the solid particles of the wax were gradually converted into a colorless oil. After refluxing for 12 hours, all of the solid particles had disappeared. The mixture was diluted with water and extracted with ether. The ethereal solution was washed with water, filtered, and the ether was distilled. The residue after drying in a current of carbon dioxide weighed 1.4 gm. or 70 per cent of the wax. It was a faintly yellow oil when warm and a hard wax-like mass when cold.

A larger quantity of the wax was hydrolyzed in the manner just described and a special effort was made to determine the nature of the water-soluble constituents. The task proved very difficult, owing to the formation of large quantities of ethereal sulfates during the hydrolysis. Prolonged boiling of the aqueous solution was necessary in order to hydrolyze the ethereal sulfates, during which boiling perceptible decomposition of the carbohydrates occurred. As a result of these experiments it may be stated that the aqueous solution contained reducing sugars which, calculated as glucose, corresponded to 4.5 per cent of the wax.
Among the other water-soluble constituents, we were able to identify glycerophosphoric acid by means of the barium salt. All of the nitrogen contained in the wax was present in the aqueous solution. Only a very small amount of the nitrogen could be accounted for as ammonia and no derivatives of choline could be isolated. In fact we were unable to isolate any nitrogen compound in solid form.

**Hydrolysis of the Purified Wax by Means of Alcohol and Hydrochloric Acid.**

In order to avoid the troublesome ethereal sulfates which interfered with the determination of the water-soluble constituents we tried to employ alcohol containing 2 per cent of hydrochloric acid. This method worked very well and hydrolysis was complete after refluxing for 7 hours. The solid wax particles had then disappeared completely and the faintly brownish alcoholic solution contained a considerable amount of a colorless oil which solidified on cooling.

The alcoholic solution was concentrated to about one-half its volume, diluted with water, and extracted with ether. The ethereal solution was washed with water, filtered, and the ether was distilled. The residue after drying in a current of carbon dioxide formed a slightly yellow oil when warm and a nearly white solid when cold.

The ether-soluble constituents were boiled for 3 hours with 5 per cent alcoholic potassium hydroxide in order to saponify any esters that had formed during the hydrolysis. The saponification mixture was diluted with hot water and extracted with hot benzene. The aqueous solutions were saved for the isolation of fatty acids. The benzene extract was washed with dilute alkali and with water, dried over sodium sulfate, filtered, and the benzene was distilled. The residue was dissolved in warm ether and freed from potassium by passing in an excess of dry hydrochloric acid. The potassium chloride and the excess of hydrochloric acid were removed by washing with water. The ethereal solution was dried with sodium sulfate, filtered, and the ether was distilled. The residue after drying in a current of carbon dioxide formed a light yellow, thick oil which solidified to a hard wax-like cake when cold. For purification it was precipitated three
times from ether by adding cold acetone, yielding a practically
snow-white amorphous powder which in all its properties corre-
sponded to the so called unsaponifiable wax obtained after saponi-
fying the purified wax with alcoholic potassium hydroxide.

Isolation of the Fatty Acids.

The fatty acids were isolated from the alkaline solution after
extracting the unsaponifiable wax with benzene. The solution
was acidified with hydrochloric acid and extracted with ether.
The ethereal solution was washed with water, filtered, and the
ether was distilled. The residue consisting of fatty acids was
dried in a current of carbon dioxide. It formed a light yellow
crystalline mass which evidently contained some liquid fatty acids.

<table>
<thead>
<tr>
<th>TABLE I. Ether-Soluble Material after Hydrolysis.</th>
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</thead>
<tbody>
<tr>
<td>Method of hydrolysis:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Time of hydrolysis, hrs...</td>
</tr>
<tr>
<td>Total ether-soluble material, per cent</td>
</tr>
<tr>
<td>Unsaponifiable wax, per cent...</td>
</tr>
<tr>
<td>Total fatty acids, per cent...</td>
</tr>
</tbody>
</table>

Summary of Ether-Soluble Constituents.

The yields of ether-soluble material, including unsaponifiable
wax and fatty acids, obtained after hydrolyzing the purified wax
with acids and alcohol, are shown in Table I.

It is evident from the data given above that the so called
unsaponifiable wax represents the principal ether-soluble compound
and that the true fatty acids were present only in small amount.
The figures also show that a loss of about 10 per cent occurred
during the second saponification and this loss is too great to be
accounted for by the mere saponification of esters. It is possible
that some water-soluble material was lost on extracting the fatty
acids with ether because the residual aqueous solution was
not examined.
Examinations of Water-Soluble Constituents.

The aqueous acid solution after extraction of the ether-soluble constituents was concentrated in vacuo until the alcohol was removed and it was then made up to a convenient volume.

The solution contained all of the nitrogen and phosphorus that originally was contained in the wax as indicated by the following figures:

<table>
<thead>
<tr>
<th></th>
<th>In wax, per cent</th>
<th>In aqueous solution, per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>0.49</td>
<td>0.41</td>
</tr>
<tr>
<td>N</td>
<td>0.77</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Practically all of the phosphorus was present in organic combination, presumably as glycerophosphoric acid.

The solution also contained reducing sugars, but the amount varied somewhat, depending upon the method of hydrolysis, as shown in Table II.

**TABLE II.**

Reducing Sugars after Hydrolysis of Wax.

<table>
<thead>
<tr>
<th>Method of hydrolysis.</th>
<th>Sulfuric acid and alcohol.</th>
<th>Hydrochloric acid and alcohol.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>Reducing sugar calculated as glucose.</td>
<td>4.49</td>
<td>6.95</td>
</tr>
</tbody>
</table>

When some of the solution was heated with orcinol or phloroglucinol and hydrochloric acid, the characteristic pentose color reaction was obtained.

When the sugar solution was treated with phenylhydrazine two osazones were obtained.

The more soluble osazone after several crystallizations from hot water and from a mixture of acetone and water was obtained as fine yellow needles. It melted at 165-166°. This melting point corresponds to that of arabinosazone but the melting point of a mixture of the osazone mentioned above with pure arabinosazone showed a large depression. Consequently the sugar could not have been arabinose.

A second, less soluble osazone, was also obtained. It was recrystallized several times from alcohol from which solvent it
separated in fine yellow needles. When heated in a capillary tube, it melted with decomposition at 210°. Unfortunately we were unable to secure any evidence regarding the nature of the nitrogen-containing compound. Only a small amount of the nitrogen could be accounted for as ammonia.

*Separation of Fatty Acids by Means of Lead Soaps.*

The various fractions of the mixed fatty acids were united, giving 10.9 gm., and dissolved in alcohol, neutralized with potassium hydroxide, diluted with water, and precipitated with a slight excess of lead acetate. The lead soaps were filtered off, washed with water, dried *in vacuo*, and extracted with ether. The ether-soluble and the ether-insoluble lead soaps were decomposed separately in the usual manner by shaking with dilute hydrochloric acid.

*Solid Saturated Fatty Acids.*

The saturated fatty acids obtained from the ether-insoluble lead soap weighed 4.3 gm. The substance was crystallized from alcohol and separated into three fractions. Fraction 1 was recrystallized four times from alcohol and was obtained in small snow-white plates that weighed 0.5 gm. The acid melted at 83-84° and evidently represents a slightly impure specimen of cerotic acid. When titrated with 0.1 N alcoholic potassium hydroxide, 0.2648 gm. of the acid required 6.68 cc. of 0.1 N KOH, which corresponds to a molecular weight of 396. For C_{36}H_{68}O_{2}, calculated molecular weight 396.

Fraction 2 melted at 55-56°, and Fraction 3 melted at 54-55°. These acids which probably represented a mixture of palmitic and stearic acid were united, dissolved in alcohol, and separated by means of magnesium acetate into three fractions. Each fraction was recrystallized twice from alcohol and once from methyl alcohol, but this treatment had not caused any separation since each substance melted at 56°. When titrated with 0.1 N alcoholic potassium hydroxide, 0.3512 gm. of acid required 12.70 cc. of 0.1 N KOH, and 0.3069 gm. of acid required 11.11 cc. of 0.1 N KOH, which corresponds to a molecular weight of 276 in each case.

It is probable, therefore, that the acid is a mixture of about
equal parts of palmitic and stearic acid. Such a eutectic mixture melts at 57° and would have a molecular weight of 270.

**Liquid Fatty Acids.**

The liquid fatty acid isolated from the ether-soluble lead soaps was obtained as a slightly yellow oil that weighed 5.0 gm. The iodine number, determined by the Hanus method, was only 11.3 and the molecular weight as determined by titration was 362. These values would indicate that a large proportion of the substance was a liquid saturated fatty acid with a higher molecular weight than that of oleic acid.

The mixture of liquid fatty acids was reduced with hydrogen in the presence of platinum oxide (19) and the reduced acid separated by means of the lead soap-ether treatment as described under phthioic acid (16).

The ether-insoluble lead soap gave a small amount of crystalline fatty acid which after being crystallized from alcohol and recrystallized from acetone melted at 68-69°. The substance was undoubtedly stearic acid and it is most probable, therefore, that the unsaturated acid present in the original mixture was oleic acid.

**Isolation of a Liquid Saturated Fatty Acid Analogous to Phthioic Acid.**

The ether-soluble lead soap was decomposed by shaking with dilute hydrochloric acid and the mixture was washed with water until the lead chloride and hydrochloric acid were removed. The ethereal solution was treated with norit, filtered, and the ether was distilled. The faintly yellow oil, after drying *in vacuo* over sulfuric acid, weighed 3.1 gm. When cooled in ice water it solidified to a white crystalline mass and liquified at 18-18.5°. At ordinary room temperature the substance was an odorless oil. The acid was saturated because in chloroform solution it did not decolorize a solution of bromine. It was miscible in all proportions with alcohol and other organic solvents.

**Molecular Weight.**—The substance was dissolved in neutral alcohol and titrated with 0.1 N alcoholic potassium hydroxide. 0.4643 gm. of acid required 12.14 cc. of 0.1 N KOH. Molecular weight 382. 0.3508 gm. of acid required 9.19 cc. of 0.1 N KOH. Molecular weight 381.
Rotation. — 0.9253 gm. of acid was dissolved in alcohol and made up to 10 cc. In a 1 dm. tube \( \alpha = -0.141^\circ \), hence \([\alpha]_D^{\text{a}} = -1.63^\circ\). In the same manner 0.8210 gm. of acid gave a reading of \(-0.136^\circ\) and \([\alpha]_D^{\text{a}} = -1.65^\circ\).

For analysis the substance was dried \textit{in vacuo} at 61° over phosphorus pentoxide but there was practically no loss in weight.

0.1520 gm. substance: 0.1763 gm. \( \text{H}_2\text{O} \) and 0.4318 gm. \( \text{CO}_2 

0.1094 " " : 0.1265 " " : 0.3098 " "

Found. C 77.47, 77.23; H 12.97, 12.93.

The acid is most likely not pure but the analysis and neutralization value indicate that it must possess a high molecular weight and that it must be analogous to the phthioic acid (16) obtained from the phosphatide. It is interesting to note that the acid is levorotatory and as has been mentioned earlier it gave a physiological reaction on injection that was identical with that given by the dextrorotatory phthioic acid.

In conclusion it is a pleasure to acknowledge the helpful cooperation of Dr. William Charles White, Hygienic Laboratory, Washington, D. C., Professor Treat B. Johnson and Professor W. L. Kulp of Yale University, Dr. F. R. Sabin and Dr. C. A. Doan of The Rockefeller Institute for Medical Research, Dr. M. C. Kahn of Cornell University Medical College, H. K. Mulford and Company, and Parke, Davis and Company.

**SUMMARY.**

The so-called wax from tubercle bacilli has been purified and analyzed. The purified wax was a white powder that melted at 200–205°. On hydrolysis this substance yielded 71 per cent of ether-soluble and nearly 40 per cent of water-soluble constituents.

The greater portion, equivalent to 56 per cent of the total wax consisted of an amorphous snow-white powder that possessed both acid and alcoholic properties. For the present this substance may be designated unsaponifiable wax.

A smaller amount of true fatty acids was isolated and these acids on separation yielded cerotic acid, probably a eutectic mixture of palmitic and stearic acids, oleic acid, and a liquid saturated fatty acid analogous to phthioic acid.
The water-soluble constituents consisted of glycerophosphoric acid, a mixture of reducing sugars that gave pentose reactions and some nitrogen-containing compound that could not be identified.

The purified wax that has been analyzed is accordingly not a wax but it represents essentially a complex phosphatide containing a large amount of carbohydrate.

BIBLIOGRAPHY.

THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI: IV. CONCERNING THE SO CALLED TUBERCLE BACILLI WAX. ANALYSIS OF THE PURIFIED WAX
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